Yen & Jaffe's Reproductive Endocrinology

Physiology, Pathophysiology, and Clinical Management

SEVENTH EDITION

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Remembrance

Samuel S. C. Yen, MD, DSc
1927–2006

In 2006, endocrinology in general, and reproductive endocrinology in particular, lost a giant in clinical and translational reproductive endocrinologic research, Samuel S. C. Yen. He was insightful and a visionary and demanded excellence from his trainees, but no more so than from himself. He was arguably the leading clinical reproductive neuroendocrinologist of his time.

He and I coedited the first four editions of this textbook, which has since been translated from English into five languages—including a pirated version from China. The genesis of our textbook was crystallized during our time at the magnificent, idyllic Rockefeller Foundation retreat, the Villa Serbelloni, at the nexus of the three legs of Lake Como in Italy. It was there, surrounded by scholars from a panoply of disciplines and countries (Sam and I were the only physicians in the group), that we had the time and freedom to finalize the chapters. We mutually selected the authors, most of whom were outstanding investigators and clinicians in the areas about which they wrote (albeit not all as expeditiously as we had hoped).

Fortunately, our first editor, John Hanley, was a true scholar who shared our passion for quality and excellence. Our original publisher, W. B. Saunders, shared that same passion.

Sam continued his insistence on excellence for each edition, and he cajoled several of our authors until they did the same.

Sam's chapters on neuroendocrine regulation of the brain and of the hypothalamic-pituitary-ovarian axis are classic. His extensive and productive collaboration with his very close friend, the Nobel Laureate Roger Guillemin, who characterized many of the hypothalamic secretagogues that Sam used in his clinical studies, enabled him to base many of his comments in the textbook on his own laboratory's studies.

Sam was hard-driving, yet charming, as demanding of himself and our authors as he was of the myriad investigators with whom he worked and trained. His was a rich, full, productive, and creative life. He was a unique and colorful individual.

His like comes along very rarely.

Robert B. Jaffe, MD
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Preface

A centerpiece of reproductive endocrinology is the utilization of assisted reproductive technologies, including *in vitro fertilization* (IVF), to build healthy families. In 1978, the first baby born from IVF, Louise Brown, was proof of the concept that successful human pregnancy was possible following *in vitro* fertilization. In the same year, the first edition of Yen and Jaffe was published and represented the birth of a field with a strong research foundation, whose findings had direct applicability to health. Three decades later, the 2010 Nobel Prize in Medicine or Physiology was awarded to Sir Robert G. Edwards, Ph.D. (b. 1925, d. 2013), for his seminal contributions to the field of reproductive endocrinology and infertility. The publication of the 7th edition of Yen and Jaffe is dedicated to Dr. Edwards and all the pioneers who have devoted their energies to advancing the field.

In the first years of the development of the field, a major focus was on the endocrine mechanisms that supported the optimal development of oocyte and sperm, their interaction and the implantation of an embryo in the developmentally prepared endometrium. Today, interest in germ cell development and germ cell biology has gained great prominence, raising the possibility of application of stem cell-based therapeutics to treat infertility and gene disorders. Fertility preservation through the cryopreservation of sperm and oocytes has emerged as a key element of care for the cancer patient and others who face concerns regarding retention of reproductive potential. The importance of these translational advances is recognized by the addition of new chapters to this edition.

The completion of the sequencing of the human genome and the reduction in cost in whole genome analysis have opened new opportunities for finding genes that affect different aspects of reproduction, such as genes that affect the age of menarche and the age of menopause; genes that predispose to endometriosis, uterine fibroids, and polycystic ovary syndrome; and genes that influence ovarian reserve and spermatogenesis. These important discoveries, which will shed light on pathophysiology and new avenues for diagnosis and treatment, are highlighted in the present edition.

Since the publication of the last edition of this text, we have achieved a better understanding of environmental factors influencing fertility, including obesity. These contemporary issues have been addressed in this edition. Epigenetic factors are thought to mediate the impact of environmental exposures on gametes and embryos and the developing fetus. They are also believed to be responsible for intergenerational effects. Although still in its infancy, the science of epigenetics holds promise for explaining reproductive phenotypes and reproductive outcomes.

We appreciate the collaboration of past and new authors for their critical evaluation of the state of their respective fields. Their contributions have enriched this text and we are grateful for their efforts. They have helped carry forward the tradition of excellence that Drs. Jaffe and Yen created when they brought forward the first edition of this text.

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CHAPTER 1

Neuroendocrinology of Reproduction

Christopher R. McCartney
John C. Marshall

The Central Control of Reproduction

Successful reproduction is essential to the survival of a species. The reproductive system represents a highly-complex functional organization of diverse tissues and signaling pathways that, when properly functioning, ensures a number of key endpoints, the most important of which are the adequate production of gametes (ova and sperm); successful delivery of gametes for fertilization; and, in women, physiological preparation for possible pregnancy. Neuroendocrine systems are the principal drivers of reproductive function in both men and women. In particular, hypothalamic gonadotropin-releasing hormone (GnRH) is the primary, if not exclusive, feedforward signal to gonadotrope cells of the anterior pituitary, stimulating the synthesis and secretion of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Together, these two gonadotropins direct the primary functions of the reproductive axis: gametogenesis and gonadal sex steroid synthesis.

Given its critical importance to a species, the reproductive system must be robust, continuing to function properly in the face of various internal and external influences. In contrast, in settings of marked physiological stress (e.g., significantly reduced energy availability), mechanisms that temporarily limit fertility—the usual outcome of which is metabolically expensive in women—are biologically advantageous for the individual and, ultimately, the species. Appropriate function (or quiescence) of the reproductive system is governed by a number of intricate relationships. For example, feedback signals from the gonads (e.g., sex steroid concentrations) communicate the status of gonadal function to the hypothalamic-pituitary axis; these signals in turn influence GnRH and gonadotropin output, rendering a coordinated and tightly regulated feedback system that maintains gonadal function within narrow limits. The reproductive system also has extensive interactions with other neuroendocrine systems, such as those involved with energy balance and adaptations to stress. The reproductive neuroendocrine network integrates these myriad feedback signals, and the GnRH-secreting neuronal network represents the final common pathway for the central control of reproduction. Thus, the regulation of GnRH secretion represents a major focus of reproductive neuroendocrinology.

Much of our understanding of reproductive neuroendocrinology has emerged from the study of rodents, sheep, and non-human primates, which largely reflects the ethical boundaries inherent to human research. Since many neurobiological principles are similar among all mammals, these animal studies have been (and continue to be) indispensable. Nonetheless, certain aspects of reproductive neuroendocrinology may differ markedly among species. Thus, when available, human data will be prioritized throughout this chapter, but animal studies will also be discussed when appropriate, recognizing that specific findings may or may not be generalizable to humans.

Neuroendocrinology: The Interface Between Neurobiology and Endocrinology

Endocrinology is the study of cell-to-cell signaling that occurs via specific chemicals (hormones) that travel through the bloodstream to influence remote targets. The term "neuroendocrinology" refers to the involvement of the central nervous system—the hypothalamus in particular—in this process. This field of study has traditionally focused on hypothalamic neuron-derived factors that influence various target tissues, either directly, as with the hormones of the neurohypophysis, or indirectly, as with hypothalamic releasing factors that control anterior pituitary hormone secretion. Neuroendocrine systems direct a wide variety of critical biological processes such as growth and development, energy and fluid homeostasis, responses to stress, and reproduction.

Neurons are highly specialized and morphologically diverse cells that transmit information via electrical impulses called action potentials. Neurons have a cell body containing the cell nucleus, mitochondria, and synthetic organelles. Neurons also have cell processes that participate in the reception and delivery of electrical impulses (Fig. 1.1). Dendrites are short processes—often extensively branched to increase surface area—that typically receive information (afferent electrical impulses). The axon is a single cell process that generally transmits efferent electrical impulses away from the cell body in a process called "neuronal firing."

In unstimulated neurons, the inner portion of the neuronal membrane is negatively charged compared to the outer membrane surface (e.g., this "resting membrane potential" is typically between -50 to -75 mV in GnRH neurons). Such electrical polarization reflects transmembrane ionic differences, which are maintained by protein channels that govern transmembrane passage of specific ions (e.g., sodium, potassium, chloride). Regulated changes of transmembrane ion differences may cause the membrane potential to become more or less negative (hyperpolarization and depolarization,

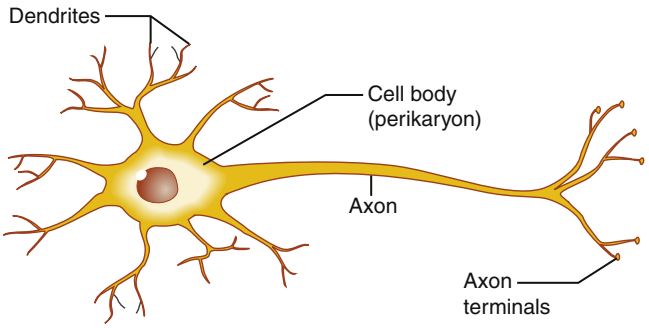


FIGURE 1.1 Morphological components of a neuron.

respectively). Depolarization to a certain threshold results in a rapid and temporary reversal of membrane potential (an action potential), which is propagated along the neuronal membrane. Notably, the amplitude of the action potential does not vary with the strength of stimulation; instead, once a threshold is reached, a full action potential occurs—the so-called "all-or-none" phenomenon. However, the degree of neuronal stimulation can alter the frequency of action potentials generated. In this way, neurons transmit information to other neurons and effector tissue cells.

Neuronal signals are transferred across neuron-to-neuron connections (synapses) via chemical neurotransmitters. This process begins with bursts of neuronal firing, which results in the opening of voltage-gated calcium channels at the axonal terminal. The influx of calcium promotes exocytosis of neurotransmitter-containing synaptic vesicles, releasing neurotransmitters into the synaptic cleft. Neurotransmitters then bind to specific ligand-dependent ion channels in the postsynaptic membrane, which can stimulate an action potential in the postsynaptic cell membrane. A wide variety of factors serve as neurotransmitters, including amino acids (e.g., acetylcholine, glutamate, γ-aminobutyric acid [GABA]), biogenic amines (e.g., norepinephrine, epinephrine, dopamine, serotonin), and neuropeptides (e.g., kisspeptin, neurokinin B, dynorphin, β-endorphin, somatostatin, proopiomelanocortin [POMC], neuropeptide Y [NPY]).

Bursts of neuronal firing can also elicit release of neuronal products into the bloodstream to influence remote targets (i.e., "neurosecretion" of "neurohormones"). Hypophysiotropic neurons are specialized hypothalamic neurons that secrete peptide releasing factors—GnRH, corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), and growth hormone-releasing hormone (GHRH)—into the hypophyseal portal circulation. These releasing factors in turn stimulate specific anterior pituitary cell populations. In contrast, hypothalamic release of dopamine into the portal circulation provides tonic inhibition of prolactin secretion. Hypothalamic neurosecretion of vasopressin and oxytocin, which are released directly into the systemic circulation, alter the function of distant tissues such as the renal tubules and uterus, respectively.

Neuroglial cells (e.g., astrocytes, ependymal cells, oligodendrocytes, and microglia) represent approximately 90% of cells in the central nervous system (CNS). Neuroglia do not conduct action potentials, but they perform critical supportive functions. For example, astrocytes form the supportive framework of the CNS; help isolate synaptic junctions (to prevent nonspecific spread of neuronal impulses); facilitate nutrient delivery to neurons; and contribute to the blood-brain barrier. In addition, astrocytes have been implicated in the control of GnRH secretion and the mechanisms underlying pubertal onset.[1] For example, astrocytes may impact neuronal activity via secretion of numerous growth factors, and astrocytes abundantly appose GnRH neurons; these contacts can influence synaptic input, and they may be influenced by estrogen in both rodents and nonhuman primates. Similarly, specialized ependymal cells (tanycytes) in the median eminence appear to modify access of GnRH neuron terminals to the hypophyseal portal blood.

Anatomy of the Reproductive Hypothalamic-Pituitary Axis

Portions of the hypothalamus and anterior pituitary gland constitute the primary effector arm of the central reproductive axis. In particular, hypothalamic neural systems regulate GnRH release into the hypophyseal portal veins, with GnRH being the signal to gonadotropes (anterior pituitary) to secrete LH and FSH. In turn, these gonadotropins direct gonadal (ovarian and testicular) function.

Hypothalamus

The hypothalamus is located at the base of the brain (Fig. 1.2). Although small (approximately 10 grams, less than 1% of total brain weight), it performs critical functions for maintenance of whole-organism homeostasis. In particular, primary functions include regulation of hunger and weight maintenance, various aspects of metabolism, growth, thirst and renal water handling, body temperature, autonomic function, sleep, circadian rhythms, and emotion. Importantly, the hypothalamus is also a primary control center for reproduction and influences sexual behavior.

As an anatomical structure, the hypothalamus does not have discrete borders; but in general, it forms the floor and inferior lateral walls of the third ventricle (Fig. 1.3). The medial portions of the hypothalamus are primarily made up of cell bodies, while the lateral portions are mostly composed of neuron fibers (axons), such as those connecting the medial hypothalamus to other areas of the brain. (Note that the hypothalamus is extensively interconnected with other brain areas.) By convention, closely associated collections of neuron cell bodies are called nuclei; and the paraventricular, dorsomedial, ventromedial, and arcuate nuclei (the latter can be called the infundibular nucleus in humans) contain a majority of the neurons that secrete hypophysiotropic hormones into the portal circulation. GnRH cell bodies do not form discrete nuclei, but are instead diffusely located throughout the preoptic area and the mediobasal hypothalamus (Fig. 1.4); the latter is situated caudal to the preoptic area, extending from the retrochiasmatic area (i.e., the area situated behind the optic chiasm) to the mamillary bodies, and including both the arcuate (infundibular) nucleus and the median eminence.

Median Eminence

Positioned at the base of the third ventricle, the median eminence is part of the anatomical link between the hypothalamus and anterior pituitary. The internal zone of the median eminence is located along the ventral floor of third ventricle

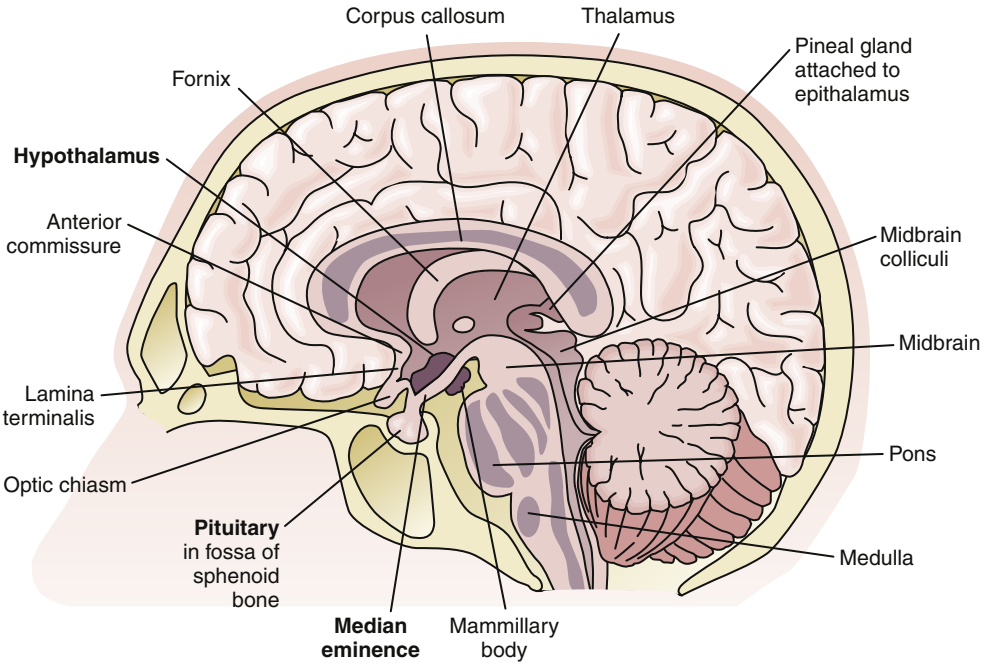


FIGURE 1.2 Cross-sectional representation of the human brain (sagittal plane), including hypothalamus, median eminence, and pituitary gland. *(Adapted from Johnson MH, Everitt BJ.* Essential reproduction*, ed 5. Blackwell Science, 2000, Fig. 6.1.)*

and is largely composed of axonal fibers from both magnocellular neurons (larger neurons that secrete vasopressin and oxytocin) and hypophysiotropic neurons as they travel from hypothalamic nuclei/areas to their final destinations—the neurohypophysis (posterior pituitary) and the external zone of the median eminence, respectively (Fig. 1.5). The external zone contains hypophysiotropic neuron terminals, which release hypophysiotropic hormones into an extensive capillary plexus—the proximal end of the hypophyseal portal system. Some nerve terminals in this zone act on other nerve terminals to influence hormone release (e.g., kisspeptin neurosecretion at GnRH neuron terminals appears to influence GnRH release).

The ependymal layer lining the third ventricle includes a population of specialized ependymal cells called tanycytes, which have a short process extending toward the ventricular surface and a long process extending into the median eminence toward areas around portal capillaries. The latter tanycyte projections envelope or retract from GnRH nerve terminals during high and low GnRH neuronal activity, respectively. Thus, tanycytes may influence GnRH secretion by physically isolating GnRH neuron terminals from portal capillaries, a regulated process.[2] Tanycytes have also been proposed to be a link between cerebrospinal fluid and events at the external zone (e.g., by transporting substances from the third ventricle to portal blood).

The median eminence is among the so-called circumventricular organs, which lie adjacent to the ventricular system and represent openings in the blood-brain barrier. While lipid-soluble molecules can diffuse in and out of the CNS relatively easily, and cellular transport mechanisms allow selective entry of ions, the blood-brain barrier functions to protect certain regions of the brain and hypothalamus from larger charged molecules, with physical protection provided by (a) tight junctions between endothelial cells and (b) neuron-capillary separation by both astrocyte foot processes and microglia. However, the CNS requires feedback signals—including hormonal, metabolic, and toxic—via macromolecules of peripheral origin that would otherwise be excluded by the blood-brain barrier; accordingly, capillaries of the circumventricular organs are fenestrated and permit transcapillary exchange of larger charged molecules (e.g., proteins, peptide hormones). Thus, the median eminence represents a key access point for central sensing of peripheral cues. Similarly, fenestrated vessels readily allow entry of hypothalamic releasing factors into portal blood.

Hypophyseal Portal Circulation

No direct neuronal connections exist between the hypothalamus and the anterior pituitary. However, the hypophyseal portal circulation (hypothalamic-hypophyseal portal system, pituitary portal system) represents the functional connection between the median eminence and anterior pituitary (Fig. 1.4). The superior hypophyseal artery (a branch of the internal carotid artery) subdivides to form an extensive capillary network in the external zone of the median eminence, with loops that reach into the inner zone. Capillary blood then drains into sinusoids that converge into the hypophyseal portal veins. Traversing the pituitary stalk to reach the anterior pituitary, the hypophyseal portal system forms the primary blood supply of the anterior pituitary. The direction of blood flow is primarily, but not exclusively, from the hypothalamus to the anterior pituitary; some retrograde flow allows for short-loop hypothalamic feedback.

Pituitary Gland (Hypophysis)

The pituitary gland appears as an extension at the base of the hypothalamus and resides cradled within the sella turcica, a saddle-like structure of the sphenoid bone (Fig. 1.2). The adenohypophysis (anterior pituitary) is of ectodermal origin, derived from an upward invagination of pharyngeal epithelium (Rathke pouch) during embryological development. The adenohypophysis is comprised primarily by the anterior lobe (pars distalis), which contains specialized

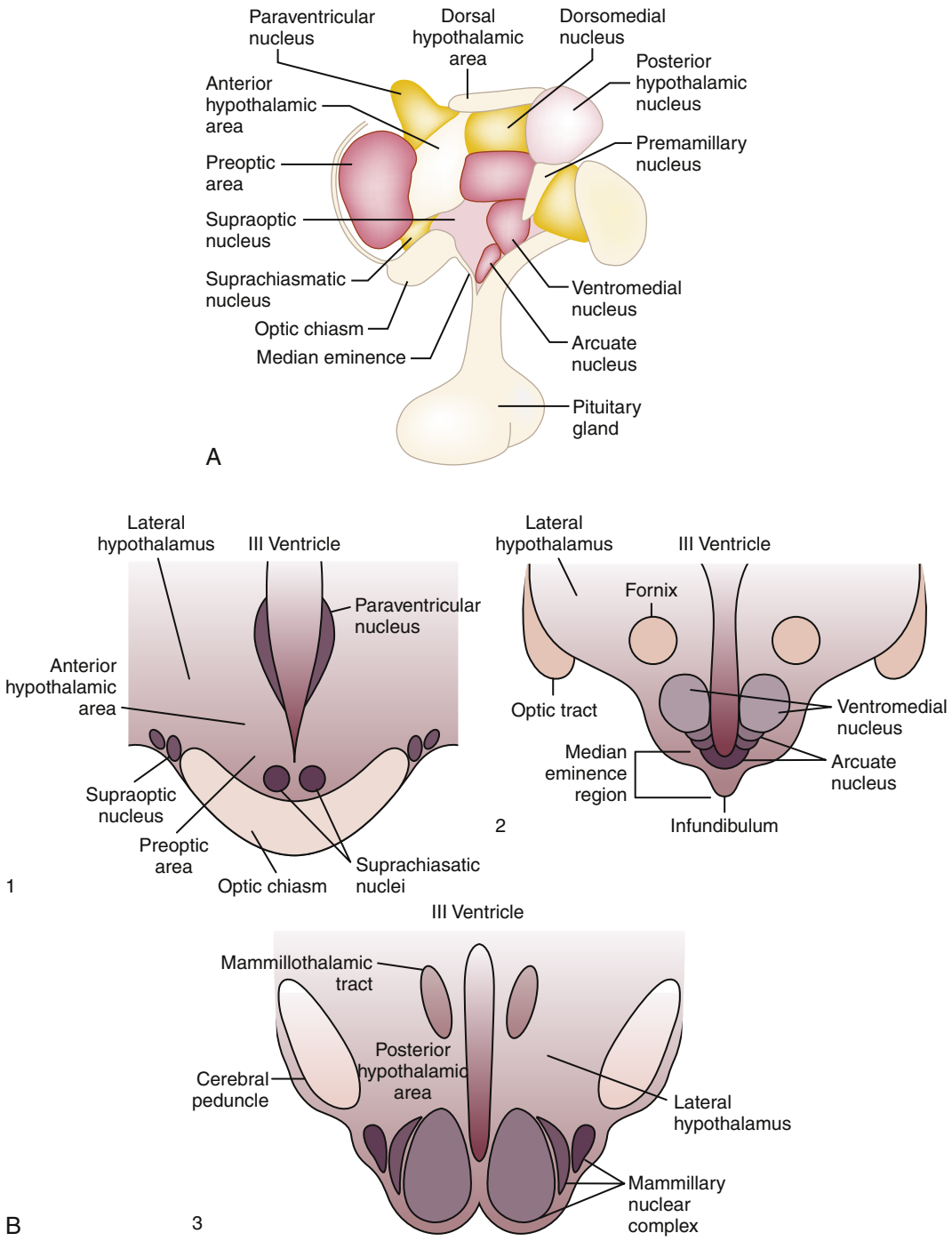


FIGURE 1.3 Nuclei and areas of hypothalamus. **A,** By custom, the nuclei and areas of the hypothalamus are often divided into three groups according to their location along the anterior-posterior plane: the anterior group, the tuberal group, and the posterior (or mamillary) group. The anterior group is formed by the paraventricular, supraoptic, and suprachiasmatic nuclei along with the anterior hypothalamic and preoptic areas. The tuberal group—so-called because of its position above the tuber cinereum (from which the infundibulum or pituitary stalk extends)—contains the dorsomedial, ventromedial, and arcuate nuclei along with the median eminence. Along with the paraventricular nucleus, the nuclei of the tuberal group contain a majority of the neurons that secrete hypophysiotropic hormones (i.e., hypothalamic hormones regulating hormone synthesis and release from cells in the anterior pituitary). Finally, the posterior group includes the posterior hypothalamic nucleus and mamillary nuclei. **B,** Cross-sectional representations (coronal planes) of the rostral (1), mid (2), and caudal (3) portions of the human hypothalamus. *(Section B is adapted from Johnson MH, Everitt BJ.* Essential reproduction*, ed 5. Blackwell Science, 2000, Fig. 6.3.)*

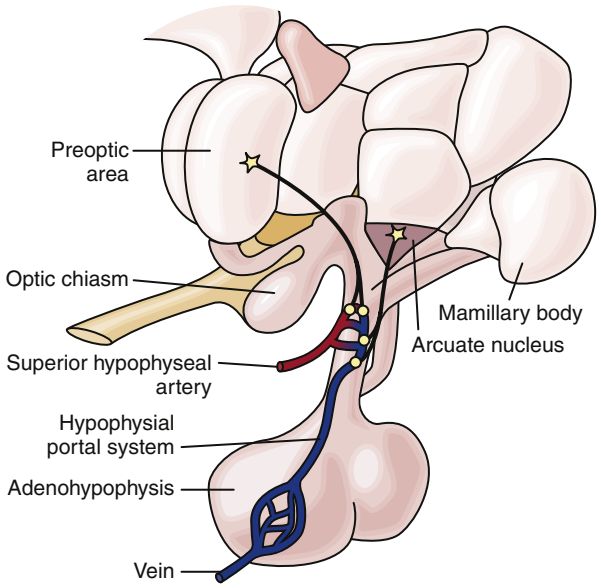


FIGURE 1.4 Anatomical relationship between hypothalamic GnRH neurons and their target cell populations in the adenohypophysis (anterior pituitary). GnRH neuron cell bodies are located in the preoptic area and the mediobasal hypothalmus. GnRH axonal projections terminate at the median eminence, where GnRH is secreted into the hypophyseal portal system. *(Adapted from Johnson MH, Everitt BJ.* Essential reproduction*, ed 5. Blackwell Science, 2000, Fig. 6.4.)*

cell populations that produce specific hormones: gonadotropes (the gonadotropins LH and FSH), mammotropes (prolactin), corticotropes (adrenocorticotropic hormone), thyrotropes (thyroid stimulating hormone [TSH]), and somatotropes (growth hormone). The intermediate lobe is vestigial in adult humans, but includes a small population of cells (e.g., POMC cells) in contact with the posterior lobe; and the pars tuberalis is a slender layer of tissue (e.g., LH-producing cells and TSH-producing cells) surrounding the infundibulum and pituitary stalk.

In contrast to the adenohypopysis, the neurohypophysis (posterior pituitary) is comprised of neural tissue and forms as a downward extension of neuroectodermal tissue from the infundibulum during embryological development. It is thus a direct extension of the hypothalamus. The neurohypophysis includes the infundibular stalk and the pars nervosa (posterior lobe of the pituitary). The supraoptic and paraventricular nuclei include magnocellular neurons that produce oxytocin and arginine vasopressin ([AVP]; also known as antidiuretic hormone [ADH]) respectively; these axons project to the posterior lobe of the pituitary, where oxytocin and AVP are secreted into a capillary network that drains into the hypophyseal veins (i.e., directly into the general circulation). The posterior lobe also includes specialized glial cells called pituicytes, which envelope or retract from magnocellular nerve terminals during high and low neuronal activity, respectively.

Gonadotropin-Releasing Hormone: The Final Common Pathway for the Central Control of Reproduction

Gonadotropin-releasing hormone, previously called luteinizing hormone-releasing hormone (LHRH), is synthesized

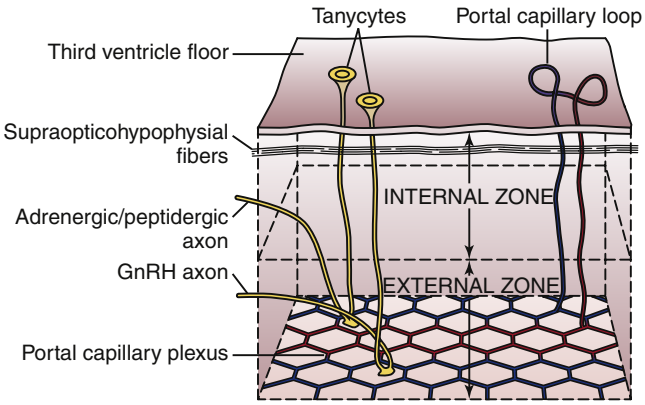


FIGURE 1.5 Diagram of the median eminence.

and released from a relatively small population of specialized hypothalamic neurons. GnRH was initially isolated from porcine hypothalami and shown to stimulate pituitary gonadotropin release.[3] Although the primary function of GnRH is to regulate pituitary gonadotropin secretion, GnRH also appears to have autocrine and paracrine functions in diverse tissues (e.g., ovary, placenta).[4]

The regulation of GnRH secretion is complex and involves overlapping pathways, which likely increases the robustness of central reproductive function. However, there are no known parallel or backup pathways for the stimulation of gonadotropin secretion. Thus, natural fertility is absolutely dependent on appropriate GnRH secretion. For example, mice with mutations of the GnRH-1 gene are hypogonadal, but reproduction can be restored via GnRH-1 gene therapy[5] or transplantation of fetal GnRH neurons.[6] Similarly, a variety of human conditions associated with absent (or near-absent) GnRH secretion lead to pubertal failure, hypogonadotropic hypogonadism, and infertility, all of which can be fully reversed with exogenous GnRH therapy.[7]

GnRH secretion is influenced by numerous factors including sex steroids, energy availability, and stress. In some mammalian species, GnRH secretion is also affected by circadian rhythms, photoperiod (e.g., seasonal breeders such as sheep), social cues, and pheromones.

GnRH Structure

Gonadotropin-releasing hormone (GnRH-1 in particular) is a decapaptide, with the amino acid structure (pyro)Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. The amino acid structure of GnRH is identical in essentially all mammalian species; and with the exception of the central Tyr-Gly-Leu-Arg segment, the amino acids of GnRH are highly conserved among vertebrate species.[8] The GnRH-1 gene *(GNRH1)* is located on human chromosome 8 (8p11.2-p21) and produces a 92 amino acid precursor peptide called prepro-GnRH, which includes a signal sequence (23 amino acids), GnRH (10 amino acids), a proteolytic processing site (3 amino acids), and GnRH-associated peptide (56 amino acids) (Fig. 1.6). The latter peptide can stimulate gonadotropin secretion and inhibit prolactin secretion, although its precise physiological role, if any, remains unclear. The actions of GnRH are mediated through the GnRH type I receptor.

Another form of GnRH (GnRH-2) and its receptor have been identified in a variety of animal species including

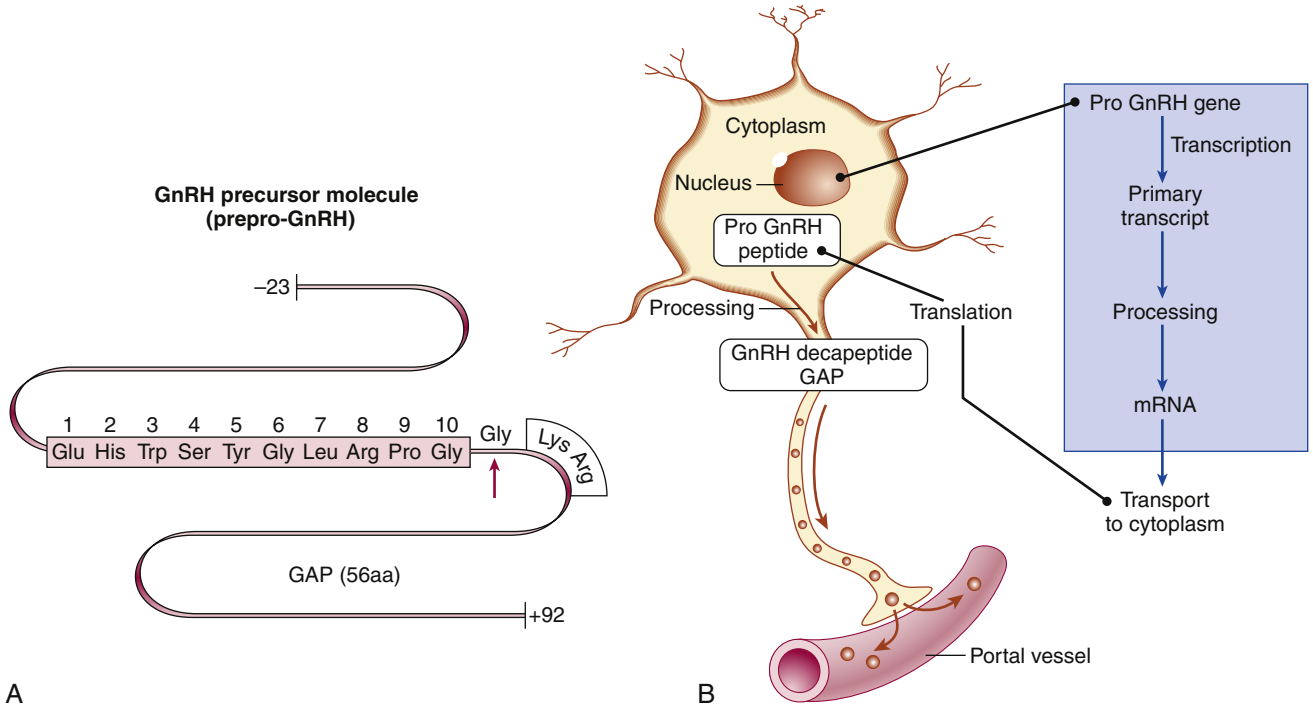


FIGURE 1.6 Schematic of GnRH synthesis. **A,** Representation of prepro-GnRH, including a 23 amino acid signal sequence, GnRH, a proteolytic processing site (Gly-Lys-Arg), and GnRH-associated peptide (GAP). The arrow indicates the site of proteolytic cleavage and C-amidation. **B,** Schematic of neuronal GnRH synthesis and secretion.

humans.[9] GnRH-2 is a decapeptide with similar structure to GnRH-1: (pyro)Glu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2 (underlined amino acids denote differences compared to GnRH-1). However, the gene for GnRH-2 is located on human chromosome 20 (20p13). GnRH-2 is widely expressed in the CNS and extra-CNS tissues, and it may contribute to reproductive behavior regulation in some species. In lower animals, GnRH-2 can act via its own receptor, which is structurally and functionally distinct from the GnRH type I receptor. Although a homologue of the GnRH-2 receptor gene has been detected in the human, it includes a frameshift and premature stop codon. Thus, GnRH-2 signaling in humans may act through the GnRH type I receptor, although the physiological role of GnRH-2 in humans remains unclear.

Anatomy of GnRH-Secreting Neurons

GnRH neurons are a heterogeneous population of hypothalamic neurons. They are relatively few, numbering approximately 1500 to 2000, and the majority of GnRH neuronal cell bodies are located in the arcuate (infundibular) nucleus (part of the mediobasal hypothalamus) and the medial preoptic area.[10] Although GnRH neurons are rather loosely affiliated anatomically, they are functionally integrated and form a complex network with numerous interconnections, in addition to connections to other neuronal populations. The GnRH neurons in the mediobasal hypothalamus appear to be requisite for gonadotropin secretion, and GnRH neuronal axons project to the median eminence via the GnRH tuberoinfundibular tract. The physiological function of other GnRH neurons, which arise from the anterior and posterior hypothalamus, and project to the limbic system and posterior pituitary, respectively, remains unclear, although some of these circuits may possibly be involved with various behavioral responses.

Embryological Development of the GnRH Neuronal Network

The ontogeny of GnRH neurons in vertebrate species is unique among neuronal systems of the CNS: nascent GnRH neurons are initially identified outside of the CNS in the nasal placode (sometimes called the olfactory placode). However, GnRH cells migrate during embryological development, as directly observed in embryonic nasal explant cultures and in embryonic head slices (mouse model).[11,12] The specific migratory pathway of GnRH neurons was first demonstrated in mice by documenting the presence of GnRH-immunoreactive cells in different areas at different stages of embryonic development (Fig. 1.7).[13-15] Specifically, GnRH expression is first observed within the nasal placode circa embryonic day 10 or 11. By embryonic day 13, GnRH cells are primarily located around the cribiform plate, and GnRH cells begin to reach the hypothalamus by embryonic day 14, approaching their final positions around embryonic day 16. This migratory pathway has been confirmed in both nonhuman primates[16] and humans.[17]

Successful migration of GnRH neurons is inextricably intertwined with olfactory system development, likely reflecting the close functional relationship between reproduction and the olfactory system (e.g., pheromones) in mammalian phylogeny. The nasal placode gives rise to nasal epithelium and olfactory sensory neurons, the latter of which extend axonal projections to the olfactory bulb. Vomeronasal neurons are a subset of olfactory neurons

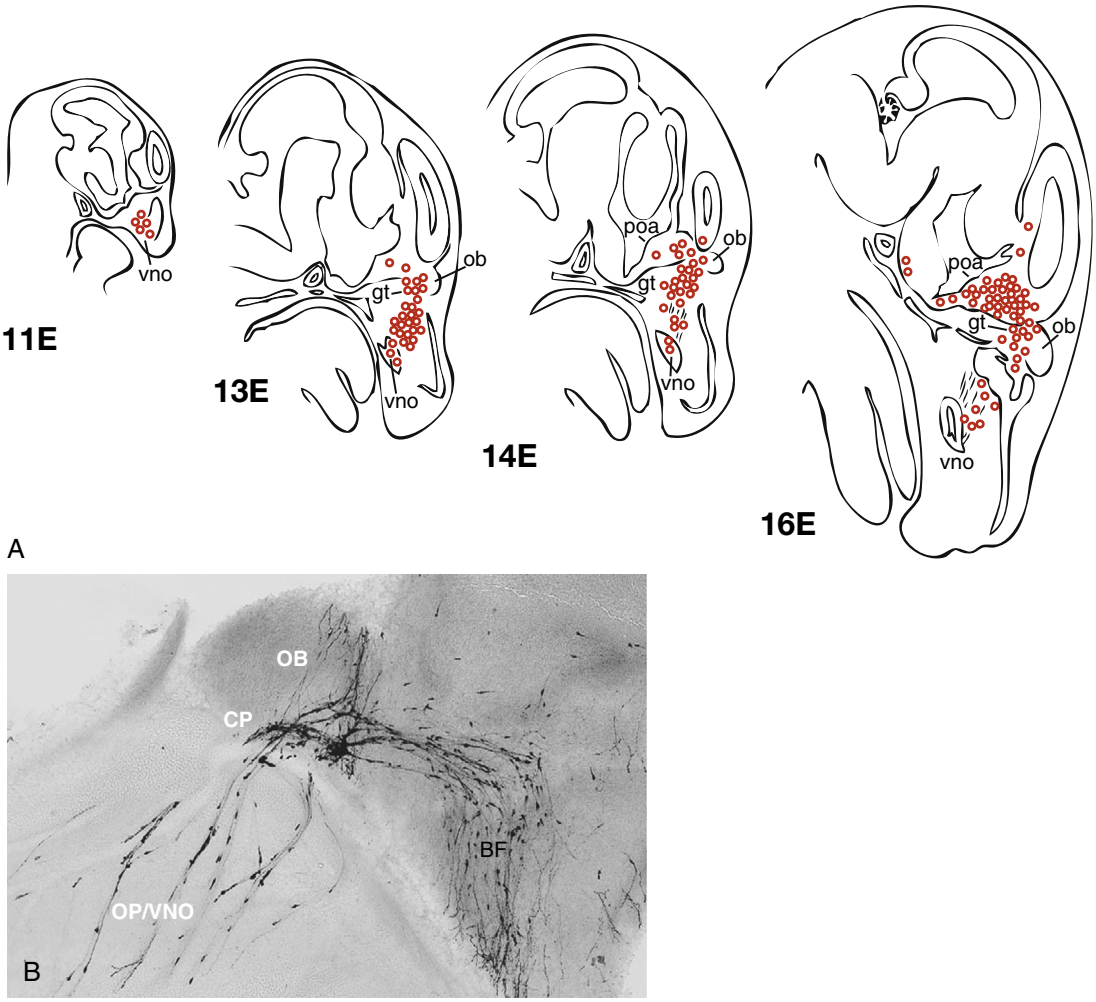


FIGURE 1.7 GnRH neuron migration during embryogenesis. **A,** Location of GnRH-immunoreactive cells (red circles) as a function of embryological age (mouse). On embryological day 11 (11E), GnRH cells are located in the nasal (olfactory) placode and presumptive vomeronasal organ (vno). GnRH cells migrate across the cribiform plate toward the olfactory bulb (ob). GnRH neurons then follow the caudal branch of the vomeronasal nerve toward the forebrain and hypothalamus. By day 16 (16E), GnRH neurons largely reside in the preoptic area (poa) of the hypothalamus. Abbreviations: gt, ganglion terminale. *(Adapted from Schwanzel-Fukuda M, Pfaff DW. Origin of Luteinizing Hormone-Releasing Hormone Neurons.* Nature *338:161-164, 1989.)* **B,** Sagittal brain slice (mouse, embryonic day 15) demonstrating the migratory route of GnRH-immunoreactive cells. Staining is for GnRH and peripherin (a neuronal intermediate filament). OP/VNO, olfactory placode-vomeronasal organ; CP, cribriform plate (CP); OB, olfactory bulb; BF, basal forebrain. *(Adapted from Wierman ME, Pawlowski JE, Allen MP, et al. Molecular mechanisms of gonadotropin-releasing hormone neuronal migration.* Trends Endocrinol Metab *15:96-102, 2004.)*

believed to be involved with pheromone detection; these axons originate in the vomeronasal organ and largely extend to the accessory olfactory bulb. At the level of the cribiform plate, some olfactory (vomeronasal) axons separate and form a branch that extends caudally into the forebrain. Of great importance, migrating GnRH neurons maintain adhesion to these axons; thus, these olfactory neurons form a critical guidance track for GnRH neuronal migration across the nasal epithelium and through the forebrain toward the hypothalamus.[18,19]

The dependence of GnRH neuronal migration on normal olfactory system development is exemplified by Kallmann syndrome, a form of congenital hypogonadotropic hypogonadism accompanied by absent sense of smell (anosmia). In this syndrome, faulty development of the olfactory system renders an inadequate guidance infrastructure for migrating GnRH neurons, leading to failure of GnRH neurons to reach the hypothalamus. The first identified cause of Kallmann syndrome was deletion of the Kallmann syndrome 1 sequence (*KAL1* gene), which is located on the X chromosome (Xp22.3) and encodes anosmin-1, a secreted matrix glycoprotein expressed in the presumptive olfactory bulb. Although precise mechanisms are unclear, anosmin-1 is believed to be important for the formation of olfactory elements that provide migratory guidance to GnRH neurons as they move out of the nasal placode. Evaluation of a 19-week-old human fetus with X-linked Kallmann syndrome demonstrated GnRH-immunoreactive cells within a tangle of olfactory and vomeronasal nerves at the dorsal surface of the cribiform plate, along with the absence of olfactory tracts and bulbs.[20] In a second human fetus (16 weeks) with X-linked Kallmann syndrome, GnRH was detected along terminal nerve fascicles in the nasal mucosa only.[21] This syndrome illustrates that, without the guidance framework provided by the olfactory neuronal system, GnRH neurons fail to migrate into the hypothalamus

and thus cannot release GnRH into the hypophyseal portal system.

A number of additional single-gene defects have been associated with Kallmann syndrome, including mutations of prokineticin 2 (PROK2) and its receptor (PROKR2),[22] fibroblast growth factor-8 (FGF8) and its receptor (fibroblast growth factor receptor 1 [FGFR1]),[23] nasal embryonic LH-releasing hormone factor (NELF),[24] and chromodomain helicase DNA binding protein 7 (CHD7).[25] The importance of these genes in GnRH neuronal development is corroborated by mouse studies. For example, in fetal mice lacking either PROK2 or PROKR2, GnRH neurons are trapped in a tangled web of olfactory/vomeronasal axons, with few, if any, reaching the forebrain.[26] Although these gene products are clearly important for GnRH neuron ontogeny, their precise roles remain uncertain.

Mouse studies suggest other important factors underlying GnRH neuron migration during prenatal development. For example, the chemokine (C-X-C motif) receptor 4 (CXCR4) is expressed on murine GnRH neurons and interacts with a secreted chemokine stromal cell-derived factor-1 (SDF-1), which is present as a gradient in the nasal mesenchyme. This gradient, with highest concentrations at the cribriform plate, provides directional information as GnRH neurons migrate toward the cribiform plate; and GnRH cell migration across the nasal compartment is markedly impaired in CXCR4 knockout mice.[27] As another example, extension of the caudal branch of the vomeronasal nerve toward the ventral forebrain involves chemoattraction via interactions between netrin-1—a chemokine expressed as a gradient in the forebrain—and its receptor, deleted in colorectal cancer (DCC). In mice without either DCC or netrin-1, the caudal branch of the vomeronasal nerve extends toward the cerebral cortex rather than the ventral forebrain; GnRH neurons follow this path, ultimately residing in the cerebral cortex.[28,29] A number of such interactions have been implicated in (a) guidance of olfactory neurons toward the forebrain and (b) the association between migrating GnRH neurons and axons of olfactory/vomeronasal nerves, but their specific roles in humans remain unclear. For example, no human mutations affecting DCC or netrin-1 have been described to date.

After reaching the hypothalamus, GnRH neurons detach from olfactory nerve axons and may disperse further before resting. A critical next step is extension of GnRH neuronal axons to the median eminence, where GnRH may gain access to the hypophyseal portal system.

GnRH Neuronal Firing and GnRH Secretion

GnRH neuronal activity is marked by bursts of action potentials (burst firing), the patterns and rates of which change across time. Changes of GnRH secretion are presumably related to changes of GnRH neuron firing rates, although the precise relationship between these events is unclear. Variable firing rate patterns (e.g., times of high and low firing rates) appear to be intrinsic to GnRH neurons, but they can also be altered by neurotransmitters and neuromodulators (e.g., glutamate, GABA, kisspeptin). Although sex steroids can markedly influence GnRH neuronal firing rates, GnRH neurons lack the primary receptors mediating sex steroid feedback (i.e., estrogen receptor alpha, progesterone receptor, androgen receptor); however, many studies suggest that sex steroid actions on GnRH neuronal activity are mediated primarily by afferent neurons (e.g., those secreting glutamate, GABA, kisspeptin, etc.).

GnRH neuron cell bodies are relatively scattered across the mediobasal hypothalamus and preoptic area, yet GnRH is secreted into the hypophyseal portal system in a coordinated, pulsatile fashion. Specifically, GnRH secretion is marked by episodic bursts of hormone release into the portal system, as demonstrated in rats,[30] sheep,[31] and monkeys.[32] Once released into the portal vascular compartment, GnRH is rapidly degraded via enzymatic proteolysis, and the half-life of GnRH in the blood is very short—approximately 2 to 4 minutes. Thus, GnRH presentation to gonadotrope cells is intermittent.

Pulsatile GnRH secretion is absolutely required for long-term stimulation of gonadotropin synthesis and secretion. Yet there is a relatively narrow window of GnRH pulse frequency and amplitude that will optimally stimulate gonadotropin secretion. Intermittent GnRH stimulation of gonadotrope cells can increase (or maintain) GnRH receptors on gonadotropes—the "self-priming" or "autopriming" effect. Thus, intermittent GnRH stimulation facilitates or maintains gonadotrope responsiveness to GnRH. However, more frequent exposure to GnRH pulses can reduce gonadotropin responses to GnRH[33]; at one extreme, continuous GnRH receptor stimulation leads to marked desensitization of gonadotropin synthesis and secretion. In a classic experiment involving rhesus monkeys with hypothalamic lesions that abolished GnRH secretion, intermittent (once an hour) exogenous GnRH administration restored pituitary gonadotropin secretion. However, changing from intermittent to continuous GnRH administration resulted in marked desensitization of gonadotropin release (Fig. 1.8).[34] This desensitization is largely related to reduced GnRH receptor expression on gonadotropes (i.e., receptor downregulation).

The foregoing phenomenon can be exploited therapeutically with the use of long-acting GnRH receptor agonists. Such agonists are peptides with structures very similar to that of GnRH, but with amino acid substitutions that enhance receptor binding affinity, increase resistance to proteolytic degradation, or both (Fig. 1.9), thus providing continuous GnRH receptor stimulation. Although initial GnRH receptor agonism temporarily increases gonadotropin release (gonadotropin "flare"), continued agonism leads to desensitization of gonadotropin secretion with accompanying reductions of gonadal sex steroid concentrations to castrate levels ("medical oophorectomy," "medical castration," "pseudomenopause"), usually over 4 to 8 weeks. These agents are useful in the therapy of gonadotropin-dependent disorders such as central precocious puberty, endometriosis, and prostate cancer.

Peptide GnRH receptor antagonists are also available for clinical use. These antagonists reversibly bind to, but do not stimulate, the GnRH receptor (i.e., competitive antagonism). Thus, these agents do not cause an initial "flare" of gonadotropin secretion, and they reduce gonadotropins more rapidly than GnRH agonists—usually within 24 to 72 hours.

GnRH Stimulation of Gonadotrope Cells

The specialized cells that synthesize and secrete gonadotropins (i.e., gonadotropes) are located mainly in the lateral

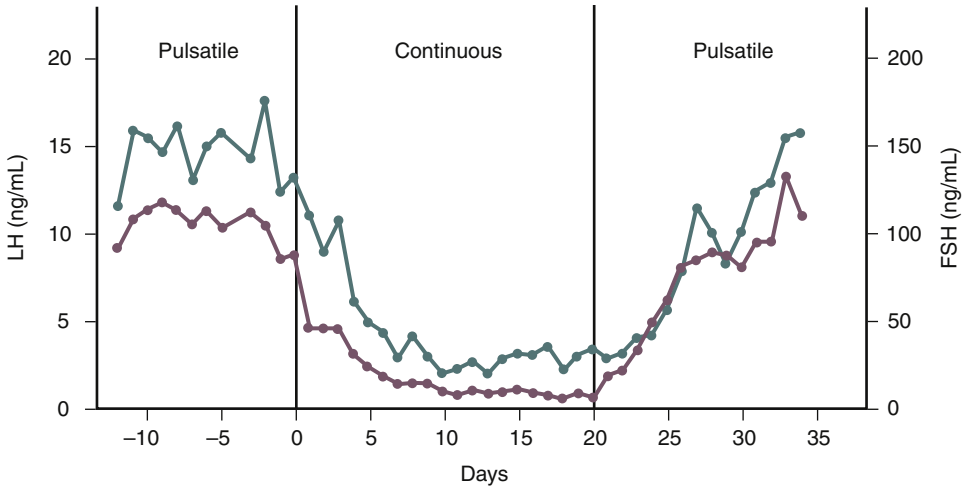


FIGURE 1.8 The influence of pulsatile vs. continuous GnRH administration to GnRH-deficient monkeys. Intermittent exogenous GnRH administration reconstitutes normal gonadotropin secretion. However, continuous GnRH infusion leads to a marked reduction (downregulation) of luteinizing hormone (green) and follicle-stimulating hormone (purple) concentrations. Resumption of pulsatile GnRH administration restores LH and FSH secretion. *(Adapted from Belchetz PE, Plant TM, Nakai Y, et al. Hypophysial responses to continuous and intermittent delivery of hypothalamic gonadotropin-releasing hormone.* Science *202:631–633, 1978.)*

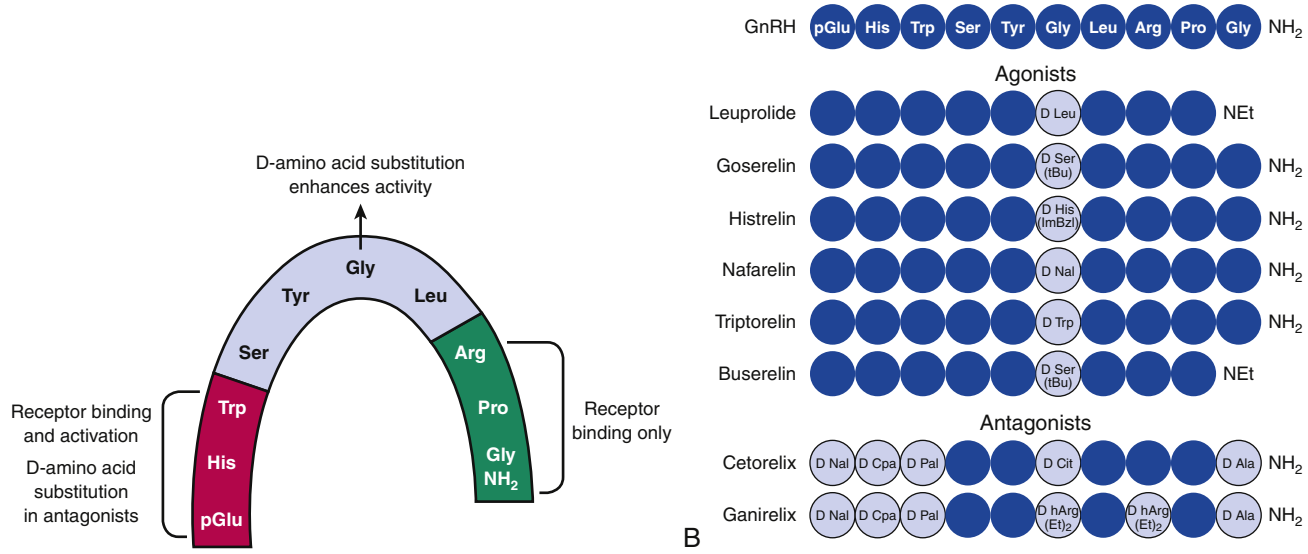


FIGURE 1.9 Structure of GnRH and GnRH receptor agonists and antagonists. **A,** Schematic of GnRH-1 in its folded conformation. Folding around the glycine in position 6 enhances GnRH receptor binding. Substitution of the glycine in position 6 with D-amino acids stabilizes the molecule in the folded conformation, which increases affinity for the GnRH receptor and reduces metabolic clearance. The amino-terminal (red) is involved with receptor binding and activation, and GnRH antagonists involve modifications of these residues that prevent receptor activation. The carboxyl-terminal (green) participates in receptor binding, but not activation. Substitution at position 10 (e.g., replacement of glycinamide by ethylamide) can increase binding affinity. **B,** Amino acid structure of GnRH along with selected GnRH receptor agonists and antagonists. Solid black circles represent amino acids that are unchanged compared to native GnRH. *(From Millar RP, et al. Gonadotropin-releasing hormone receptors.* Endocr Rev. *25:235–275, 2004.)*

portions of the anterior pituitary gland and constitute 7% to 10% of the adenohypophysis cell population. GnRH action at the pituitary gonadotrope begins with GnRH binding to the GnRH type I receptor on the plasma membrane.[8] The GnRH type I receptor is a member of the seven-transmembrane receptor family, a G protein-coupled receptor, and encoded on chromosome 4. GnRH receptor density varies in different physiological conditions and exhibits a positive correlation with gonadotrope responsiveness to GnRH (e.g., with both being high in rodents during preovulatory gonadotropin surges[35]). GnRH receptor density appears to be modulated primarily by GnRH, with intermittent GnRH stimulation leading to increased GnRH receptor expression; this is a central facet of the self-priming effect of GnRH, and an important mechanism by which GnRH action is modulated in different physiological states.

A majority of gonadotropes synthesize and secrete both LH and FSH. A detailed description of intracellular mechanisms of GnRH action on the gonadotrope is provided in Chapter 2. Briefly, GnRH receptor binding activates the guanosine

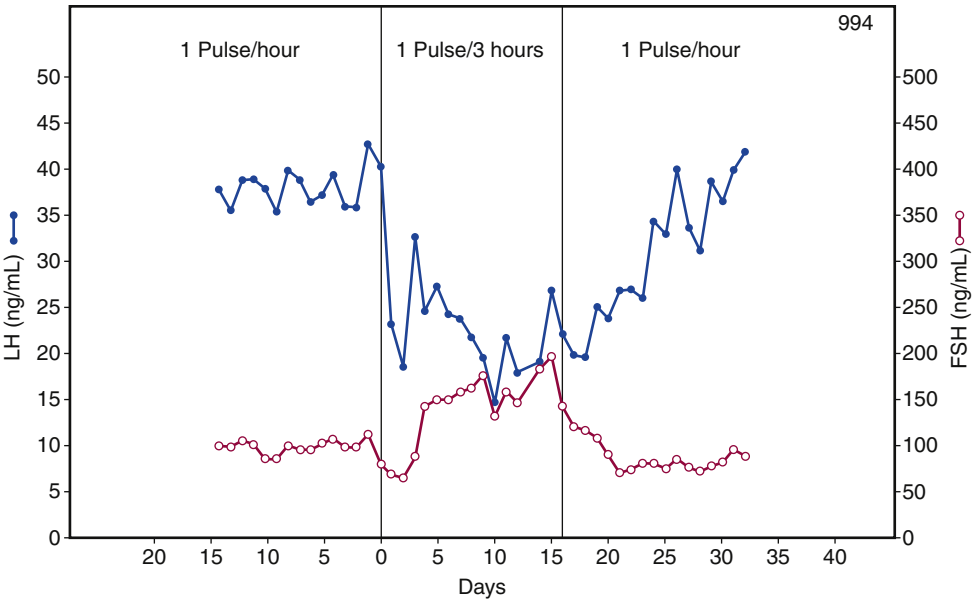


FIGURE 1.10 LH and FSH concentrations in gonadectomized (but sex steroid-replaced) monkeys after arcuate nucleus ablation—a model of isolated GnRH deficiency. Exogenous GnRH administered in a pulsatile fashion every hour reconstituted LH and FSH secretion. Changing GnRH pulse administration from a relatively high frequency (hourly) to a relatively low frequency (every 3 hours) resulted in decreased LH but increased FSH secretion. *(Adapted from Wildt L., et al., Frequency and amplitude of gonadotropin-releasing hormone stimulation and gonadotropin secretion in the rhesus monkey.* Endocrinol *109:376–385, 1981.)*

triphosphate (GTP)-binding protein $G_{q/11}$ leading to an increase of second messengers inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). Further intracellular signaling involves increased intracellular calcium and activation of various protein kinase C (PKC) isoforms, mitogen-activated protein kinases (e.g., extracellular signal-regulated kinase [ERK], c-Jun NH_2-terminal kinase [JNK], and p38), calcium/calmodulin-dependent kinase II (Ca/CaMK II), and adenylate cyclase.

Each gonadotropin consists of two protein subunits, α and β. The 92 amino acid α-subunit is common to both LH and FSH—in addition to human chorionic gonadotropin (hCG) and TSH. β-subunits for LH (LHβ) and FSH (FSHβ) are 121 and 117 amino acids in length, respectively, and account for the biological specificity of these two hormones. GnRH stimulates gene expression of LHβ, FSHβ, and α-subunit, and the latter noncovalently dimerizes with either LHβ or FSHβ to form LH or FSH, respectively. Gonadotropins also undergo variable post-translational modification, primarily glycosylation (addition of oligosaccharide moieties to specific amino acids), which influences bioactivity and elimination half-life.[36] The gonadotropins are then packaged into secretory granules for eventual secretion.

Although GnRH is the primary stimulus for LH and FSH synthesis and release from a common cell type, concentrations of these two gonadotropins vary differentially throughout ovulatory cycles, with FSH predominance in the early follicular phase and LH predominance in the late follicular phase. This sequential pattern of FSH and LH predominance is important for normal follicular maturation, ovarian steroid production, and subsequent ovulation. At least two mechanisms govern differential gonadotropin secretion throughout ovulatory cycles. First, both estradiol and inhibins selectively inhibit FSH release from gonadotropes during the midfollicular and luteal phases.[37,38] Second, different patterns of pulsatile GnRH release differentially affect gonadotropin synthesis and secretion. Specifically, rapid (high frequency) GnRH pulses favor LH, while slower (low frequency) GnRH pulses favor FSH synthesis and secretion. For example, studies in ovariectomized, GnRH-deficient monkeys

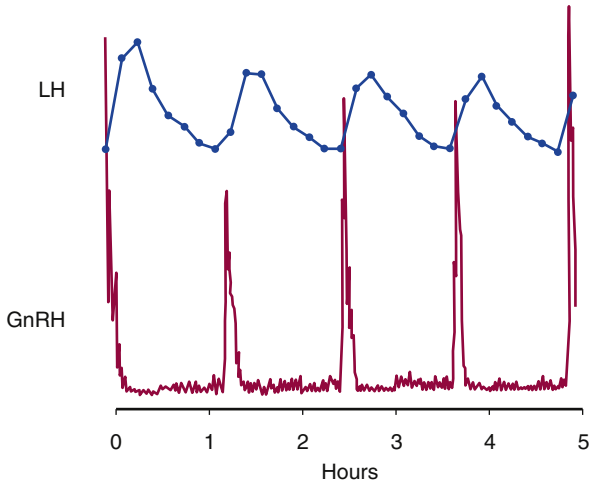


FIGURE 1.11 Close temporal relationship between pulses of LH (jugular vein) and GnRH (pituitary portal system) in the sheep model. *(Adapted from Moenter SM et al. Dynamics of gonadotropin-releasing hormone release during a pulse.* Endocrinology. *130:503–510, 1992.)*

reveal that a decrease in the frequency of exogenously-administered GnRH pulses from one pulse per hour to one pulse every 3 hours results in a 65% increase in plasma FSH, despite a 50% decrease in LH (Fig. 1.10).[33] Similar findings have been described in sheep[39] and humans.[40,41] Detailed studies in rats demonstrate that rapid GnRH pulse stimulation favors α-subunit and LHβ mRNA expression, while slow GnRH pulses favor FSHβ mRNA expression.[42] The mechanisms effecting differential LH and FSH expression in response to changes of GnRH pulse frequency include variations of GnRH receptor number on the gonadotrope cell surface[43] and alterations of gonadotrope activin β_B and follistatin expression (discussed later in the chapter).[44]

A pulse of GnRH release stimulates a pulse of LH release on a one-to-one basis, and LH (or α-subunit) pulse patterns, as assessed by frequent sampling of peripheral blood, accurately mirror GnRH pulse patterns in animal studies (Fig. 1.11).[31,45] Similarly, exogenous GnRH pulses elicit LH

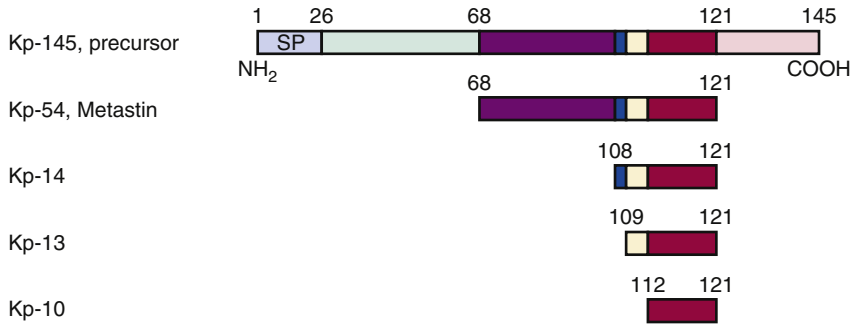


FIGURE 1.12 Schematic of the precursor kisspeptin-145 and the functional kisspeptin (Kp) fragments, including size and cleavage sites. Note that all functional kisspeptin fragments maintain amino acids 112-121. SP, signal peptide. *(From Roseweir AK, Millar RP. The role of kisspeptin in the control of gonadotrophin secretion.* Human Reproduction Update *15:203–212, 2009.)*

pulses in GnRH deficient patients. Since measurable GnRH is effectively confined to the hypophyseal portal system, which is inaccessible in humans, GnRH pulse frequency is inferred from LH pulse frequency (or α-subunit pulse frequency[46,47]) in human studies. While pulses of GnRH stimulate pulsatile release of FSH, the longer serum half-life of FSH renders FSH pulses more difficult to identify via frequent sampling of peripheral blood. Also, while short-term LH secretion is very closely tied to continued GnRH stimulation, FSH secretion is less acutely dependent on GnRH stimulation.[48,49] For example, with GnRH antagonism, the percentage reduction of LH exceeds that of FSH.[50]

Neuronal Inputs into GnRH Neurons

The governance of GnRH neurons is highly complex and involves numerous interacting neural systems utilizing various neurotransmitters and neuromodulators. The neuronal populations upstream of the GnRH neuron play key roles in puberty and are important mediators of sex steroid feedback and the influence of nutritional cues and stress on the GnRH pulse generator. Numerous neurotransmitters appear to be involved in the regulation of GnRH secretion, including dopamine, norepinephrine, glutamate, GABA, and nitric oxide. The control of GnRH secretion has been the subject of intense investigation, and the recent discovery of several neuronal populations upstream of the GnRH neuron (e.g., kisspeptin neurons) has markedly enhanced our understanding of reproductive neuroendocrinology.

Kisspeptin

The kisspeptin system is believed to be requisite for normal GnRH secretion, serving as a "gatekeeper" of puberty and helping to mediate the effects of sex steroids and metabolic cues on GnRH secretion. Kisspeptin was originally called metastin because of its ability to suppress metastatic spread of human melanomas and breast carcinomas. However, in recognition of its discovery at Pennsylvania State University in Hershey, Pennsylvania, it was later named kisspeptin after Hershey's chocolate KISSES®. Herein we will use the following abbreviations[51]: *KISS1* and *Kiss1*, the human and nonhuman kisspeptin genes, respectively; *KISS1R (Kiss1R)* and KISS1R (Kiss1R), the human (nonhuman) kisspeptin receptor genes and gene products, respectively.

The *KISS1* gene product is a 154 amino acid precursor protein (kisspeptin 1-145). Variable proteolytic modification yields kisspeptins of different lengths: kisspeptin-54, -14, -13, and -10, with the numbers referring to the amino acid length of bioactive kisspeptin fragments (Fig. 1.12). Importantly, all functional kisspeptins maintain the 10 amino acids of the carboxy-terminal (kisspeptin amino acids 112 to 121), which are important for receptor binding and function. Kisspeptin is the natural ligand of KISS1R—also known as the G-protein coupled receptor 54 (GPR54)—a seven transmembrane domain, G protein-coupled receptor.

The importance of the kisspeptin system in reproduction was initially revealed by members of two consanguineous families with *KISS1R* mutations leading to pubertal failure and normosmic hypogonadotropic hypogonadism.[52,53] Inactivating *KISS1* mutations leading to pubertal failure and normosmic hypogonadotropic hypogonadism have also been described in four sisters.[54] Mouse models with targeted knockouts of *Kiss1* and *Kiss1R* exhibit hypogonadotropic hypogonadism with impaired sexual maturation, reduced gonadal size, failure of estrous cyclicity (females), impaired spermatogenesis (males), and infertility.[53,55,56] However, the notion that kisspeptin is an absolute requirement for puberty and reproductive function in mice is somewhat controversial.[57,58] *KISS1R* and *KISS1* mutations neither interrupt GnRH neuron migration to the hypothalamus nor impair GnRH synthesis.

Single boluses of kisspeptin markedly stimulate LH release in rodents, sheep, monkeys, and humans. This effect of kisspeptin is mediated by stimulation of GnRH neurons, as supported by the following: kisspeptin fibers appear to project to and form synaptic contacts with GnRH neurons[59,60]; the kisspeptin receptor is expressed by a majority of GnRH neurons[61]; kisspeptin can directly depolarize GnRH neurons[62,63]; and kisspeptin stimulation of gonadotropin secretion is completely blocked by GnRH antagonists.[64,65] However, kisspeptin may also work indirectly, as kisspeptin increases GABAergic and glutamatergic postsynaptic currents onto GnRH neurons in the presence (but not absence) of estrogen in the mouse model.[66] Kisspeptin does not stimulate LH secretion in Kiss1R knockout mice,[56] suggesting that kisspeptin acts exclusively through its cognate receptor.

It remains unclear to what degree kisspeptin acts at GnRH cell bodies versus nerve terminals, but some studies suggest that kisspeptin neurons can form synapses with GnRH neuron terminals in the external zone of the median eminence, and that kisspeptin can stimulate GnRH release (exocytosis) from GnRH neuron terminals.[67,68] Although kisspeptin may possibly have direct effects on gonadotropes, available data suggest that this does not play a major role in kisspeptin's ability to stimulate gonadotropin secretion. For example, pulsatile GnRH can restore normal reproductive function in patients with *KISS1R* mutations.[69]

The numbers of kisspeptin neurons are high in the human arcuate (infundibular) nucleus, similar to findings in monkeys.[65,70,71] Extensive study in the rodent model discloses two primary populations of kisspeptin-expressing neurons in the hypothalamus: one in the arcuate nucleus (mediobasal hypothalamus) and the other in the anteroventral periventricular nucleus (AVPV) of the preoptic area.[72] Of interest, kisspeptin expression in the AVPV is much higher in female compared to male rodents, which appears to reflect organizational effects of sex steroids during early development[73,74]; and the kisspeptin neurons in the AVPV appear to be specifically important for LH surge generation in rodents. Sexual dimorphism of kisspeptin expression has also been described in sheep[75] and humans.[71] However, in primates, including humans, the majority of the kisspeptin cell bodies reside in the arcuate nucleus; and although a study of adult women revealed rare kisspeptin neurons in the medial preoptic area, a population homologous to the rodent AVPV has not been identified.[70]

Neurokinin B

Neurokinin B (NKB), a peptide encoded by the tachykinin 3 gene (*TAC3*), is a member of the tachykinin family—a family that also includes substance P and neurokinin A (products of the *TAC1* gene). There are several neurokinin receptors (NK1R, NK2R, NK3R), and although NKB can produce some agonism at NK1R and NK2R, NKB binds preferentially to and acts primarily via its cognate receptor NK3R (*TACR3* gene).[76] Studies of patients with idiopathic hypogonadotropic hypogonadism from consanguineous families revealed that homozygous loss-of-function mutations of either *TAC3* or *TACR3* can cause pubertal failure and severe hypogonadotropic hypogonadism—highlighting the importance of NKB in human reproduction.[77,78] In contrast to *Kiss1* and *Kiss1R* knockout mice, *Tacr3* knockout mice remain fertile, although they can demonstrate reproductive defects.[79,80]

Animal studies demonstrate that a selective NK3R agonist (senktide) stimulates LH secretion in the rat,[81] sheep,[82] and monkey,[83] albeit not as potently as kisspeptin. This effect appears to be influenced by the sex steroid milieu. For example, senktide administration to ewes increases LH secretion during the follicular phase, but not during the luteal phase.[82] In addition, senktide increases LH release in the presence of physiological estradiol in rodents, but it reduces LH release in estradiol-deficient animals.[81] Mechanisms underlying these apparently paradoxical observations are unclear.

Stimulation of LH secretion by NKB is mediated by GnRH secretion, as GnRH receptor antagonism abolishes LH responses to senktide in the monkey.[83] Yet, there are few or no NKB receptors on GnRH neurons, and LH is not rapidly stimulated with central senktide administration. However, kisspeptin neurons express NK3R; and in ovariectomized and estradiol-replaced rats, senktide increases c-fos expression in kisspeptin neurons.[81] Additionally, desensitization of Kiss1R markedly reduces GnRH responsiveness to senktide in monkeys, while similar desensitization of NK3R does not impair GnRH responsiveness to kisspeptin.[84] Moreover, a recent study suggests that continuous kisspeptin infusion can restore pulsatile LH secretion in patients with loss-of-function mutations of *TAC3* or *TACR3*.[85] Taken together, these studies support the contention that NKB primarily influences pulsatile GnRH secretion indirectly by stimulating kisspeptin release.[86,87]

Endogenous Opioid Peptides

Endogenous opioid peptides (EOP), which include endorphins, enkephalins, and dynorphins, participate in myriad processes such as motor activity, cognitive functions, water and food intake, and regulation of neuroendocrine function.[88] Most active EOPs share a common sequence (Tyr-Gly-Gly-Phe-[Met or Leu]) at the amino-terminal, although endorphins, enkephalins, and dynorphins are derived from different precursor proteins that undergo regulated post-translational processing (Fig. 1.13).[89] Endorphins such as β-endorphin are products of the precursor protein proopiomelanocortin (POMC). POMC can be preferentially processed to produce adrenocorticotropin hormone (ACTH) and β-lipotropin, as occurs in corticotropes (adenohypophysis) under the control of corticotropin-releasing hormone (CRH). However, in the hypothalamus, POMC processing primarily yields β-endorphin and α-melanocyte-stimulating hormone. Hypothalamic β-endorphin participates in the regulation of reproduction, temperature, and cardiovascular and respiratory functions, and it acts primarily via μ (micro)-opioid receptors. Enkephalins are derived from proenkephalin, and their primary functions appear to relate to autonomic nervous system modulation, mainly via δ (delta)-receptor activation. Dynorphins are products of the precursor prodynorphin and act chiefly at κ (kappa)-opioid receptors. Importantly, while β-endorphin, enkephalins, and dynorphins acts primarily via μ-, δ-, and κ-opioid receptors, each can act as agonists at more than one receptor subtype.

Numerous studies provide evidence that hypothalamic opiates partly mediate sex steroid negative feedback on GnRH release. For example, GnRH neurons express few if any progesterone receptors, but β-endorphin concentrations increase in hypophyseal blood during the luteal phase—when sex steroids suppress GnRH secretion—in monkeys.[90,91] Moreover, naloxone and naltrexone (opiate receptor antagonists acting primarily at μ- and κ-opioid receptors) increase LH pulse frequency when administered to luteal phase women[92] or progestin-treated postmenopausal women.[93] Similarly, morphine suppresses GnRH secretion from mediobasal hypothalami isolated from fetal and adult humans—an effect that is reversed by naloxone[94]; and chronic high-dose opiate administration can cause hypogonadotropic hypogonadism by suppressing GnRH and LH secretion.[88]

Several animal studies implicate dynorphin as a principal mediator of progesterone negative feedback on GnRH pulse frequency in females.[95] For example, dynorphin neurons in the arcuate nucleus co-localize with progesterone receptors in ewes,[96] and dynorphin-containing varicosities are closely associated with GnRH neuron cell bodies in the mediobasal hypothalamus.[97] Progesterone treatment in ewes increases dynorphin A concentrations in third ventricle cerebrospinal fluid,[98] and central infusion of dynorphin in goats reduces volleys of multiple-unit activity in the mediobasal hypothalamus and reduces LH pulses.[87] In luteal phase ewes, specific κ-opioid receptor antagonists—but not antagonists to δ- or μ-opioid receptors—reversed progesterone inhibition of LH secretion and LH frequency when locally administered into the mediobasal hypothalamus.[97] However, other EOPs (e.g.,

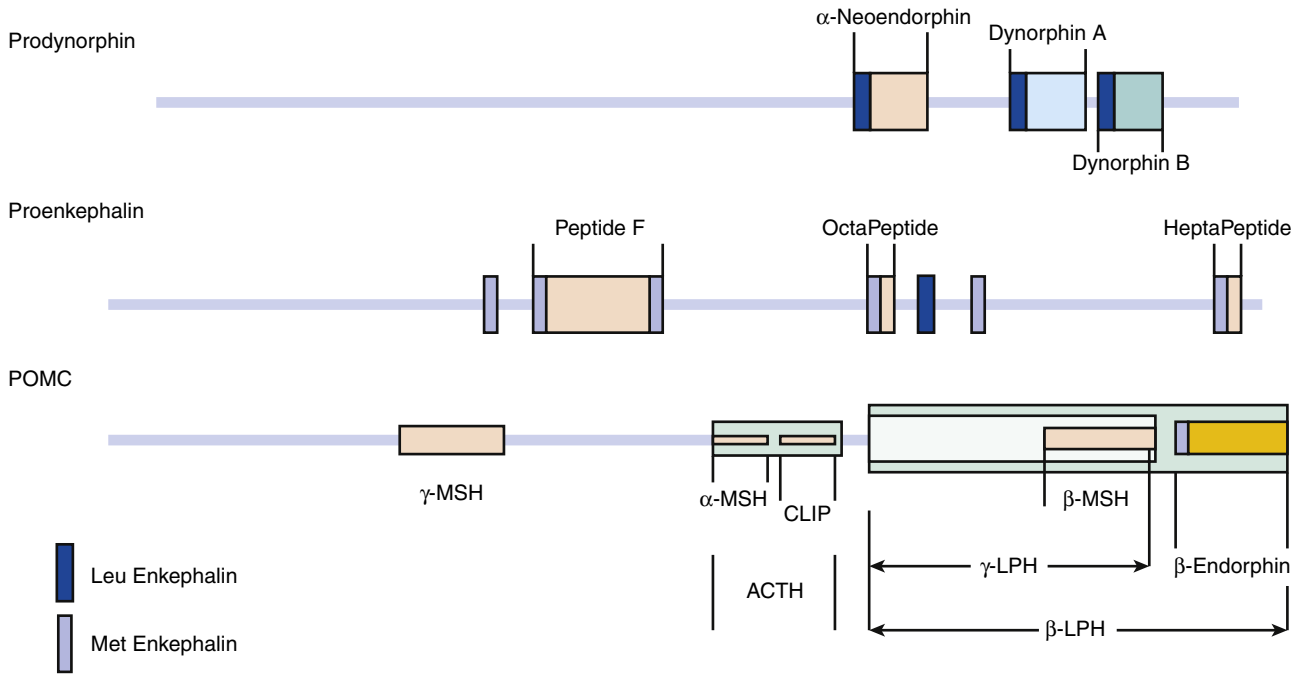


FIGURE 1.13 Schematic of endogenous opiate precursors. POMC, proopiomelanocortin; MSH, melanocyte-stimulating hormone; CLIP, corticotropin-like intermediate lobe peptide; ACTH, adrenocorticotropic hormone; LPH, lipotropin. *(From Akil H, et al. Endogenous opioids: overview and current issues.* Drug Alcohol Depend. *51:127–140, 1998.)*

β-endorphin) in other hypothalamic areas may be involved as well; for example, in the aforementioned study,[97] κ- and μ-receptor antagonists locally administered into the preoptic area increased LH and LH pulse frequency.

Kisspeptin, Neurokinin B, Dynorphin (KNDy) Neurons

In the arcuate nucleus, kisspeptin, NKB, and dynorphin are frequently coexpressed in the same neuron. For example, kisspeptin neurons in the arcuate nucleus have been found to coexpress NKB and dynorphin in mice,[86] goats,[87] and sheep.[99] For convenience, and as a playful nod to kisspeptin (namesake of Hershey's chocolate KISSES®), such neurons are increasingly being called KNDy neurons (Kisspeptin, Neurokinin B, Dynorphin). Available data from human autopsy studies are consistent: 77% of kisspeptin cell bodies (and 56% of kisspeptin axon fibers) in the arcuate (infundibular) nucleus coexpress NKB.[71]

Kisspeptin, Neurokinin B, Dynorphin (KNDy) neurons in the arcuate nucleus form an extensively interconnected network surrounding the third ventricle. KNDy axons also appear to project to the median eminence: in rats, Kiss1/NKB fibers from the arcuate nucleus project to the internal zone of the median eminence where they are in close proximity to GnRH fibers.[100] As with kisspeptin neurons, KNDy neuron neuroanatomy exhibits sexual dimorphism in sheep, possibly related to perinatal sex steroid exposure.[75]

As discussed further below, KNDy neurons appear to be intimately involved with sex steroid feedback on GnRH secretion, and some investigators have suggested that the KNDy neuronal network represents a fundamental component of the GnRH pulse generator, with NKB stimulating and dynorphin inhibiting kisspeptin release.

Gonadotropin-Inhibitory Hormone

The role of gonadotropin-inhibitory hormone (GnIH) and its orthologues, also called RF-amide related peptides (RFRP), in the central control of reproduction has been recently reviewed.[101] Briefly, GnIH immunoreactive cells have been identified in hypothalami of a number of species including monkeys, and GnIH-immunoreactive fibers can be found in close proximity to GnRH neurons and in the median eminence. GnIH can reduce GnRH neuronal activity, and GnIH also appears to have hypophysiotropic actions to directly inhibit pituitary gonadotropin release. A recent study in sheep revealed reduced GnIH expression in the preovulatory period in ewes, suggesting a reciprocal relationship with GnRH release, and infusion of GnIH blocked the estrogen-induced LH surge.[102] GnIH has also been implicated in the regulation of food intake (increase), sexual motivation (decrease), and the influence of stress on reproduction. In sum, a growing body of data suggests that GnIH is an important factor controlling GnRH and gonadotropin secretion in a number of animal species, although an understanding of its role in humans awaits further investigation.

The GnRH Pulse Generator

As described above, intermittent GnRH receptor stimulation is an absolute requirement for physiological maintenance of gonadotropin secretion. Although the precise basis of pulsatile GnRH release remains unclear, a number of observations support the concept that neuronal systems within the mediobasal hypothalamus effect pulsatile release of GnRH into the hypophyseal portal system. In animal models, volleys of multiple unit electrical activity (i.e., detection of activity in multiple neurons near an electrode)

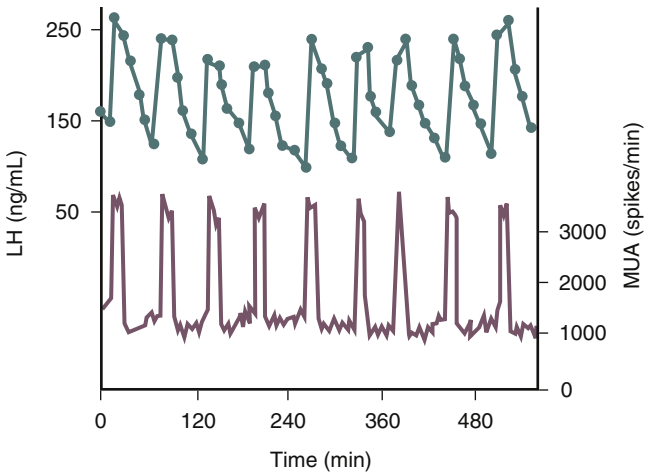


FIGURE 1.14 Temporal association between volleys of multiple unit activity (MUA) in the hypothalamus and luteinizing hormone (LH) pulses (green) detected in peripheral blood in an ovariectomized monkey. *(Adapted from Knobil E. The electrophysiology of the GnRH pulse generator in the rhesus monkey.* J Steroid Biochem *33:669–671, 1989.)*

in the mediobasal hypothalamus coincide with the initiation of LH pulse secretion (Fig. 1.14).[103] Similarly, electrical stimulation via electrodes placed in the mediobasal hypothalamus stimulates GnRH release into the hypophyseal portal system in monkeys.[104] Mediobasal hypothalami isolated from fetal (20 to 23 weeks gestation) and adult humans release GnRH in discrete pulses, with a frequency of approximately one pulse per 60 to 100 minutes[94]; and mediobasal hypothalami separated from the remainder of the brain can maintain pulsatile LH secretion in monkeys.[105] Lastly, selective radiofrequency ablation of the arcuate nucleus (part of the mediobasal hypothalamus) in adult female monkeys obliterates gonadotropin secretion.[106] These data suggest that the mediobasal hypothalamus, the arcuate nucleus in particular, houses all requisite components for GnRH pulse generation (i.e., the GnRH pulse generator) and that pulsatile GnRH release does not require innervation from outside of the mediobasal hypothalamus. Nonetheless, mechanisms underlying episodic GnRH pulse generation, and what neuroanatomical components constitute the GnRH pulse generator, are uncertain.

Several studies suggest that pulsatility is an intrinsic property of GnRH neurons. For example, pulsatile GnRH release is exhibited by immortalized GnRH-secreting neurons[107,108] and by cultured GnRH neurons obtained from fetal rats, sheep, and monkeys.[109-111] If GnRH pulse generation is an intrinsic property of GnRH neurons, then coordination of GnRH release could be facilitated by cell-to-cell interconnections among GnRH neurons.[112,113]

Recent data suggest that afferent inputs (e.g., kisspeptin neurons) are important for normal GnRH secretion, and some investigators have suggested that kisspeptin (KNDy) neurons represent a key component of the GnRH pulse generator, with NKB and dynorphin influencing kisspeptin stimulation of GnRH neurons. As described above, volleys of multiunit activity in the arcuate nucleus are temporally associated with LH pulses; however, in addition to GnRH neurons, the arcuate nucleus contains numerous kisspeptin (KNDy) neurons. Also, kisspeptin release into the hypophyseal portal circulation appears to be pulsatile in sheep and monkeys. Although these kisspeptin pulses were not clearly coincident with peripheral LH pulses in ovariectomized ewes,[114] kisspeptin pulses occurred approximately once per hour and corresponded to GnRH pulses 75% of the time in midpubertal rhesus monkeys.[115]

Although kisspeptin administration did not influence the frequency of LH pulses or volleys of multiple unit electrical activity in the arcuate nucleus in one rat study,[116] administration of a selective kisspeptin antagonist into the arcuate nucleus suppressed LH pulse frequency in another.[117] Also, central administration of dynorphin in goats inhibits both MUA volleys in the mediobasal hypothalamus and pulsatile LH release, whereas NKB provokes MUA volleys.[87] Human studies also imply that kisspeptin may play a role in the GnRH pulse generator. For example, continuous intravenous infusion of a relatively low-dose of kisspeptin can increase LH pulse frequency in adult men.[118] Another recent study in adult men suggested that a single injection of kisspeptin may reset the GnRH pacemaker.[119] In the latter study, the interval between the kisspeptin-induced LH pulse and the immediately preceding endogenous LH pulse was variable, but on average shorter than the normal LH interpulse interval (as expected). In contrast, the interval between the kisspeptin-induced LH pulse and the subsequent endogenous LH pulse was similar to normal interpulse intervals (circa 2 hours), suggesting that kisspeptin administration reset the hypothalamic GnRH clock.

Hypothetical models regarding how KNDy neurons may be involved with GnRH pulse generation are shown in Figure 1.15. However, some data suggest that kisspeptin may not be requisite for GnRH pulse generation. In particular, frequent sampling studies reveal that humans with *KISSR* mutations demonstrate pulsatile LH release, albeit at low amplitude.[53,120] Similarly, a recent study suggested that puberty occurs and fertility is preserved in female mice with either (a) congenital absence of kisspeptin neurons or (b) congenital absence of neurons expressing Kiss1R.[57] When taken as a whole, available data imply that kisspeptin action may not be an absolute requirement for pulsatile GnRH secretion, but that kisspeptin is required for normal GnRH pulse secretion and normally exerts an important influence on GnRH pulse generation.

Physiologic Development of Reproductive Neuroendocrine Function

Patterns of GnRH secretion change markedly across human development. Reproductive neuroendocrine events throughout early maturation, including both before and during the establishment of reproductive competence, are discussed in detail in Chapter 18. Briefly, GnRH and gonadotropin secretion is robust in utero, peaking in midgestation. In males, gonadotropin secretion markedly stimulates testicular androgen secretion, which is important for normal genital differentiation. The gestational increase of sex steroid (e.g., estradiol) production from the fetoplacental unit provides negative feedback to limit fetal GnRH and gonadotropin secretion. Birth is followed by a marked but transient (3 to 9 month) increase of GnRH and gonadotropin secretion (the "mini-puberty of infancy"), perhaps related to the withdrawal of fetoplacental sex steroids.

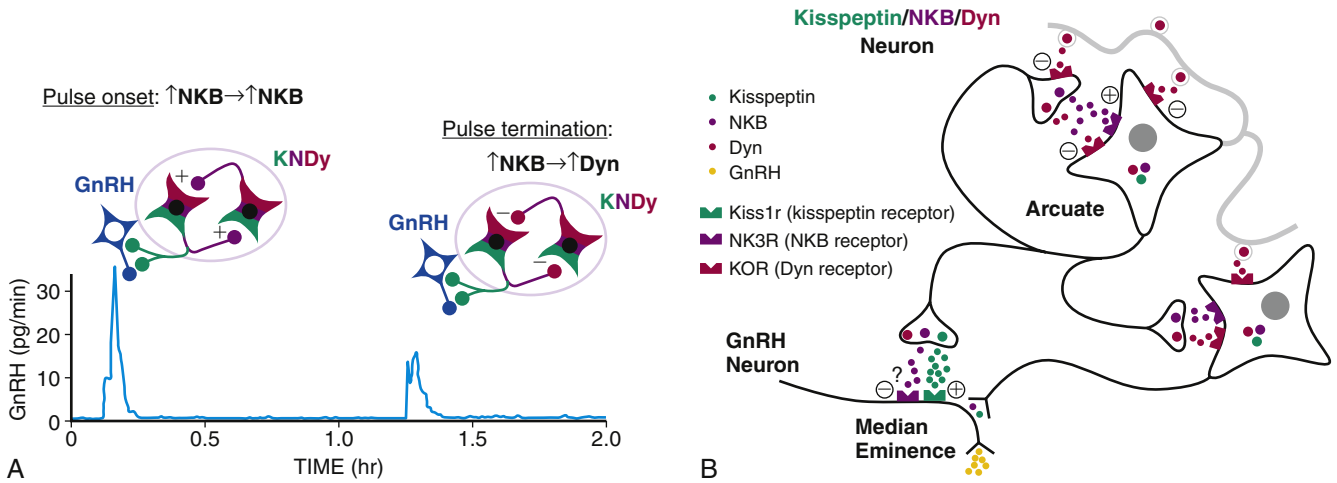


FIGURE 1.15 Working model regarding how KNDy neurons may participate in the generation of GnRH pulses proposed by Lehman and colleagues **(A)** and Wakabayashi and colleagues **(B)**. **A,** By this model, neurokinin B (NKB, magenta) stimulates and dynorphin (DYN, red) suppresses kisspeptin release, with kisspeptin (green) stimulating GnRH neuronal firing. The onset of a GnRH pulse is triggered by an initial increase of NKB, which stimulates further NKB release (positive feedback loop) and increases kisspeptin output. NKB stimulation of KNDy neurons also stimulates DYN release; and after a short period of time, the increase of DYN suppresses kisspeptin (and NKB) release. This withdrawal of kisspeptin stimulation terminates the GnRH pulse. *(From Lehman MN, Coolen LM, Goodman RL. Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion.* Endocrinology. *151:3479–3489, 2010.)* **B,** By this model, KNDy neurons in the arcuate nucleus form a neural circuit, within which neurokinin B (NKB, magenta) accelerates and dynorphin (Dyn, red) reduces KNDy neuron activation. These reciprocal effects of NKB and Dyn produce episodic activation of KNDy neurons, with KNDy neuronal activation increasing kisspeptin release at the median eminence. Kisspeptin in turn stimulates GnRH release into the hypophyseal portal system. KOR, kappa opiate receptor. *(Adapted from Lehman MN et al. Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion.* Endocrinology, *151:3479-3489, 2010; and Wakabayashi Y et al. Neurokinin B and dynorphin A in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat.* J Neurosci. *30:3124-3132, 2010.)*

A marked sex difference of gonadotropin release is evident at this time, with LH concentrations being higher in males and FSH levels higher in females. The possibility that kisspeptin is important for the minipuberty of infancy is suggested by a patient with a compound heterozygote mutation of *KISSR*, who had micropenis, undescended testes, and undetectable serum gonadotropins at 2 months of age—a time usually marked by robust gonadotropin secretion.[121]

By late infancy or early childhood (earlier in boys than in girls), GnRH and gonadotropin secretion markedly decreases, leading to a hypogonadotropic phase of childhood marked by low sex steroid concentrations—the "juvenile pause." Studies of gonadotropin secretion in children reveal low LH and FSH concentrations, a high FSH-to-LH ratio, and low LH pulse amplitude and frequency.[122] Mechanisms accounting for low GnRH secretion during this time appear to include inhibition of the GnRH pulse generator ("neurobiological brake") by higher-order neuronal systems (e.g., involving γ-aminobutyric acid [GABA]- and neuropeptide Y-secreting neurons) and a developmental removal of stimulation (e.g., involving neurons secreting glutamate and norepinephrine).

Near the close of the first decade, a marked nocturnal amplification of pulsatile LH secretion indicates the neuroendocrine beginnings of puberty. A majority of studies suggest that early pubertal subjects demonstrate sleep-entrained increases of LH (GnRH) pulse frequency and amplitude.[123] Gonadotropin concentrations rise across puberty,[124,125] stimulating gametogenesis, gonadal sex steroid secretion, and the development of secondary sexual characteristics. Mechanisms underlying puberty are poorly understood, but likely reflect developmental remodeling of inhibitory and stimulatory neural circuits in the hypothalamus. For example, puberty has been associated with reductions of GABAergic inhibitory neurotransmission and an increase of excitatory neurotransmitters such as glutamate. Kisspeptin and NKB also appear to play important roles in human puberty, as inactivating mutations of *KISS1*, *KISS1R*, *TAC3*, or *TACR3* result in pubertal failure; and a gain of function *KISS1R* mutation has been reported as a cause of central precocious puberty.[126] In addition to these transsynaptic mechanisms, neuroglial cells may contribute to the pubertal reactivation of GnRH secretion (e.g., by secretion of growth factors).[1]

Patterns of Pulsatile GnRH Secretion in Adults

Human studies employing frequent blood sampling and pulse detection analysis have documented significant changes of LH (and by inference GnRH) pulse frequency throughout ovulatory cycles. Briefly, average LH (GnRH) pulse frequency is around one pulse every 90 minutes in the early follicular phase, and this gradually increases to approximately one pulse per hour by the late follicular phase. Although monkey studies suggest that GnRH pulse frequency slows during the mid-cycle surge,[127] human studies suggest no change in either LH or α-subunit pulse frequency at midcycle.[128,129] LH pulse frequency slows markedly during the luteal phase, approximating one pulse every 3 to 8 hours. These day-to-day changes of GnRH pulse frequency appear to be important for normal hormonal changes across ovulatory cycles.[130,131]

In adult humans and nonhuman primates, GnRH pulses occur approximately once per hour in the (near) absence of sex steroid negative feedback (e.g., after surgical or natural menopause).[132-134] Similarly, the isolated human mediobasal hypothalamus secretes GnRH pulses every 60 to 100 minutes,[94] and LH pulses do not appear to exceed a once hourly frequency during any phase of the cycle.[133,135] These findings have contributed to the concept that a pulse frequency of approximately one per hour (a "circhoral" frequency) may be an inherent characteristic of the adult GnRH pulse generator, and that day-to-day changes of GnRH pulse frequency in women reflect the imposition or removal of sex steroid (primarily progesterone) negative feedback.

LH pulse amplitude also changes across the menstrual cycle. LH pulse amplitude decreases slightly across much of the follicular phase, but it is greatly amplified at the midcycle LH surge. During the luteal phase, LH pulse amplitude is variable, but in general approximately twofold higher than that of the follicular phase. It is important to note that the amplitude of LH pulses can be modulated centrally via changes of GnRH released per pulse, at the pituitary gonadotrope via changes of gonadotrope responsiveness to GnRH, or both. Also of interest, LH pulse amplitude varies inversely with the preceding LH interpulse interval.[136]

In women, dynamic changes of gonadotropin secretion are required to achieve follicular development, ovulation, and preparation for possible pregnancy. In contrast, young men demonstrate consistent daily patterns of GnRH and gonadotropin secretion, with GnRH pulse frequency approximating 1 pulse every 2 hours. This achieves continuous spermatogenesis, and healthy men are prepared for fertilization at all times. Additionally, day-to-day testosterone secretion remains relatively constant, although testosterone concentrations may exhibit diurnal changes with peaks in the morning.

Feedback Regulation of GnRH and Gonadotropin Secretion

After puberty, gonadal hormones continually relay information about the state of gonadal function to the hypothalamo-pituitary unit. Hypothalamic areas involved with the regulation of GnRH secretion (and pituitary gonadotropes) express receptors for estrogen, progesterone, and androgen; and sex steroid feedback plays a predominant role in the physiological modification of GnRH and gonadotropin secretion. These steroid feedback signals can in turn alter gonadotropin feedforward signals to the gonads by influencing GnRH secretion, modulating pituitary (gonadotrope) responses to GnRH, or both. Under normal circumstances, these regulatory feedback loops maintain appropriate gonadal function. The negative feedback actions of pharmacological doses of sex steroids (e.g., combined oral contraceptives) suppress gonadotropins and can be employed for temporary contraception in women; similar strategies are being developed for men.

Negative Feedback Regulation of GnRH and Gonadotropin Secretion in Women

In women, estradiol concentrations correspond to follicular development during the follicular phase and corpus luteum function in the luteal phase. When concentrations are relatively low (i.e., excluding preovulatory concentrations), estrogens inhibit gonadotropin secretion. This effect is most dramatically illustrated by markedly increased gonadotropin secretion in states of estrogen deficiency (i.e., the open loop condition) such as menopause[137] and aromatase deficiency.[138] The negative feedback effects of estradiol appear to be mediated primarily at the hypothalamus.[139] GnRH release (by direct measurement) is increased in ovariectomized sheep and monkeys, and this is reversed with estrogen replacement.[140,141] In human studies, GnRH release can be estimated using GnRH antagonists, with the premise that the degree of LH suppression after incomplete GnRH antagonism is inversely related to endogenous GnRH secretion. In postmenopausal women, the percent reduction of LH concentrations after incomplete GnRH receptor blockade is increased by estradiol replacement.[142]

Overall, studies indicate that estradiol reduces GnRH pulse amplitude, but not GnRH pulse frequency.[141,143] Although one study in ovariectomized monkeys suggested that estradiol reduces the frequency of both hypothalamic multiple unit electrical activity and LH pulses,[144] available studies in postmenopausal women imply that estrogen replacement primarily reduces LH pulse amplitude rather than LH pulse frequency.[134,145] In addition, LH pulse frequency is maximal at 1 pulse per hour during the late follicular phase in women, when estradiol concentrations are relatively high.

Estrogens may also decrease pituitary LH responses to GnRH, although available data are mixed. For example, although estradiol acutely reduces LH release in GnRH-deficient monkeys and sheep receiving fixed-dose exogenous pulsatile GnRH,[146,147] relatively low (i.e., not preovulatory) doses of estradiol do not markedly influence LH release in GnRH-deficient women receiving fixed-dose exogenous pulsatile GnRH.[37] Interestingly, initial reductions of LH release with estradiol may be followed by increased LH release[148]; this biphasic pattern presumably reflects initial negative feedback and later positive feedback.

Progesterone is the primary negative feedback regulator of day-to-day GnRH pulse frequency in women. LH pulse frequency slows in tandem with increases of progesterone (from the corpus luteum) in the early luteal phase,[135] and LH pulse frequency is inversely correlated with progesterone (but not estradiol) concentrations during the luteal-follicular transition.[149] Moreover, administration of progesterone to women during the follicular phase, when progesterone concentrations are usually low, slows LH pulse frequency.[150] Similarly, progesterone plus low-dose estradiol (but not estradiol alone) slows LH frequency in postmenopausal women.[134,145] Importantly, the ability of progesterone to slow GnRH pulses appears to require the permissive presence of estradiol,[151,152] which likely reflects the ability of estrogen to increase hypothalamic progesterone receptor expression.[153,154] In contrast, progesterone inhibition of GnRH pulse frequency appears to be antagonized by androgens. For example, androgens increase GnRH neuronal firing rates in the mouse model[155]; and in hyperandrogenic women with polycystic ovary syndrome (PCOS), high GnRH pulse frequencies are relatively resistant to negative feedback suppression by progesterone and estradiol[156]—a defect that can be reversed by androgen receptor blockade.[157] These findings may be a consequence

of androgen-mediated reductions of hypothalamic progesterone receptor expression.[158]

Positive Feedback and the Mid-Cycle Gonadotropin Surge

Most examples of endocrine feedback regulation involve negative feedback loops. However, the ovulatory menstrual cycle is unique in that it also depends on the positive feedback effects of sex steroids on the hypothalamic-pituitary unit. Specifically, high estradiol concentrations from the dominant ovarian follicle can produce a marked increase in gonadotropin release—a midcycle (or preovulatory) gonadotropin surge. In effect, estradiol from the preovulatory follicle signals to the hypothalamo-pituitary unit that follicular development is adequate for ovulation. Estradiol positive feedback appears to be related to both achieved estradiol concentrations and the duration of estradiol elevation, as demonstrated in both monkeys[159] and women.[160] Although the midcycle surge is characterized by a marked discharge of both gonadotropins, the increase of LH release exceeds that of FSH, with blood concentrations increasing approximately tenfold vs. fourfold, respectively; thus, it is often called the LH surge.

The mid-cycle gonadotropin surge uniformly involves positive feedback at the pituitary, markedly increasing gonadotrope responsiveness to GnRH stimulation.[161,162] However, whether the gonadotropin surge involves positive feedback at the hypothalamus is likely species dependent. GnRH secretion is augmented during the LH surge in rats[163] and sheep[164]; such "GnRH surges" appear to be physiologically important in these species. Similarly, GnRH release appears to increase in response to estradiol positive feedback in female monkeys.[165,166] However, LH surges can be induced by high estradiol concentrations in GnRH-deficient monkeys receiving constant dose (exogenous) GnRH delivered as once hourly pulses,[167] implying that a GnRH surge is not essential for LH surge generation in these animals.

One study using incomplete GnRH receptor antagonism to estimate GnRH secretion in women suggested that GnRH secretion may actually be reduced at midcycle compared to the late follicular and early luteal phases.[168] Similarly, pulsatile administration of constant-dose exogenous GnRH produces LH surges in GnRH-deficient women[162]; indeed, LH surges can occur in such women even when pulsatile GnRH doses are reduced at midcycle.[169] Also, while pituitary metabolic activity (by positron emission tomography) increases in women at midcycle, hypothalamic metabolic activity does not.[170] Thus, although continued GnRH stimulation plays a critically important permissive role in LH surge generation in women (e.g., the surge can be prevented with GnRH receptor antagonists[171,172]), available data do not suggest that the gonadotropin surge is accompanied by increased GnRH release in women.

Progesterone increases pituitary gonadotropin responses to GnRH[173,174]; when given in the luteal phase, the progesterone receptor antagonist mifepristone decreases mean LH and LH amplitude[175,176] and reduces LH responses to exogenous GnRH.[176] Nonetheless, progesterone by itself is unable to induce gonadotropin surges; indeed, progesterone can block LH surge generation when administered before estradiol in postmenopausal women.[177] However, progesterone augments gonadotropin secretion in estradiol-primed women.[160,178] Although estradiol alone can provoke an LH surge, the late follicular rise in progesterone, which begins approximately 12 hours before the LH surge,[160,179] may be important for the full expression of the midcycle gonadotropin surge. For example, progesterone may increase the duration of the surge,[160] and the progesterone-receptor antagonist mifepristone can delay the surge.[180] Some studies suggest that progesterone may be important for the increase of FSH at midcycle,[177,178] while others suggest that estradiol alone is sufficient to produce an FSH surge.[160,181]

Of interest, although humans demonstrate sexual dimorphism of hypothalamic neuronal populations (e.g., kisspeptin), the circuitry required for LH surge-like activity appears to be present in male primates. For example, in male monkeys orchiectomized after puberty, estradiol administration can induce LH surges,[182] and ovarian transplants can induce LH surges and other neuroendocrine changes that maintain cyclic function of the transplanted ovary.[183] Estradiol and progesterone positive feedback can be experimentally induced in adult men,[184,185] but this is not a normal occurrence in male physiology.

Negative Feedback Regulation of GnRH and Gonadotropin Secretion in Men

In contrast to cyclic changes in women, normal postpubertal males demonstrate a relatively constant average LH pulse frequency of approximately one pulse every 120 minutes, related to relatively stable day-to-day sex steroid concentrations and corresponding negative feedback effects (tonic inhibition). In male monkeys, bilateral orchiectomy increases mean LH, LH pulse frequency, and LH pulse amplitude—effects that are prevented by physiologic testosterone replacement.[186] Similarly, testosterone deficient men (e.g., related to either primary testicular failure or "medical castration" with ketoconazole) exhibit elevated mean LH, LH pulse frequency, and LH pulse amplitude; changes that are at least partially reversed by testosterone replacement.[187-189]

The importance of the androgen receptor in mediating testosterone negative feedback of LH secretion is suggested by elevated LH concentrations in the setting of androgen insensitivity[190] and androgen receptor blockade.[191,192] Moreover, mean LH is reduced in men by administration of dihydrotestosterone (DHT), a potent androgen that cannot be aromatized to estradiol.[193-195] However, a portion of synthesized testosterone is aromatized to estradiol, either in testicular Leydig cells or in nongonadal tissues, and estrogens can exert negative feedback actions at the hypothalamo-pituitary unit. For example, estradiol administration reduces LH secretion in normal and agonadal men.[191,193,196,197] Taken together, these findings suggest that both androgens and estrogens exert negative feedback actions at the hypothalamo-pituitary unit. However, the relative roles of androgen and estrogen binding at their respective receptors at the hypothalamus and anterior pituitary remain controversial.

Many studies suggest that sex steroid negative feedback on the GnRH pulse generator is partly mediated by the androgen receptor. For example, DHT can reduce LH pulse frequency in men,[195] and some[191,192] but not all[198] studies suggest that androgen receptor blockade increases

LH pulse frequency in men. Similarly, the marked increase of LH pulse frequency with high-dose ketoconazole (which inhibits both testicular/adrenal steroidogenesis and aromatase activity) is completely reversed by testosterone replacement despite low estradiol concentrations, while LH frequency is only partly normalized with estradiol add-back (with persistently low testosterone concentrations).[189] On the other hand, aromatase inhibitors[199] and antiestrogens[196,200-202] increase LH pulse frequency in normal men, and estrogen treatment in men with aromatase deficiency reduces LH pulse frequency.[203] Overall, these data suggest that both androgens and estrogens mediate negative feedback of hypothalamic GnRH secretion.

In contrast to dual (androgen, estrogen) steroid feedback on GnRH pulse secretion, a number of studies suggest that estradiol is a primary mediator of negative feedback at the pituitary in men.[189,193,197,204] For example, androgen receptor blockade does not alter LH responses to exogenous GnRH in some studies,[192,198] and aromatase inhibition appears to prevent testosterone's ability to reduce GnRH-stimulated LH and FSH secretion.[188] Perhaps the most compelling studies were performed in GnRH-deficient men receiving pulsatile exogenous GnRH in constant doses. Under this "GnRH clamp" paradigm, testosterone alone and estradiol alone (but not DHT alone) reduced LH and FSH concentrations.[204] In another "GnRH clamp" study, LH amplitude and mean LH increased after high-dose ketoconazole, and LH parameters were normalized with estradiol, but not testosterone, add-back.[189] Overall, these study results imply that estradiol is the primary mediator of testosterone negative feedback at the pituitary gonadotrope.

Kisspeptin and KNDy Neurons as Mediators of Sex Steroid Feedback

Evidence in rodents, sheep, and monkeys suggests that sex steroid feedback on GnRH secretion is mediated by KNDy neurons and kisspeptin, at least in part. For example, kisspeptin and KNDy neurons show a high degree of colocalization with estrogen receptors,[72] progesterone receptors,[96] and androgen receptors.[205] The numbers of kisspeptin neurons are high in the arcuate nucleus of humans and monkeys.[65,70,71] In the monkey, kisspeptin expression is markedly reduced by estrogen or estrogen plus progesterone.[70] Also, in animal models and in postmenopausal women, sex steroid deficiency is associated with increased kisspeptin expression in the arcuate nucleus (in parallel with circulating gonadotropins), and this is reversed by estradiol or testosterone replacement.[70,72] In addition to influencing kisspeptin expression, estrogen modulates neuronal responsiveness to kisspeptin in mice.[63] A model regarding the influence of KNDy neurons in negative feedback is shown in Figure 1.16.

Kisspeptin also appears to play a key role in the midcycle LH surge in the female rodent. *Kiss1* and *Kiss1R* null mice do not exhibit LH surges,[206] and the LH surge can be prevented by a kisspeptin antagonist[207] or a monoclonal antibody to kisspeptin.[208] Of particular interest, available data in rodents suggest that kisspeptin neurons in the AVPV are stimulated by estradiol, while those in the arcuate nucleus are inhibited by estradiol.[72] Also, estradiol administration directly into the medial preoptic area (location of the AVPV) induces LH surges, while estradiol administration directly into the mediobasal hypothalamus—which also raises estradiol levels in the pituitary—does not.[209] These and other findings in rodents have led to a model in which kisspeptin neurons in the arcuate nucleus regulate tonic GnRH release by mediating estrogen negative feedback, while kisspeptin neurons in the AVPV mediate the positive feedback effects of estrogen (Fig. 1.17).

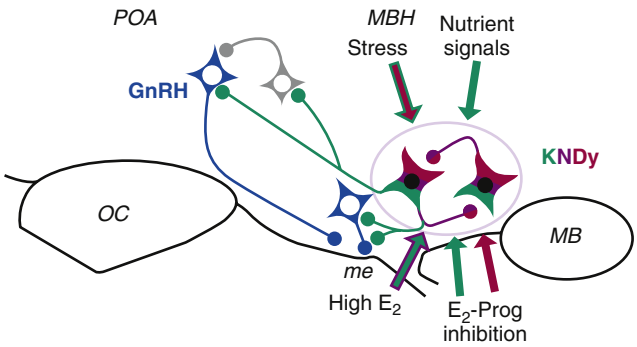


FIGURE 1.16 Model of KNDy signaling to GnRH neurons, largely based on data obtained in sheep. KNDy peptides are kisspeptin (green), which stimulates GnRH neurons; neurokinin B (NKB, magenta); and dynorphin (red). The major influences on GnRH secretion are shown, with putative effects on KNDy peptides denoted by the color of the arrow. For example, estradiol (E_2) inhibition may involve reductions of kisspeptin (green arrow), while progesterone (Prog) inhibition likely involves an increase of DYN. Arrows with two colors signify that more than one KNDy peptide may mediate a given effect (e.g., in the ewe, stimulation of GnRH secretion by high E_2 may involve an increase of both kisspeptin and NKB). The possibility that kisspeptin stimulation of GnRH neurons is mediated by interneurons is shown by the gray cell. POA, preoptic area; MBH, mediobasal hypothalamus; MB, mammillary bodies; ME, median eminence; OC, optic chiasm. *(Adapted from Lehman MN, Coolen LM, Goodman RL. Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion.* Endocrinology. *151:3479–3489, 2010.)*

It remains unclear whether a population of kisspeptin neurons homologous to those in the AVPV of rodents plays a similar role in primates. While LH surge generation requires an intact preoptic area in rodents,[210] monkeys retain the ability to produce LH surges after isolation of the mediobasal hypothalamus from the remainder of the brain.[105] Also, LH surges persisted after destruction of the preoptic area, which included the AVPV and suprachiasmatic nuclei, in one monkey study,[211] but not in another.[212] While human and monkey studies suggest the presence of kisspeptin neurons in the preoptic area,[70,71,213] it is unclear whether these are truly analogous to those of the AVPV in rodents. Of interest, kisspeptin expression increases in a caudal portion of the arcuate nucleus during the preovulatory period in both monkeys and sheep,[213-215] and some have suggested that kisspeptin neurons in this region could possibly represent a special population important for surge generation. Regardless, it is not certain that a unique population of kisspeptin neurons need be invoked in women, as increased GnRH secretion at mid-cycle (i.e., a GnRH surge) does not clearly occur in women (described earlier in the chapter). Thus, how these intriguing observations in animal models relate to human neurophysiology during midcycle remains unclear.

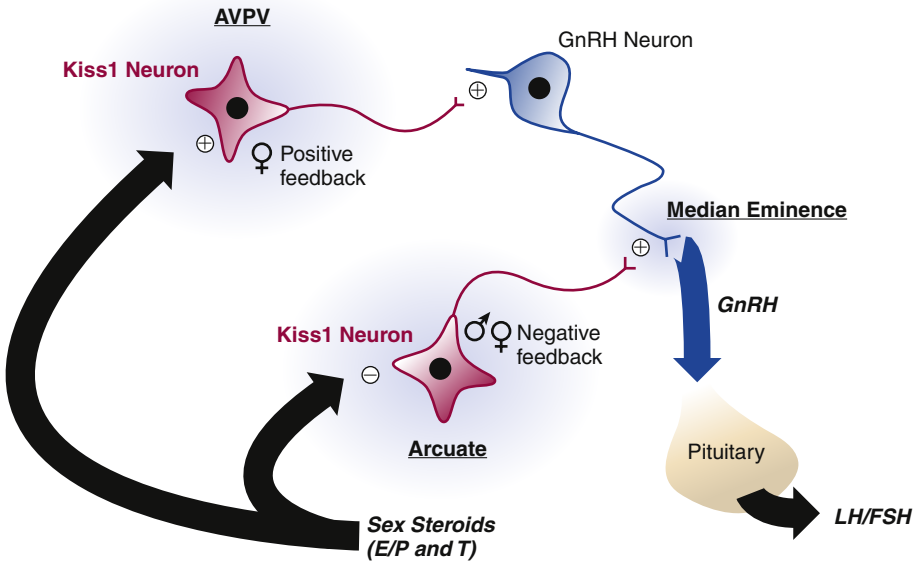


FIGURE 1.17 Model of kisspeptin-mediated feedback regulation of GnRH and gonadotropin secretion in rodents. By this model, kisspeptin (Kiss1) neurons in the arcuate nucleus of males and females project to and stimulate GnRH neurons. This population of kisspeptin neurons is inhibited by sex-appropriate gonadal steroids (i.e., estradiol [E], progesterone [P], and testosterone [T]). Thus, tonic GnRH secretion is primarily regulated by relatively low concentrations of estradiol via kisspeptin neurons in the arcuate nucleus. Females have another population of kisspeptin neurons in the anteroventral periventricular nucleus (AVPV) that also projects to and stimulates GnRH secretion. However, estradiol stimulates kisspeptin neurons in the AVPV—in contrast to estradiol inhibition of kisspeptin neurons in the arcuate nucleus. Thus, while high estradiol concentrations inhibit arcuate kisspetin neurons in females, they stimulate AVPV kisspeptin neurons, resulting in a GnRH surge. LH, luteinizing hormone; FSH, follicle-stimulating hormone. *(From Oakley AE, Clifton DK, Steiner RA. Kisspeptin signaling in the brain.* Endocrine Reviews *30:713–743, 2009.)*

Selective Regulation of Pituitary FSH Secretion

Inhibins, activins, and follistatin preferentially influence FSH secretion and contribute to divergent release of LH and FSH throughout the menstrual cycle.[38] During the midfollicular to late follicular phase and the luteal phase, both estradiol and inhibins selectively inhibit FSH release from gonadotropes. Inhibins are heterodimer peptide members of the transforming growth factor (TGF)-β superfamily with two isoforms, inhibin A and inhibin B, which contain identical α-subunits but different β-subunits (βA for inhibin A, βB for inhibin B). Most inhibin is derived from the ovaries: inhibin B is secreted by ovarian granulosa cells, mainly during the early follicular phase in response to FSH stimulation; and inhibin A is primarily produced by the corpus luteum during the luteal phase in response to LH stimulation. The chief function of both inhibins is to inhibit FSH release from pituitary gonadotropes. In men, inhibin B is produced from Sertoli cells and is a key negative feedback regulator of pituitary FSH release, although estradiol also inhibits pituitary FSH release.[216]

Activin is a dimer peptide with three isoforms: activin A (βAβA), activin B (βBβB), and activin AB (βAβB). The activin β-subunits and the inhibin β-subunits are identical. Activin produced in pituitary gonadotropes stimulates production of FSH in a paracrine fashion. Follistatin is a monomer peptide synthesized by the anterior pituitary (including folliculostellate cells); it inhibits pituitary FSH secretion by binding activin, thus rendering it inactive. Of interest, gonadotrope follistatin production varies in parallel with GnRH pulse frequency, one of the mechanisms contributing to the differential effects of GnRH pulse frequency on LH and FSH release.[44,217] In contrast to inhibins, which act primarily via endocrine signaling, activin and follistatin produced in the pituitary influence FSH secretion via autocrine-paracrine signaling.

Interface Between Reproduction and Energy Availability

Organisms require metabolic energy to support a number of processes, including maintenance of cellular function, muscle contraction (e.g., cardiac function, locomotion), thermogenesis, and growth. For various reasons, energy availability can be limited—sometimes severely so. For example, low energy availability may result from short-term or long-term reductions of calorie intake (e.g., famine, anorexia nervosa), insufficient calorie intake for metabolic demands (e.g., in the setting of significant exercise loads), or reduced ability to utilize energy sources (e.g., as may occur in severe diabetes). In such situations, energy use has opportunity costs: energy used for one process is no longer available for another. Thus, energy-requiring processes are prioritized to favor those that are life-sustaining.

Reproduction in women, pregnancy and lactation in particular, is metabolically demanding. For example, pregnancy requires an estimated additional 80,000 kilocalories.[218] Since reproduction is not imperative for individual survival, it is metabolically gated: reductions of energy availability can suppress reproductive function (nutritional infertility). This is biologically advantageous for the individual and, ultimately, the species. As such, it can be seen as an appropriate adaptive response. This process is believed to

be at the center of functional hypothalamic amenorrhea, a reversible condition of suppressed hypothalamic-pituitary function occurring in the absence of anatomical or organic abnormalities and often accompanied by reduced body weight, disordered eating (e.g., restrictive eating patterns), excessive exercise, and/or psychological stress. Prototypical examples include the amenorrhea of anorexia nervosa, a disorder marked by weight loss, altered body image, and fear of weight gain; and the "female athletic triad," which classically refers to the clinical association of amenorrhea, osteoporosis, and reduced energy availability related to vigorous exercise and disordered eating. The functional relationships between metabolic status and reproductive function are mediated by neural systems located in the hypothalamus.

Functional hypothalamic amenorrhea is characterized by impaired GnRH and gonadotropin secretion. Although a majority of women with functional hypothalamic amenorrhea demonstrate low LH pulse frequency,[219,220] such patients may demonstrate variable LH pulse patterns as a group—including absent pulses, low frequency and amplitude, low frequency only, low amplitude only, and normal frequency and amplitude; and patterns can change across time in the same woman.[7,219,220] In these patients, GnRH and gonadotropin secretion is inadequate for normal follicular development, estrogen production, and mid-cycle gonadotropin surges, but cyclic ovulation and fertility can be restored with pulsatile administration of exogenous GnRH.[7]

Some investigators have proposed that reduced reproductive function in functional hypothalamic amenorrhea primarily reflects insufficient body fat stores—the "critical fatness hypothesis."[221] This idea has been controversial, and a substantial body of data suggests that reduced energy availability, rather than body fat per se, is a primary variable driving reduced reproductive function in these settings. For example, there is marked variability of body fat percentage in athletes with and without amenorrhea, and body fat does not reliably distinguish amenorrheic from eumenorrheic athletes.[222] Also, calorie restriction can result in amenorrhea before weight loss; and in those with a history of eating disorders, amenorrhea can persist after weight restoration.[223] Likewise, findings consistent with functional hypothalamic amenorrhea can be observed shortly after bariatric surgery for severe obesity; importantly, the onset of amenorrhea can occur while still obese (e.g., body mass approximately 35 kg/m^2), albeit in the setting of negative energy balance.[224] Moreover, altered LH pulsatility is observed very quickly (within 5 days) of controlled reductions of energy availability.[225] Overall, these findings suggest that altered reproductive function in this setting reflects altered energy availability rather than body composition. It is also important to note that energy balance (and thus body weight) can be maintained in the face of calorie restriction by reducing metabolic rate[226]; this situation involves reduced energy availability for "noncritical" functions such as reproduction. Also of interest, experiments in women and monkeys suggest that reproductive function may not be impaired until energy availability is reduced by more than 30% (Fig. 1.18).[225,227]

While some have posited a specific influence of exercise, calorie supplementation to maintain adequate energy availability appears to prevent alterations of LH secretion despite significant daily exercise loads.[228] Similarly, while amenorrhea can be induced in monkeys by gradually increasing daily exercise in the setting of constant food intake,[229] providing supplemental calories reverses amenorrhea despite continued exercise.[230]

The neurobiological mechanisms underlying functional hypothalamic amenorrhea, and mechanisms underlying the influence of nutritional status on the reproductive system in general, remain poorly understood. Chronic energy deprivation is associated with myriad neuroendocrine adaptations and hormonal changes, including reductions of leptin, insulin, insulin-like growth hormone-1 (IGF-1), and thyroid hormone concentrations; increases of growth hormone and ghrelin levels; and activation of the hypothalamic-pituitary-adrenal axis.[231] A number of these alterations can influence GnRH and gonadotropin secretion and may mediate the influence of low energy availability on reproductive function. Much interest has centered on the permissive role of leptin, a hormone derived from adipose tissue that functions to signal metabolic status to central systems, influencing feeding, energy expenditure, and reproduction. Humans and mice lacking leptin or the leptin receptor (LepR) (e.g., ob/ob and db/db mice, respectively) have pubertal failure and infertility; in the setting of leptin deficiency, these manifestations can be reversed with leptin administration.[232,233] Low leptin levels have also been observed in women with functional hypothalamic amenorrhea,[234] and a specific role for leptin was suggested by a small study in which recombinant human leptin administration improved LH pulse secretion and parameters of ovarian function (e.g., estradiol concentrations) in women with functional hypothalamic amenorrhea; leptin administration was also associated with ovulation in a subgroup of these subjects.[235] While serum leptin concentrations generally correlate with fat mass, leptin levels can change rapidly in response to reduced energy availability; and leptin concentrations are suppressed with maneuvers known to suppress LH secretion, such as marked short-term energy restriction.[236] Of interest, exogenous leptin administration prevents the fall of LH release and testosterone concentrations associated with short-term fasting in men.[237] Although the precise role of leptin in regulating reproductive neuroendocrine function is unclear, it is believed that leptin has an important permissive role in normal GnRH secretion.

Similarly, a number of neuropeptides have been implicated in the influence of energy availability on reproductive function, including kisspeptin, neuropeptide Y (NPY), galanin-like peptide (GALP), beta-endorphin, corticotropin-releasing hormone (CRH), ghrelin, and polypeptide YY. While metabolic signals could potentially act directly on GnRH neurons, many studies suggest that afferent neural circuits are involved. For example, recent studies in mice suggest that metabolic signals can be relayed to the GnRH pulse generator from different areas in the brain, such as the ventral premammillary nucleus (e.g., leptin effects)[238] and the area postrema (e.g., in the absence of utilizable glucose).[239] Some studies suggest that increased opioid tone may contribute to slow GnRH pulses in functional hypothalamic amenorrhea.[220,240-242]

Even though successful reproduction is not metabolically costly for males, male reproductive function can also be affected by metabolic stress. For example, healthy young

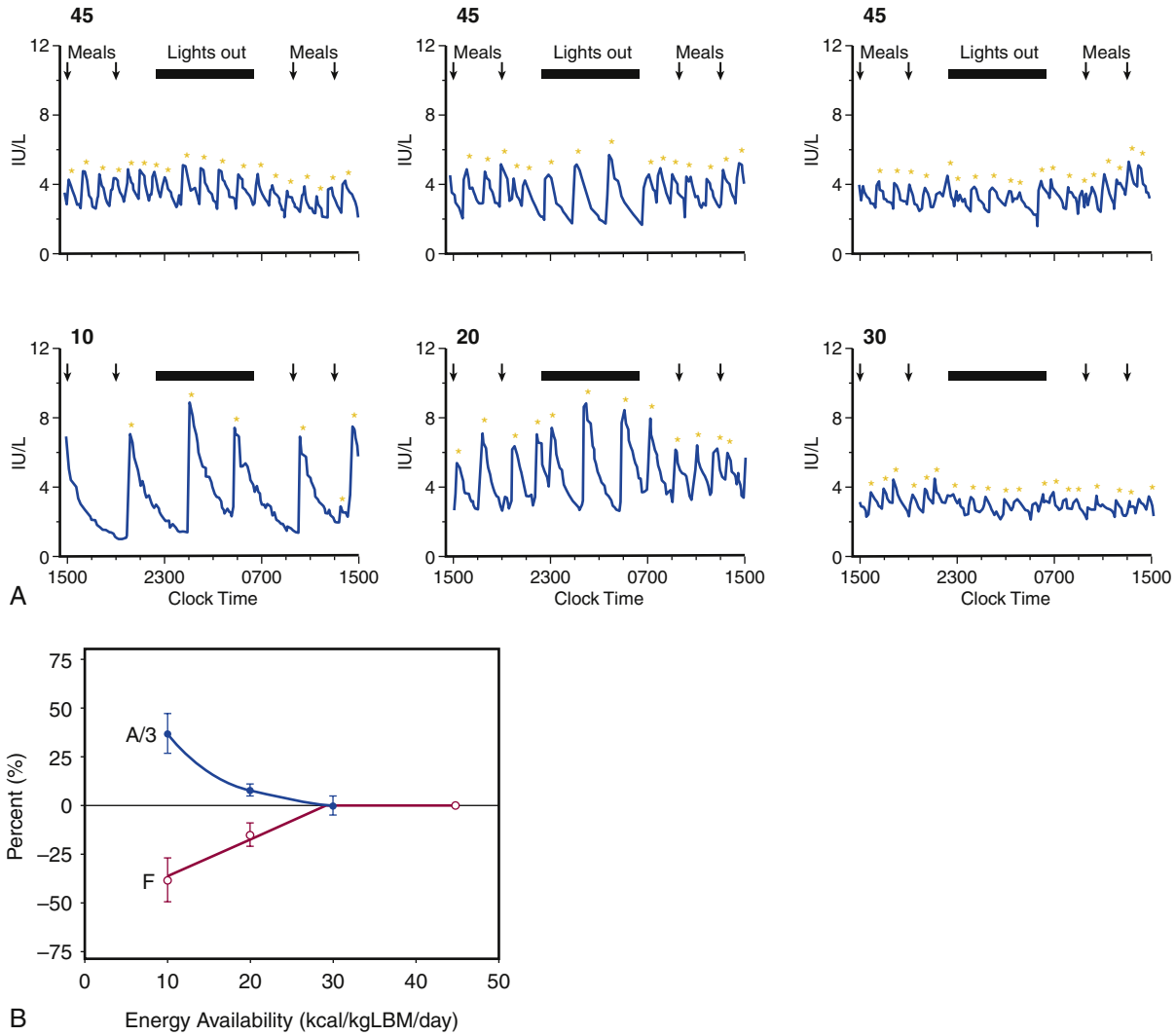


FIGURE 1.18 Influence of energy availability on LH pulsatility in healthy women. **(A)** Representative 24-hour LH time series in three women under different conditions of energy availability. These studies were designed to assess the effects of exercise and reduced energy availability in habitually sedentary women with regular menstrual cycles. In this study, exercise energy expenditure was substantial for all participants at approximately 840 kcal/day, and energy availability was altered via variable calorie intake. When energy availability is defined as dietary energy intake minus exercise energy expenditure—an estimate of the energy available for non-exercise related functions—and normalized to fat-free mass (thus expressed as kcal/kg lean body mass [LBM] per day), 45 kcal/kg LBM per day approximates balanced energy availability, and LH profiles under this condition are shown on top. Conditions of restricted energy availability (i.e., 10, 20, and 30 kcal/kg LBM per day) are shown along the bottom. Significant LH pulses are denoted by asterisks; arrows denote the timing of meals; and black bars denote lights out periods. **(B)** Association of energy availability and LH pulse characteristics. Energy availability is shown on the x-axis; on the y-axis, LH pulse amplitude (solid circles) and LH pulse frequency (open circles) are expressed as changes relative to values observed at 45 kcal/kg LBM per day. (Note that changes of LH pulse amplitude are divided by three.) While energy availability reductions to 30 kcal/kg LBM per day did not alter LH pulse characteristics, reductions below 30 kcal/kg LBM per day were associated with progressive reductions of LH pulse frequency and corresponding increases of LH pulse amplitude. *(Adapted from Loucks AB, Thuma JR. Luteinizing hormone pulsatility is disrupted at a threshold of energy availability in regularly menstruating women.* J Clin Endocrinol Metab *88:297–311, 2003.)*

men participating in U.S. Army Ranger training, which involves multiple stressors including intermittent extreme calorie restriction and weight loss (10 to 12 kg on average), can experience reductions of LH and suppression of testosterone concentrations to near castrate levels.[243] Of interest, increased calorie intake allowed prompt recovery of testosterone in this study, even without altering other associated stressors (e.g., exercise, sleep deprivation). Additionally, anorexia nervosa in adolescent boys and men can be associated with marked hypogonadotropic hypogonadism.[244] Energy-sensitive reproductive function in males would also be expected to delay reproduction during times of reduced energy availability; these regulatory pathways may have developed in males because of advantages imparted to other members of the species (e.g., mates and offspring).

Impact of Stress on Reproductive Function

Functional hypothalamic amenorrhea in the setting of reduced energy availability represents a particular form of stress-related reproductive suppression. A "stressor" represents a real or potential threat to homeostasis, such as

predator proximity, injury, illness (e.g., systemic infection), temperature extremes, reduced energy availability (e.g., fasting), and situations that provoke psychological distress. The nature of the stress response depends on the precise nature of the stressor, but typically involves both neural and neuroendocrine responses. A group of neurons in the hypothalamic paraventricular nucleus project to the median eminence, where they secrete corticotropin-releasing hormone (CRH) into the hypophyseal portal system. CRH (and, to some degree, cosecreted arginine vasopressin [AVP]) stimulates corticotrope cells in the anterior pituitary to release adrenocorticotropic hormone (ACTH), which in turn stimulates adrenal glucocorticoid (cortisol) production. A subset of paraventricular neurons is also involved with the regulation of the autonomic sympathetic nervous system, which includes neural pathways linked to the brain stem, spinal cord, and adrenal medulla (e.g., sympathoadrenal axis). Other components of the stress response include central arousal systems and the locus ceruleus, a nucleus in the brainstem involved with emotional and cognitive responses to stress. Thus, stressors trigger integrated neural, endocrine, and behavioral responses that promote short-term maintenance of homeostasis and survival. For example, activation of the sympathoadrenal axis leads to increased epinephrine secretion—an important component of the "fight-or-flight" response—while activation of the hypothalamic-pituitary-adrenal (HPA) axis with increased cortisol secretion enhances energy mobilization.

Chronic stress and marked acute stress can inhibit reproductive function—an appropriate adaptive response when homeostasis is threatened. For example, critical illness is associated with a reversible hypogonadotropic hypogonadism.[245,246] Mechanisms underlying the suppression of reproductive function during stress are highly complex; and while suppression of GnRH secretion is a major component, direct pituitary and gonadal effects may occur as well. Notably, the specific effects of stress on various aspects of reproductive function appear to depend on a number of factors including species, sex, hormonal milieu (e.g., intact vs. castrate), and the specific type of stress experienced.

A number of mediators have been implicated in stress-related inhibition of GnRH secretion, including CRH (which activates the HPA axis but also appears to have central effects), CRH-like peptides called urocortins, AVP, ACTH, endogenous opioid peptides (e.g., β-endorphin), and cortisol, in addition to noradrenergic, GABAergic, and serotoninergic neural pathways. For example, intracerebroventricular injection of CRH reduces multiple unit electrical activity in the mediobasal hypothalamus in monkeys[247]; and CRH antagonists can prevent some forms of stress-related LH suppression.[248] Naloxone can block CRH-related LH suppression in monkeys,[249] suggesting the involvement of endogenous opioid peptides as well. Noradrenergic afferents from the locus ceruleus have also been implicated in GnRH pulse generator suppression.

Notably, some data suggest that stress plays a role in functional hypothalamic amenorrhea. For example, amenorrheic athletes and women with anorexia nervosa demonstrate evidence of HPA axis activation (e.g., elevated cortisol concentrations).[231,250] Also, functional hypothalamic amenorrhea in women may be associated with evidence of higher psychological stress, including perfectionism, a history of unfavorable childhood experiences, and difficulty coping with stressors[251,252]; and reproductive function may be improved in some with cognitive behavioral therapy[253] or hypnotherapy.[254] Studies in female monkeys provide corroborating evidence: in one study, while very few monkeys demonstrated altered reproductive function when exposed to either (a) psychosocial stress (relocation to new housing setting with unfamiliar monkeys) or (b) mild dietary restriction plus daily exercise, the combination was associated with altered cycle length or anovulation in a majority.[255]

For unclear reasons, the degree to which stressors (e.g., reduced energy availability) interrupt reproductive function is variable among individual women and in female monkeys (i.e., "hypothalamus robustus" vs. "hypothalamus fragilis," "stress sensitive" vs. "stress resilient"). As suggested above, it is likely that a number of factors (e.g., reduced energy availability, stress) can interact to impact GnRH secretion. Also, a recent study suggested that mutations in genes associated with hypogonadotropic hypogonadism, including Kallmann syndrome 1 sequence gene *(KAL1)*, fibroblast growth factor receptor 1 gene *(FGFR1)*, prokineticin receptor 2 gene *(PROKR2)*, and the GnRH receptor gene *(GNRHR)*, are more likely to be identified in women with functional hypothalamic amenorrhea compared to normally cycling women.[256] Thus, it seems likely that genetic variants (e.g., those related to GnRH neuron biology), and perhaps epigenetic modifications as well, play a role in reproductive susceptibility to reduced energy availability and stress.

Lactation

High prolactin concentrations during pregnancy and suckling in the postpartum period stimulate milk production, which, for much of human history, was effectively the only source of nutrition for infants. Suckling also leads to posterior pituitary release of oxytocin, which stimulates contraction of myoepithelial cells within mammary gland acini, causing milk ejection. Lactation is also associated with amenorrhea and subfertility. The likelihood of pregnancy during the first 6 months postpartum is low (less than 2%) in fully-breastfeeding, amenorrheic women,[257] and some lactating women may remain amenorrheic for years. Since a short interval between births can place infant well-being at risk, lactational amenorrhea has ostensibly been an important adaptation enhancing infant survival in many cultures both past and present.[258]

During pregnancy, high placental sex steroid (estradiol, progesterone) and prolactin concentrations markedly suppress GnRH and gonadotropin secretion and prevent follicular development. In the absence of lactation, cyclic hypothalamic-pituitary-ovarian activity typically resumes in the 8 weeks after parturition. In the setting of lactation, however, pulsatile GnRH remains suppressed (e.g., low frequency pulses), with consequent impairment of LH secretion and estradiol production.[259] The reduction of GnRH secretion during lactation is suggested by a marked reduction of multiple unit electrical activity in the mediobasal hypothalamus in nursing monkeys,[260] and by the ability of pulsatile exogenous GnRH to restore ovarian function in amenorrheic lactating women.[261]

Mechanisms underlying lactational amenorrhea are incompletely understood. Lactation is associated with a very high metabolic cost: daily production of 750 to 1000 mL of human milk requires approximately 500 to 600 kilocalories a day,[262] some of which is obtained from fat stores and increased food intake. Nonetheless, the high energy requirements of lactation—which are approximately twice that of pregnancy—may induce some or all of the aforementioned mechanisms that inhibit pulsatile GnRH secretion in the setting of reduced energy availability. As recently reviewed,[263] animal (chiefly rodent) studies reveal that lactation is associated with activation of orexigenic neural systems (e.g., neuropeptide Y [NPY]) and inhibition of anorexigenic neural systems within the hypothalamus—changes that may partly relate to alterations of peripheral metabolic cues (e.g., leptin, insulin). Such alterations increase food intake and may suppress GnRH neuronal activity, either by direct effects on the GnRH neuronal network or by modification of key afferent systems such as kisspeptin. As an example of the former, NPY neuronal activation during lactation may directly inhibit GnRH neuronal activity.[264] As an example of the latter, expression of both kisspeptin and NKB in the arcuate nucleus is reduced in lactating rodents.[100,265]

Hyperprolactinemia suppresses GnRH pulsatility, at least in part via increased hypothalamic opioids,[266] and suppression of GnRH secretion during periods of lactation may partly reflect high prolactin concentrations. However, prolactin levels gradually decrease to normal despite continued breastfeeding; thus, the activity of the hypothalamic-pituitary-ovarian may not correlate well with circulating prolactin concentrations. Importantly, the intensity (e.g., frequency, duration) of suckling appears to be an important determinant of contraceptive effectiveness in women, and the suckling stimulus may inhibit the hypothalamic-pituitary-ovarian axis through additional (e.g., neural) mechanisms.

Miscellaneous Physiological Influences on GnRH Secretion

Circadian Changes

Diurnal rhythms (i.e., those that cycle once a day) are frequently observed in endocrinology, and this includes the reproductive system. Diurnal rhythms are called circadian rhythms if they are internally (endogenously)-driven rhythms, although such rhythms are usually entrained (synchronized) to environmental cues (e.g., light-dark cycle). Circadian rhythms are dictated by a "master clock" located in the suprachiasmatic nucleus of the hypothalamus and are derived from complex intracellular interactions involving the so-called "clock genes," which participate in feedback interactions that generate recurring cyclic activity.[267] Such clocks in the suprachiasmatic nucleus can be synchronized by light-dark signals received from the retina and transferred to the suprachiasmatic nucleus via the optic nerve (retinohypothalamic tract).

Depending on factors such as species and sex, both basal gonadotropin secretion and LH surges may exhibit diurnal rhythms. As a prominent example, LH surges in female rats are specifically confined to the late afternoon, which is shortly before rats become active (i.e., when copulation is most likely).[268] It is believed that this reflects a daily stimulus generated by the suprachiasmatic nucleus, but only relayed to the GnRH neuronal network in the presence of preovulatory estradiol concentrations. Thus, ovulation in rats is optimally timed to coincide with sexual opportunity and receptivity.

In contrast to rodents, LH surges do not appear to be constrained to a specific time of day in monkeys; for example, LH surges can be advanced by 12 to 18 hours with supraphysiological estradiol administration.[159] Some studies in women suggest that LH surges tend to be initiated in the morning. For example, in one study of 19 ovulatory women, LH surges were initiated in the early morning hours (circa 0400 to 0800 hours)[269]; and in one study of 155 spontaneous cycles, the estimated time of LH surge initiation was between midnight and 8:00 AM in 85% of cycles.[270] These studies do not confirm a true circadian signal, and putative confinement of ovulation to the morning hours could reflect diurnal environmental cues. In addition, the potential relevance of a specific daily timing of ovulation in women is uncertain since ovulation typically occurs some 36 hours after the LH surge; and the likelihood of conception when sexual intercourse occurs on the day before ovulation (circa 40%) is similar to conception rates when intercourse occurs on the day of ovulation.[271] Although humans demonstrate clear diurnal changes of gonadotropins, recent studies in women who were carefully assessed during the early follicular phase[272] or after menopause[273] suggest that LH and FSH secretory parameters (including LH pulse frequency and amplitude) do not exhibit circadian changes after controlling for sleep status, body position, light exposure, activity level, and nutritional cues.

Sleep

Although precise mechanisms are unclear, sleep can have a major influence on pulsatile LH (and presumably GnRH) secretion. For example, the nocturnal amplification of LH pulsatility during puberty is specifically related to sleep: it generally begins within an hour of sleep onset, and it follows sleep reversal.[274,275] Although the data relating LH pulses to sleep stage are incomplete, sleep-related pulses during puberty appear to occur primarily during non-rapid eye movement (REM) sleep.[274,275]

Sleep also influences LH pulse secretion in adults, primarily in the form of sleep-related slowing of LH pulse frequency, which is most prominent during the early follicular phase,[133,135,276,277] but also occurs in the late follicular phase.[225,278] Nocturnal slowing of LH pulse frequency in women during the early follicular phase is specifically related to sleep, as it follows sleep reversal.[279] During this time, LH pulses are uncommon during REM and slow wave sleep, and more common following brief awakenings.[279] Such slowing may be mediated by hypothalamic opioids, since naloxone prevents the sleep-associated decrease of LH pulse frequency.[280]

Taken together, these data imply that sleep influences LH pulse frequency, and that the effect of sleep can be modulated by factors such as developmental stage and sex steroid milieu. The physiological relevance of sleep-associated changes of GnRH secretion remains unclear, but such changes have been postulated to contribute to normal gonadotropin production across puberty[123] and to the early follicular prominence of FSH secretion in postpubertal women.[279]

Pheromones

Pheromones are chemicals transmitted through the air that can influence reproductive function and sexual behavior (e.g., to produce stereotyped behaviors, neuroendocrine changes) in other individuals within many mammalian species. For example, the presence of a novel and sexually mature male mouse can synchronize estrous cycles among female mice; this so-called "Whitten effect" is presumably mediated by pheromones.[281] Similarly, pheromones produced by male sheep and goats can induce out-of-season ovulation in females—the "male effect."[282]

The role of pheromones in humans remains unclear. Menstrual synchrony (sometimes called the McClintock effect) is a putative phenomenon in which menstrual cycles of women living in very close proximity become synchronized.[283] This has been cited as physiological evidence of pheromone functionality in humans, and axillary secretion from late follicular women has also been reported to advance ovulatory timing in recipient women.[284] However, supportive research has been criticized on methodological grounds, and the existence of this phenomenon remains controversial.[285] Also, while the vomeronasal organ—believed to be responsible for pheromone detection in animals—develops in utero in humans, it subsequently regresses and is largely believed to be nonfunctional in adults.[286]

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 2

The Gonadotropin Hormones and Their Receptors*

Mario Ascoli
Prema Narayan

Gonadal functions, gametogenesis and steroidogenesis, are regulated by a hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH), and two anterior pituitary gonadotropins: luteinizing hormone (LH) and follicle stimulating hormone (FSH). Along with placental-derived human chorionic gonadotropin (hCG), these three gonadotropins regulate androgen, estrogen and progesterone synthesis, oogenesis, spermatogenesis, ovulation, and the maintenance of early pregnancy.

Structurally, the three gonadotropins, and the highly related thyroid-stimulating hormone (TSH) exist as heterodimers; sharing a common α subunit and homologous hormone-specific β subunits. They are highly elongated molecules with two intertwined subunits that have similar conformations. They are also members of the larger cystine knot-containing growth factor family that includes transforming growth factor β (TGFβ), activin and others.

The three gonadotropins act via two G protein-coupled receptors (GPCRS). The gonadotropin receptors (as well as the TSH receptor [TSHR] and several others) are characterized by the presence of a large extracellular domain containing several leucine-rich repeats (LRR). This large extracellular domain is responsible for the recognition and high-affinity binding of the appropriate hormones. The luteinizing hormone receptor (LHR) recognizes both LH and hCG, and the follicle stimulating hormone receptor (FSHR) is specific for FSH. The LHR is expressed in Leydig, theca, granulosa, and luteal cells whereas the FSHR is expressed in granulosa and Sertoli cells. Expression of the gonadotropin receptors in non-gonadal tissues has also been reported but these findings are still controversial and the physiological significance, if any, of the extragonadal expression of the gonadotropin receptors is debatable.

This chapter focuses on the three gonadotropins and their two receptors.

Gonadotropins

Gonadotropin Proteins (LH, hCG, and FSH)

Amino Acid Sequences and Three-Dimensional Structures

The three gonadotropin proteins (LH, hCG, and FSH) and TSH comprise the better characterized members of a family of complex proteins known as the glycoprotein hormones.[1] They are non-covalently bound heterodimers composed of a common α subunit and distinct β subunits. The common α gonadotropin subunit (α-GSU), encoded by the *CGA* gene contains 92 amino acid residues, and LHβ, FSHβ, and hCGβ are, respectively, 121, 110, and 145 amino acid residues in length. The additional length of hCGβ is due to a carboxy-terminal extension arising from a frameshift mutation in an ancestral LHβ gene resulting in a read-through into an untranslated region of LHβ and an extension of the open reading frame.[2-4] This extension is known as the carboxy-terminal peptide (CTP). The amino acid sequences of the human (h) subunits are shown in Figure 2.1, and it can be seen that the α and β subunits are relatively rich in Cys residues and that considerable homology exists in the β subunits.

Crystal structures are available for deglycosylated hCG,[5,6] glycosylated, antibody-bound hCG,[7] a partially deglycosylated hFSH[8] and a partially deglycosylated complex of a single chain hFSH bound to a large N-terminal fragment (residues 1-268) of the hFSHR ectodomain (ECD).[9] Figure 2.2 shows the crystal structures of hCG and hFSH.

The conformations of hCG and hFSH are quite similar, each being highly elongated molecules with the two subunits intertwined one with another in a slightly twisted manner. Despite the absence of any striking sequence homology, the two subunits in both heterodimers have similar folds, characterized by three major loops, and each subunit contains a cystine knot motif, consisting of three disulfides located in the core of each subunit. The α and β subunits contain, in addition to the three disulfides in the cystine knot, two and three disulfides, respectively. A 20-amino acid residue region of the β subunit, denoted as the seatbelt, wraps around and latches a portion of the α subunit. A major difference in the structures of hCG and hFSH is in the C-terminal portions of the seatbelts that exhibit distinct conformations. In both hCG and hFSH, the two subunits are associated in a head-to-tail arrangement (Fig 2.2). Although there are no structures available for LH and TSH, the similarities of the hCG and FSH structures engender confidence that the overall conformations of LH and TSH will be closely related to the known structures. Solution structures have also been obtained for deglycosylated human α-GSU[10,11] using nuclear magnetic resonance (NMR) spectroscopy. The overall ensemble of

*The authors would like to acknowledge the contributions of Dr. David Puett, who was an author of this chapter in the previous edition.

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hFSHβ  1  ------NSCELTNITIAIEKEECRFCISINTTWCAGYCYTRDLVYKDPARPKIQKTCTFKELVYETVRVPVCAH 68
hLHβ   1  SREPLRPWCHPINATLAVEKEGCPVCITVNTTICAGYCPTMMRVLQAVLPPLPQVVCTYRDVRFESIRLPGCPR 74
hCGβ   1  SKEPLRPRCRPINATLAVEKEGCPVCITVNTTICAGYCPTMTRVLQGVLPALPQVVCNYRDVRFESIRLPGCPR 74

hFSHβ 69 HADSLYTYPVATQCHCGKCDSDSTDCTVRGLGPSYCSFGEMKE----------------EMKE--------- 111
hLHβ  75 GVDPVVSFPVALSCRCGPCRRSTSDCGGPKDHPLTCDHP--------------------QLSG-----LLFL 121
hCGβ  75 GVNPVVSYAVALSCQCALCRRSTTDCGGPKDHPLTCDDPRFQDSSSSKAPPPSLPSPSRLPGPSDTPILPQ 145

α  1  APDVQDCPECTLQENPFFSQPGAPILQCMGCCFSRAYPTPLRSKKTMLVQKNVTSESTCCVAKSYNRVTVMG 74
   75 KVENHTACHCSTCYYHKS                                                        92
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FIGURE 2.1 Amino acid sequences of human α *gonadotropin subunit* (α-GSU), luteinizing hormone (LH)β, chorionic gonadotropin (CG)β, and follicle-stimulating hormone (FSH)β. Amino acid sequences were obtained from the Ensembl web site *(http://www.ensembl.org/index.html),* and the β subunits are aligned to maximize homology. Identical, highly conserved, and semiconserved residues among the three β subunits are highlighted by the blue, green, and yellow boxes, respectively. All cysteines participate in disulfide bond formation in the native proteins. hCG, human chorionic gonadotropin; hFSH, human follicle-stimulating hormone; hLH, human luteinizing hormone. *(Copyright © 1999–2008 The European Bioinformatics Institute and Genome Research Limited, and others. All rights reserved.)*

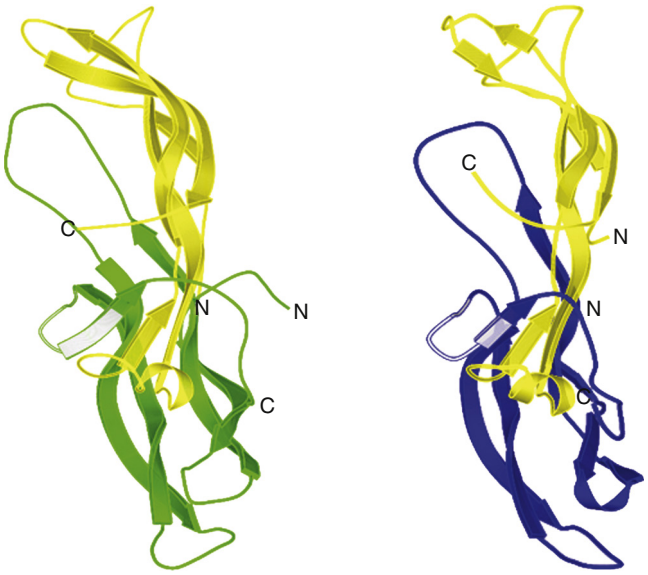


FIGURE 2.2 Crystal structures of human chorionic gonadotropin (hCG; *left*) and human follicle-stimulating hormone (hFSH; *right*). The structures[5-8] show that the two subunits are highly elongated and intertwined (hCGα, *yellow;* hCGβ, *green;* and hFSH, *blue*), forming a relatively large contact surface area. As discussed in the text, there are several interesting features associated with the structures: each subunit contains a cystine knot motif; the β subunit wraps around a portion of the α subunit, forming what is termed a seatbelt (shown in *white*); although having little sequence homology, the two subunits adopt similar folding patterns; hCG and hFSH form very similar structures, but the respective subunits of each exhibit subtle differences in their conformations. C, C-terminus; N, N-terminus.

structures determined for α-GSU is similar to that obtained in the crystal structures of hCG and hFSH.

With crystal structures available for FSH[8] and an FSH-FSHR ECD complex[9] (discussed elsewhere in this review), it is possible to delineate the conformational changes of the free heterodimer, and that bound to receptor. The unbound form of the hormone is more flexible than that of the bound form. It is the C-terminal region of the α-GSU, however, that undergoes the greatest change in conformation. In addition, the two C-terminal residues in α-GSU are unordered in the crystal structure of FSH, but in the complex with receptor they are fully ordered.

Glycosylation

The gonadotropins are trafficked from the endoplasmic reticulum to the cis-Golgi and undergo glycosylation as they traverse the Golgi reaching the trans-Golgi, to yield the mature hormones. The human subunits contain N-linked glycans: two on α-GSU at Asn52 and Asn78, two on CGβ (Asn13 and Asn30), two on FSHβ (Asn7 and Asn24), and one on LHβ at Asn30. In addition, hCGβ contains four mucin-type O-linked glycans at serines 121, 127, 132, and 138, located on the CTP (Fig. 2.3). The carbohydrate moieties appear to be important in subunit assembly and stabilization, secretion, and circulatory half-life. Although earlier studies suggested a role of the N-linked glycan at Asn52 on α-GSU in receptor activation, more recent evidence indicates that the glycan acts more as a conformational or stabilizing determinant of the protein.[12,13] Moreover, there is growing evidence that the particular type of glycosylation may influence biological activity.[14-20]

The biantennary N-linked glycans on hFSH and hCG terminate in sialic acid (and to a lesser extent sulfate), although the number of such moieties varies from zero to two, accounting in large part for the microheterogeneity of these glycoprotein hormones. In LH the biantennary N-linked structures tend to terminate mainly in sulfate. These are but generalizations since, for example, hFSH also contains triantennary and tetrantennary N-linked glycans, and some hFSHβ subunits lack N-linked structures completely.[21-24] A variety of glycosyltransferases are responsible for N- and O-glycan biosynthesis; notably, sulfation in the pituitary requires N-acetylgalactosamine transferase and sulfotransferase, both of which are missing in the placenta.[21,22] Also, fucose is often found in the glycoprotein hormones. As summarized, nearly 50 different N-linked and O-linked glycans have been reported in hCG α and β preparations.[25-27] The major gonadotropin N-linked and C-linked glycans are shown in Figure 2.3.

Folding and Assembly

Investigations into the kinetic folding pathways of the hCG subunits led to the interesting suggestion that disulfide

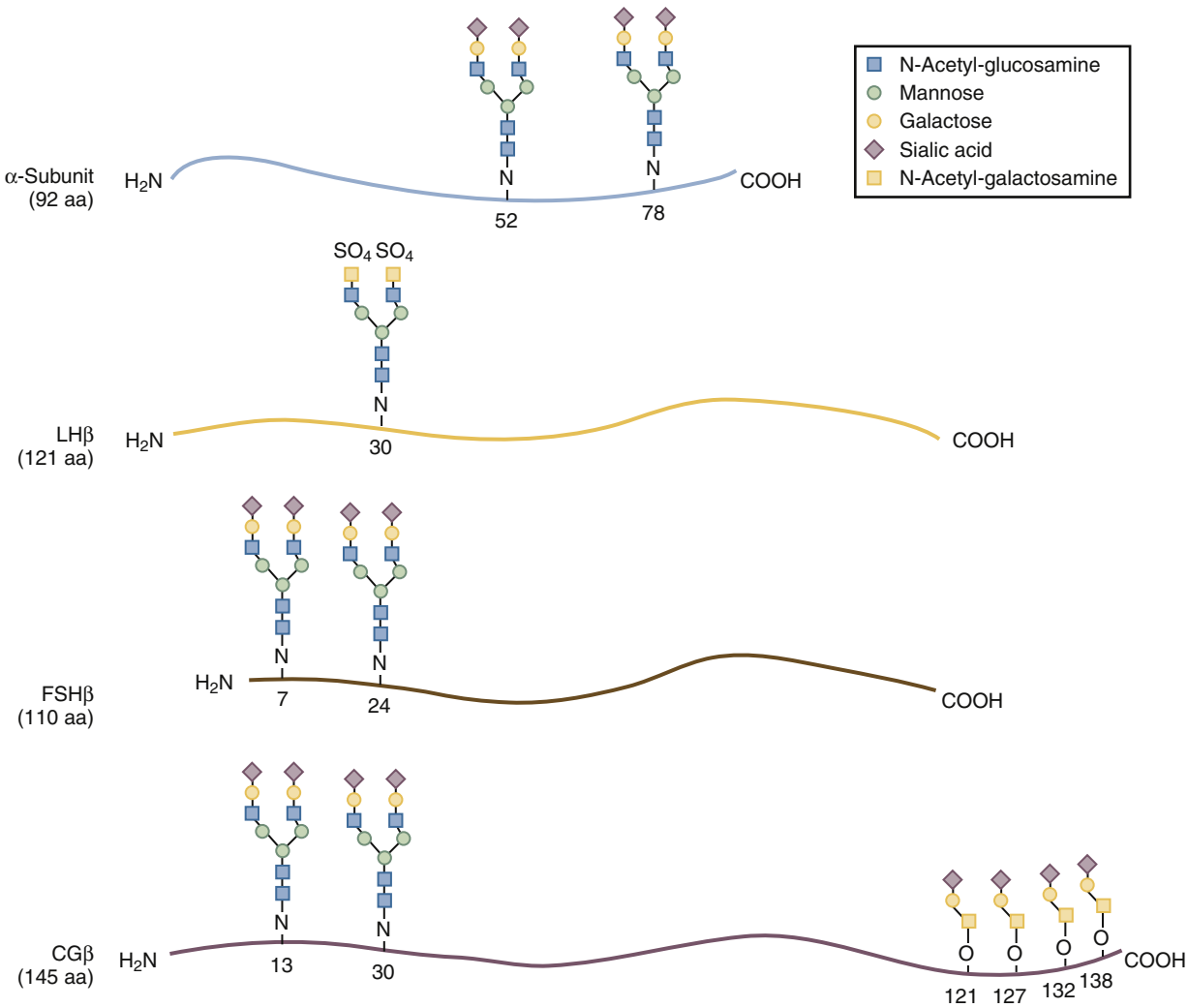


FIGURE 2.3 Location and typical structures of the N-linked and O-linked glycans on the gonadotropins. The sites of glycosylation on each of the gonadotropin subunits and representative structures of the various N-linked glycans on human luteinizing hormone (hLH), human chorionic gonadotropin (hCG), and human follicle-stimulating hormone (hFSH). As discussed in the text, scores of different structures have been identified, often with fucose present; moreover, some sites are devoid of glycosylation in various gonadotropin preparations. A representative structure of type 1 O-linked glycans on the carboxy-terminal peptide of hCGβ is shown; type 2 structures have been reported as well, and some sites show no glycosylation in various gonadotropin preparations. *Blue squares,* GlcNAc; *yellow squares,* GalNAc; *green circles,* mannose; *yellow circles,* galactose; *purple diamonds,* sialic acid.

exchange occurs during the maturation process and that subunit association occurred before completion of protein folding and disulfide formation.[28-32] Moreover, it was posited that subunit association occurred before the seatbelt was latched by closure of Cys26 and Cys110. In contrast to these reports, a different mechanism in which subunit assembly involved closure of the seatbelt latch, followed by threading of α loop 2, has been proposed.[33-37] Others have also studied the folding patterns of hCG and reported that subunit association occurred between an almost completely folded α subunit and an immature β subunit.[38-41]

In addition to the heterodimeric nature of the hormones, homodimers have also been found for LHβ,[42,43] and for the α subunit.[40,41,44] Whether these homodimeric forms of the glycoprotein hormones have any associated bioactivity remains to be shown, although it has been reported that the free α subunit potentiates progesterone-mediated decidualization.[45]

Other Heterodimeric Glycoproteins Related to the Gonadotropins

A search of the human genome revealed two glycoproteins, GPA2 and GPB5, similar to the glycoprotein hormone α and β subunits, respectively.[46] Heterodimers can be formed between GPA2 and GPB5, and the complex is capable of stimulating the TSH receptor. This heterodimer, named thyrostimulin, is postulated to act in a paracrine manner to activate the pituitary TSHR.[47] Comparative structure analysis of the glycoprotein hormone subunits and GPA2 and GPB5 suggests that GPA2 and GPB5 cannot form stable heterodimers at low concentration in circulation as they lack the cystine residues potentially involved in the cystine knot structure and GPB5 does not possess the sequence homologous to the seat belt.[48] This suggests a paracrine, rather than endocrine role for thyrostimulin. Phylogenetic studies suggest that the thyrostimulin may in fact be an "old" hormone

with the subunits appearing with the emergence of bilateral metazoa more than 500 million years ago.[46,49] In the basal chordate, amphioxus, thyrostimulin appears to play a role in embryonic development and heterodimerization may not be required for its activity.[49] A recent study suggests that thyrostimulin, expressed in the oocyte, is a paracrine regulator of TSHR in the granulosa cells of the rat ovary.[50]

Structure-Function Studies

Amino Acid Mutations

As discussed elsewhere in this review, only a limited number of naturally occurring mutations have been identified in the α and β subunits of the glycoprotein hormones. In contrast, there is a wealth of information available from site-directed mutagenesis followed by biological characterization of the mutant hormones. Few mutants have been described in which there was a significant increase in bioactivity; most mutations either have no effect or induce a loss-of-function, either disrupting folding, subunit assembly, or receptor binding/activation. Gain-of-function mutations in hCG were obtained by replacing single or multiple amino acid residues at the N-terminal region of the α subunit with Lys.[51] A 2-fold increase in potency of hCG was obtained with a single replacement of Phe with Thr at position 18 of α-GSU.[52] Mutant forms of α-GSU missing the N-linked oligosaccharide at Asn52 are capable of associating with the hCGβ or FSHβ subunit giving a heterodimer that binds to the cognate receptor but has diminished signaling efficacy.[53] Although still controversial, it appears that the role of N-linked glycosylation at Asn52 is to stabilize the active conformation of the heterodimer by formation of a hydrogen bond with a Tyr on the β subunit.[8] Mutations in the central region of α-GSU[54-56] and at the C-terminus[57,58] yielded mutants that associated with the β subunits of hCG and hFSH, but displayed compromised functionality in receptor binding.

A large number of β subunit mutations have been prepared and characterized.[59-67] As with mutations in α, many interfere with folding, subunit assembly, or receptor binding. The data, overall, being consistent with the structures of hCG and FSH. Unlike α-GSU mutants, however, there have been no reports of β mutants that retain the ability to form heterodimers and bind to receptor, yet do not signal.

Protein Engineering

Protein engineering has been used to produce a variety of subunit deletion derivatives, single chain hormones and chimeric hormones yielding interesting results. As mentioned earlier, C-terminal deletion mutants of the α subunit result in large N-terminal fragments that retain the ability to bind to the β subunit, but the resulting heterodimers exhibit minimal, if any, receptor binding capability. Deletion mutants at the N- and C-termini of hCGβ have been reported by several groups,[59,60,63,68] and the shortest form that retains minimal functionality in subunit assembly and subsequent receptor binding and activation is a fragment consisting of residues 8-100.[63]

A number of glycoprotein hormone chimeras have been designed and characterized, providing useful information on specific amino acid residues involved in receptor binding and activation.[69-75] In general, these results emphasize the role of the β subunit seatbelt region in receptor binding, although different portions are important in receptor specificity.

A novel approach to the study of gonadotropin structure-function relationships involved the design of single chain hormones (i.e., fusion proteins of the α and β subunits). The first reports of a yoked or tethered hCG demonstrated that the single chain gonadotropin, in the configuration N-hCG β-α-C, was bioactive.[76,77] Subsequently, similar fusion proteins of LH and FSH were also found to be bioactive.[78,79] Later, single chain proteins of hCG in the N-α-hCGβ-C configuration and proteins with different linkers were expressed and characterized, in many cases with interesting mutations in one or both subunits.[80,81] From these studies it was concluded that the N-α-hCGβ-C configuration was also bioactive and, quite surprisingly, that, each disulfide of the subunits could be eliminated without a loss of activity.[82-84] The single chain hormones also displayed increased stability and heat resistance *in vitro* compared to their heterodimeric counterparts.[78] Extending the approach of covalently linking the two subunits, others designed and expressed disulfide-linked heterodimers.[85-89] These results further substantiated the possibility that α-Asn52 contributed to heterodimer stability and was not involved directly in signal transduction,[12] and also led to suggestions that the C-terminus of the α-GSU is not required for LHR binding. This latter finding is consistent with the observation that the five C-terminal residues can be removed from α-GSU without a loss of LHR binding. The use of single chain gonadotropins, particularly in the N-α-β-C configuration, also raises interesting questions about the role of the C-terminal region of α-GSU in FSH where the structure of the FSH-FSHR ECD complex shows a large movement of α-GSU in the receptor complex compared to the heterodimer.[9] Of great interest was the report that single chain hCGβ-βhomodimers bind to LHR with an affinity about three times lower than wild type hCG; this engineered homodimer does not elicit a biological response and blocks hCG binding to LHR.[90] A second single chain hCG antagonist was designed by mutating three of the four N-linked glycosylation sites that are associated with LHR activation (Asn 13 and 30 in the β subunit and Asn 52 in the α subunit). Asn 78 in the α subunit, associated with receptor binding, was left intact. This analog behaved as a competitive antagonist and suppressed ovarian hyperstimulation syndrome in rats.[91]

The approach of converting glycoprotein hormones to single chains has been extended to produce fusion proteins with dual and triple activities. For example, a three domain fusion protein of the form, N-FSHβ-hCGβ-α-C, exhibited both LH and FSH activities.[92-94] Interestingly, the bifunctional gonadotropin is secreted from cells as two species, one with LHR, and the other with FSHR activities.[95] A four domain fusion protein, N-TSHβ-FSHβ-hCGβ-α-C, although secreted inefficiently, was found to exhibit three distinct bioactivities both in cellular and whole animal studies.[96,97] These results raise intriguing questions regarding subunit association and conformation as manifested in receptor binding and activation. A most provocative finding was based on a bifunctional, triple domain fusion protein of the form: N-FSHβ-hCGβ-α-C.[84] These investigators showed that disruption of heterodimer formation by mutation of either Cys10-Cys60 or Cys32-Cys84 did not

eliminate bioactivity, and thus concluded that αβ contacts are not required for receptor binding and activation. These selected results, along with others not covered here, indicate that single chain glycoprotein hormones exhibit some properties distinct from those in heterodimers. The single chain proteins with increased stability, the single chain hCG antagonists and single chain molecules with dual LH/hCG-like and FSH-like bioactivities, are attractive candidates for clinical utility.

The concept of single chain gonadotropins was extended to produce fusion proteins of hCG and LHR (i.e., covalent attachment of a single chain hCG to LHR) which when expressed, led to constitutive receptor activation in transfected cells and transgenic mice.[98-102] This model was also used to demonstrate that the individual subunits are devoid of bioactivity.[103]

Gonadotropin Subunit Genes and Transcripts

Gonadotropin Subunit Genes

The three gonadotropins, LH, CG, and FSH, are encoded by one gene each for the common α subunit and the β subunits of LH and FSH; in contrast, the CGβ subunit, expressed in primates and equids, is encoded by six genes.[104,105] It has been suggested that the glycoprotein hormone α and β subunits diverged from a common ancestral gene over 900 million years ago[104] with the β gene undergoing duplications and mutations to yield the current family. In humans, the gene encoding the common alpha subunit is on chromosome 6, that for FSHβ on chromosome 11 and those for LHβ and CGβ on chromosome 19 (http://www.ensembl.org). The gene for human α is 9.4 kb and contains four exons and three introns; that for FSHβ is 4.2 kb with three exons and two introns; for LHβ the gene is 1.1 kb with three exons and two introns; and those for CGβ are variable in length.

The seven genes for LHβ and CGβ exist in a large cluster spanning about 52 kbp.[104,105] The six genes encoding the β subunit of CG (i.e., *CGB*, *CGB1*, *CGB2*, *CGB5*, *CGB7*, and *CGB8*) exist as tandem and inverted repeats.[106] The genes for CGβ have been analyzed in detail.[104,105] Four of the CGβ genes, *CGB*, *CGB5*, *CGB7*, and *CGB8*, exhibit 97%-99% sequence identity while their identity with LHβ is 92%-93%. These gene similarities lead to protein amino acid sequences that are 98%-100% identical for the four CGβ genes and 85% identical with LHβ. The genes for *CGB*, *CGB5*, *CGB7*, and *CGB8* encode proteins with a carboxy-terminal extension (CTP), that appears to have arisen from the LHβ gene by frameshift mutations leading to a read-through into a previously untranslated region, thus extending the reading frame.[2,3,107] The CTP contains mucin-type O-glycans (Fig. 2.3), resulting in properties of hCG not found in LH.[4]

Gonadotropin Subunit Transcripts

The available evidence indicates single transcripts for the gonadotropin genes, with the exception of the human *FSHB* gene for which four messenger RNA mRNA species have been described, arising from alternate splicing and the utilization of two polyadenylation sites.[108] The CGβ family is interesting in that all six genes appear to be expressed giving transcripts of varying lengths. *CGB5* and *CGB8* are highly expressed in the placenta.[104,105,109] Although expressed in placenta,[104,105] pituitary,[110] testis,[111] and breast cancer,[112] no proteins have yet been identified for the *CGB1* and *CGB2* genes. The predicted sizes of CGβ1 and CGβ2 are smaller than that of hCGβ; this observation, coupled with the distinct amino acid sequences predicted, suggests that these proteins, if biosynthesized, may have quite different functions than those of hCG. Using transgenic mice expressing a 36 kb cosmid insert that contained the six *CGB* genes, transcripts of *CGB1* and *CGB2* genes were found to be present in brain at levels comparable to those of the other four *CGB* genes.[113]

Naturally Occurring Mutations in *CGA*, *LHB*, and *FSHB* Genes

The only mutation reported in the *CGA* gene is that from a human carcinoma, Glu56Ala, resulting in a mutant form of α-GSU that does not associate with LHβ.[114] In contrast, there are several reports of mutations in the genes encoding the β subunits, resulting in loss-of-function and thus hypogonadism.[105,115,116] The first report of a mutation in the *LHB* gene was that of a missense mutation in a male presenting with delayed puberty and hypogonadism.[105,115,116] This mutant led to a replacement of Gln54 with Arg; while subunit assembly could occur, the heterodimer was unable to bind to LHR. Other studies showed that LHβ and hCGβ subunits with Gln54 replacements formed heterodimers with α-GSU, but these heterodimers exhibited reduced binding to LHR.[64,67] Another missense mutation reported in LHβ was that of Gly36 to Asp, reported on a male with delayed puberty and infertility.[105,115,116] Gly36 is part of the CAGYC sequence in LHβ that is critical to the formation of the disulfide knot; presumably, an Asp at this position prevents at least one of the disulfides from forming.

Two additional mutations in the *LHB* gene were recently reported. The first reported mutation,[117] a G-C substitution at the +1 position of intron 2 (a 5′ splice-donor site), leads to a hypothetical aberrant protein with a 79 amino acid residue insert beginning after Met41 and a frameshift in exon 3, thus removing the essential seat belt loop of β and important cysteines. The offspring of consanguineous parents (second cousins) who were heterozygous for the mutation were analyzed. Three homozygous siblings presented with hypogonadism and infertility, and interestingly, the sister underwent normal pubertal development and menarche at age 13 years. Three heterozygous siblings were fertile. Analysis of serum hormone concentrations of the three siblings homozygous for the LHβ gene mutation showed undetectable levels of LH and high levels of free α-GSU; the two males had elevated FSH and low testosterone while the female had FSH, estradiol and progesterone values in the normal range, albeit on the low end of normal for the sex steroids.

The second recently reported mutation[118] was a 9 bp deletion in exon 2 resulting in deletion of amino acid residues 10-12 of LHβ in a man and his sister. Both were homozygous for the deletion while two additional unaffected siblings were heterozygous. In spite of undetectable levels of LH, low serum and intratesticular testosterone, the man had complete spermatogenesis and normal sperm count. Presumably, the low activity by the mutant LH detected *in vitro* was sufficient for normal spermatogenesis. The sister underwent normal puberty and menarche, but subsequently had amenorrhea, infertility, ovarian cysts, and low estradiol levels.

Recently, rare missense heterozygous mutations in the hCGβ subunit have been identified in a Northern European population.[105,119,120] The Val56Leu mutation in the *CGB5* gene was identified in a patient with recurrent miscarriage (RM). Although the amount of mutant hCGβ subunit assembled into the heterodimer was very low, it elicited a strong signaling response on binding to the receptor.[119] Arg8Trp mutation, also found in an RM patient did not affect assembly. The Pro73Arg mutation found in five individuals (three RM and two controls) caused about half of the secreted β-subunits to acquire an alternate conformation, but did not affect biological activity. Individuals homozygous for these mutations have not been identified, perhaps because such genotypes would result in complete pregnancy failure and suggests that only mutations with mild functional consequences can be tolerated in the major *CGB* genes.

Several mutations have been described in exon 3 of *FSHB* that lead to an absence of pubertal development, amenorrhea, and infertility in females and azoospermia in males.[105,115,116] The first report was on a 27-year-old woman presenting with the above symptoms and undetectable serum FSH both before and after administration of GnRH. She was found to be homozygous for a two base-pair deletion at codon 61 (Val61Stop) of *FSHB* causing a frameshift, namely an alteration of codons 61-86, and premature termination of the β subunit. A similar mutation was found in an 18-year-old male who was evaluated for delayed puberty.

Other reports identified a compound heterozygous mutation at codon 51 leading to a Cys51 to Gly replacement and Tyr76Stop nonsense mutation.[105,115,116] A missense mutation was found in a 28-year-old male who presented with infertility and was found to have Cys82 replaced with Arg[105] and recently a frameshift at codon 79 (Ala79Stop) caused by a 1 bp deletion and premature termination.[121] One case of hypoglycosylation was reported for FSH, resulting in a hormone with diminished activity.[122]

Overall, the observed phenotypes associated with the naturally occurring mutations in *LHB*, *CGB*, and *FSHB* are consistent with the known structures and actions of the gonadotropins, although fertility in men is not totally dependent on FSH as it is in women.

Polymorphisms in *LHB, CGB,* and *FSHB* Genes

A fairly well characterized polymorphism in LHβ appears in variable frequencies in ethnic groups throughout the world and results from two single nucelotide polymorphisms (SNPs) leading to replacements of Trp8 with Arg and Ile15 with Thr.[105,115,116] There is, however, no association of this polymorphism with assessed phenotypes of infertility, polycystic ovarian syndrome or cancer,[105,115,116] although biopotencies are increased *in vitro* with altered half-life in circulation.[123,124] Individuals harboring this double polymorphism appear to exhibit low, if detectable, levels of immunoreactive LH, but this results from the use of antibodies that do not recognize the altered LH. The Thr15 mutant has an additional site of glycosylation, but Arg8 may be responsible for most of the altered properties.

Another LHβ variant is a replacement of Gly102 with Ser, resulting in reduced LH biopotency *in vitro*.[105,115,116] The frequency of this polymorphism, however, seems to be quite low.

An unusual polymorphic variant of LHβ involves an Ala to Thr replacement three residues before the signal peptide cleavage site.[115,116] Using *in vitro* assays, it was found, rather surprisingly, that the mature protein from the variant appears less potent than wild type LH in cAMP production, but more potent in inositol phosphate production. The SNP-related alteration may interfere with proper processing of the β subunit, although studies have not addressed this possibility.

A polymorphism has been reported in exon 3 of *CGB5*, resulting in a Val79 replacement with Met.[105,115,116] This SNP results in a β subunit deficient in folding and interaction with the α subunit. The frequency and physiological consequences of this polymorphic variant are unknown; one sampling of just under 600 samples from four European groups failed to detect a single case.[115,116] Other polymorphic variants have been detected, but these were silent or located in intron regions.[115,116] A case study in which the *CGB5* and *CGB8* genes were analyzed in RM (n=184) and control fertile (n=195) patients from Estonia and Finland, 71 polymorphisms were identified of which 48 were novel.[120] A protective effect against RM was associated with two single nucleotide polymorphisms located at identical positions in *CGB5* and *CGB8* and with four *CGB5* promoter variants. These findings suggested that polymorphisms in the *CGB* gene might contribute to the susceptibility to RM. These polymorphisms can be found in the SNP database (http://www.ncbi.nlm.nih.gov/SNP/), accession nos. ss105106983-ss105107053.

A very limited number of silent polymorphic variants of *FSHB* have been reported.[105,115,116] Recently, a *FSHB* promoter polymorphism (-211G/T) identified in a cohort of Estonian men has been associated with reduced serum FSH levels in GT heterozygotes and TT homozygotes.[125] This polymorphism was found to be more prevalent among infertile men.[126] The *LHB* and *FSHB* polymorphisms can be found at the SNP database: (www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=3972 and www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=2488).

Regulation of Expression and Secretion of the Gonadotropins

Transcriptional Regulation

The neurocrine reproductive axis, composed of the hypothalamus, anterior pituitary and gonads, is now known to be regulated, apparently in large part, by kisspeptin, a product of *KISS1*, acting via the G protein-coupled receptor, GPR54, located on GnRH neurons.[127,128] The three gonadotropin subunit genes in the pituitary are differentially responsive to GnRH pulse frequency and magnitude: *CGA* being preferentially transcribed at high GnRH pulse frequencies, *LHB* at intermediate frequencies, and *FSHB* at lower frequencies.[129] Sex steroid-mediated regulation of the gonadotropin genes may result from direct effects on the hypothalamus and pituitary, and recent evidence suggests a critical role of the kisspeptin-GPR54 system in sex steroid action.[130-132]

Binding of GnRH to its receptor in the gonadotropes primarily activates $G\alpha_{q/11}$ resulting in the activation of phospholipase Cβ, calcium influx, activation of protein kinase C (PKC), and calcium-calmodulin kinase II.[133,134] PKC

mediates the downstream activation of the mitogen activated protein kinase (MAPK) cascades: extracellular signal regulated kinase (ERK1/2), jun N-terminal kinase (JNK) and P38.[134-136] The MAPK pathway can also be activated by GnRH stimulation of $G\alpha_s$[137,138] and while both the $G\alpha_s$ and $G\alpha_{q/11}$ pathways are responsive to pulses of GnRH, only the $G\alpha_{q/11}$ pathway is responsive to constant GnRH.[138] A recent study suggests that the different G proteins regulate the pattern of FSH and LH expression by GnRH. FSHβ induction depends predominantly on $G\alpha_{q/11}$ while LHβ expression depends on $G\alpha_s$. Interestingly, $G\alpha_s$ signaling induced inhibin α, which acted as an autocrine regulatory factor to suppress FSHβ expression.[139]

Our understanding of the mechanisms that mediate transcription of the pituitary gonadatropin genes has primarily come from studies on the rodent genes in two murine immortalized gonadotrope cell lines, αT3-1 and LβT2[134,140] and several excellent recent reviews on the regulation of these genes are available.[134,141-146] GnRH activation of the MAPK signaling cascade increases transcription of the immediate early genes including *Egr1*, *Jun*, and *Atf3* which encode the DNA proteins early response protein 1 (EGR1), JUN and activating transcription factor 3 (ATF3), respectively. The orphan nuclear receptor, steroidogenic factor 1(SF1), ATF3/JUN heterodimer, LIM homeobox protein 3 (LHX3), and paired-like homeodomain transcription factor 1 (PITX1) are required to activate C*ga*.[145,146] EGR1, SF1, and PITX1 form a tripartite complex that bind to a highly conserved proximal promoter sequence to synergistically activate the *LHB/Lhb* gene and βcatenin serves as a co-activator of SF1 to maximally activate the *Lhb* gene in response to GnRH.[134,144,145] Activator protein 1 (AP1), SF1, nuclear factor Y, PITX1, and LHX3 bind to and activate the *Fshb* promoter.[134,141,143,145,146]

Activin induces *Lhb* and *Fshb* gene expression by interaction of the Smads with the activin response elements. Binding of FOXL2, a member of the forkhead family of transcription factors, to the *Fshb* promoter is essential for activin responsiveness and the runt-related transcription factors RUNX are negative regulators of *Fshb* gene expression.[147]

The gonadal steroids can act on gonadotropin promoters in the pituitary to positively or negatively regulate their expression. The ligand-bound estrogen receptor α (ERα) indirectly binds to the *Lhb* promoter sequence through interactions with SF1 and PITX1 to induce expression.[134,145] There is little evidence to support regulation of *Fshb* expression by estrogen in rodents. Androgens suppress transcription of *Lhb* and increase transcription of *Fshb* via interactions of the androgen receptor (AR) to the hormone response elements on the promoter sequences.[134,145] Progesterone suppresses *Lhb* gene expression in gonadotrope cells and this is thought to be mediated by indirect binding of the progesterone receptor (PR) to the promoter.[134] In contrast, progesterone induces the expression of *Fshb* in gonadotrope cells by binding of PR directly to the *Fshb* proximal promoter. Recent studies have shown that activin and progesterone act synergistically to mediate *Fshb* transcriptional regulation in gonadotrope cells[134] and FOXL2 is necessary for the full synergistic response.[148]

It has been proposed that in the trophoblast layer of the placental villous, association of the cAMP response binding protein (CREB) and v-ets erythroblastosis virus E26 oncogene homolog 2 (ETS2), augmented by protein kinase A, regulates C*GA* expression. An upstream regulatory element (URE) on C*GA* contains binding sites for several transcription factors, and a second control element, alpha-activating elements (αACT), binds a GATA factor and AP2γ. The C*GB5* promoter binds both CREB and ETS2, and an AP1-like site has been identified.[149-151]

Post-Translational Regulation (Glycosylation)

It is now well established that the glycosylation patterns of at least some of the glycoprotein hormones change during various physiological states.[14] Examples include a shift in the structures of hCG N-linked oligosaccharides during pregnancy in the differentiation of cytotrophoblasts to syncyciotrophoblasts,[18,152-155] changes in LH and FSH N-linked glycosylation during the menstrual cycle[156] and alterations in FSH N-linked glycans during adolescence in boys.[14,17] Indeed, a portion of FSH contains a non-glycosylated β subunit.[24,157]

The circulatory half-life of the sulfated gonadotropins, notably LH, is invariably less than that of the sialic acid-containing hormones. This arises from an hepatic receptor that recognizes the terminal N-acetyl galactosamine-sulfate, rapidly removing it from circulation.[158] Indeed, ablation of the gene encoding GalNAc-4-sulfotransferase, the enzyme responsible for modifying the terminal GalNAc in LH with sulfate, resulted in mice with increased half-life and circulating levels of LH.[159]

Various laboratories have shown that the hyperglycosylated variant of hCG is produced in early pregnancy, i.e., from cytotrophoblasts, and in gestational trophoblastic diseases whereas hCG is secreted by syncytiotrophoblast.[153,154,160,161] Hyperglycosylated hCG molecules have enhanced branching into triantennary and unusual biantennary N-linked oligosaccharides and a prevalence of the more complex Core 2, as opposed to Core 1 O-linked sugars.[161,162] In clinical studies, hyperglycosylated hCG is identified by the binding of a monoclonal antibody B152 that specifically recognizes the Core 2 O-linked sugars at Ser 132.[163,164] It has been suggested that in addition to the canonical physiological function of "rescuing" the corpus luteum, hyperglycosylated hCG acts in an autocrine/paracrine manner to promote trophoblast invasion of the placenta.[18,19,165,166] There are also data suggesting that the hCG produced by cytotrophoblasts and choriocarcinoma have distinct carbohydrate moieties.[167] The N-linked oligosaccharides on hCG from invasive mole and testicular cancer are characterized by both biantennary and triantennary structures and often more heavily fucosylated glycans, while the four O-linked units tend to have more core-2 type structures.[27]

Samples from patients with choriocarcinoma, testicular cancer and invasive mole showed interesting differences in their glycans. Triantennary N-linked glycans increase in choriocarcinoma[161] at Asn30 but not at Asn13,[27] while the reported increase in monoantennary N-linked glycans was observed at both Asn13 and Asn30.[27] The status of hCG fucosylation in pregnancy and cancer patients has been investigated by several groups[168] with some conflicting results. In malignancies,

fucosylation was recently reported to increase at Asn13, but not at Asn30.[27]

Regulation of Secretion

The pathways of secretion and polarity of hormone release are different for the various gonadotropins. Although LH and FSH are synthesized by the same cell, LH is packaged in dense storage granules[169] with regulated secretion occurring from the basolateral surface under the control of the pulsatile secretogogue, GnRH.[170-172] In contrast, FSH secretion is constitutive, linked to its synthesis and exhibits no apparent polarity of secretion.[170-173] The sorting determinant for the regulated secretion of LH lies in the hydrophobic heptapeptide at the carboxy terminus of LH[174,175] and a single leucine residue at position 118 contributes to the sorting of LH via the regulated pathway. Recent studies have demonstrated that sulfation of LH does not play a role in its regulated secretion,[176] but may be important for its extracellular bioactivity.[159]

Although LHβ and CGβ share 85% sequence identity and are functionally interchangeable, hCG is not stored in granules, but rather is secreted constitutively into the maternal circulation at the apical side of trophoblasts.[177] The CTP sequence, unique to hCG, is the important determinant in the constitutive secretion[178] and the O-linked oligosaccharides in the CTP are essential for the apical release of hCG.[179]

Expression in Physiological and Pathophysiological Conditions

The accepted physiological action of hCG is in the initiation and maintenance of pregnancy. In the gonads it maintains functionality of the corpus luteum and progesterone production, particularly during the first trimester of pregnancy. It also mediates multiple placental, uterine, and fetal functions including trophoblast invasion; development of syncytiotrophoblast cells, uterine growth and differentiation; placental development and localized suppression of the immune system.[180] In addition, hCG is located in pituitary,[181] but its physiological significance is unknown. As with pituitary hCG, there are reports of small amounts of glycoprotein hormone synthesis in various non-pituitary and non-placental tissues,[44,182] but specific functions have not been ascribed to these ectopically produced hormones. The major forms of circulating LH and hCG have been delineated, along with their patterns in normal physiological conditions and various disorders.[156]

There is ample evidence supporting ectopic production in a variety of disorders. It is well known that hCG is expressed in malignant forms of gestational trophoblastic disease, e.g., invasive mole and choriocarcinoma.[155,168,183,184] In men and women, hCGβ, hyperglycosylated hCGβ, and only occasionally intact hCG, is expressed in a variety of other malignancies, including breast, bladder, colorectal, gynecological, head and neck, hematological, lung, neuroendocrine, oral/facial, pancreatic, prostate and testicular cancer.[184-188] The detection of free CGβ subunit in these malignancies is generally associated with poor prognosis.

In an analysis of human *GCA*, *LHB*, and *CGB* gene expression in breast cancer, studies showed that most normal tissues expressed only *CGB7*, while *CGB3*, *CGB5*, and *CGB8* were expressed in trophoblastic tissues and correlated with the malignant transformation of breast cancer and other non-trophoblastic malignancies.[112,189] Human *CGA*, *LHB*, *CGB1*, *CGB2*, and *CGB7* were not, however, up-regulated in breast cancer.

A possible role of LH in the etiology and progression of Alzheimer disease has been postulated.[190-193] Of interest was the observation that LH modulates the processing of the amyloid-β precursor protein yielding deposition of amyloid-β peptide.[194] Genetic ablation of *Lhcgr* in amyloid precursor protein transgenic mice improved the amyloid pathology suggesting that chronic elevation of LH may promote the amyloid β plaque formation.[195]

Diagnostic and Therapeutic Applications of the Gonadotropins

Immunoassay-based measurements of the serum concentrations of pituitary-derived gonadotropins have been used extensively to monitor functionality of the hypothalamic-pituitary-gonadal axis, and measurements of urinary concentrations of hCG are widely used for pregnancy determinations and management, as well as monitoring trophoblastic malignancies. Hyperglycosylated hCG is the preferable form for the early detection of pregnancy or its complications.[163] In addition, studies have shown that hyperglycosylated hCG can be used to detect Down syndrome pregnancies,[164,196] particularly when coupled with other serum markers, α-fetoprotein and estradiol.[197] hCGβ and hyperglycosylated hCGβ are used as tumor markers for the detection of malignancies.[168,184] Immunocytochemistry is also commonly used in evaluating expression of hCGβ in suspected tumor tissue sections. It has been recognized for decades, that multiple variants of hCG are present in normal pregnancy, giving rise to microheterogeneity and macroheterogeneity.[198] These include intact or heterodimeric hormone, nicked hCG, heterodimeric hormone with bond cleavages in the hCGβ 43-48 region, free α and β subunits, nicked hCGβ core fragment (i.e., free β subunit with bond cleavages in the 43-48 region), and hCGβ core fragment, consisting of two disulfide-linked fragments, 6-40 connected to 55-92.[154] Hyperglycosylated hCG can present with a similar number of derivatives. Some of these variants may be useful diagnostic tools for germ cell and gynecological malignancies.[168] Therefore, hCG, assays must be capable of distinguishing the variants. Several reviews, workshop proceedings, and reports have addressed this issue and the challenges of obtaining and using appropriate standards.[18,167,168,183,198] The preparation and adoption of universal standards, coupled with complete characterization and disclosure of antibody specificities, will greatly facilitate standardization of glycoprotein hormone immunoassays.

While immunoreactivity is the primary technique for determining hormone concentrations in body fluids, it is often necessary to measure bioactivity. The earlier cumbersome *in vivo* assays for the glycoprotein hormones have, by and large, been replaced with radioreceptor and signaling assays in transfected cells. Such measurements provide quantitative data on hormone-receptor binding and efficacy of signal transduction, but they give no information on circulatory half-life and, thus, *in vivo* potency. For this, animal and human studies are obviously required.

Therapeutically recombinant gonadotropins expressed in Chinese hamster ovary cells are used in the treatment of infertility. The longer circulatory half-life of hCG, attributed to the β subunit, CTP, has been utilized most effectively in producing long acting analogs of FSH and TSH with a CTP engineered to the β C-terminus.[199-202] Another approach that has proven successful in extending the half-life of FSH is an engineered extension at the α N-terminus with two sites of N-glycosylation.[203] The resulting analog was glycosylated as judged by mobility on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and exhibited increased circulatory half-life and *in vivo* potency.

Recently, a number of reports have appeared suggesting that hCG, in particular, could be used therapeutically in the treatment of cancer. A recent phase I clinical trial has shown that administration of hCG to postmenopausal breast cancer patients led to a reduction in the proliferative index (Ki67) and the levels of estrogen and progesterone receptors.[204] In contrast, female transgenic mice overexpressing hCG or LH exhibit multiple sites of tumorigenesis.[205] A conjugate of the lytic peptide, hecate (a 23-amino acid residue peptide, similar to bee venom mellitin, that disrupts cell membranes), to a 15-amino acid residue peptide from hCGβ (residues 81-95) was found to kill cultured prostate and ovarian cancer cells and reduce the tumor xenografts in nude mice.[206-209] In a transgenic mouse model, it was found that the hecate-hCGβ 15-amino acid residue peptide reduced malignant Leydig and granulosa cell tumors via necrosis or necrosis-like cell death.[210] While it is surprising that such a short hCGβ peptide is capable of LHR binding, the results are quite dramatic and may lead to specific therapies of LHR-positive tumors.

Gonadotropin Receptors

Gonadotropin Receptor Proteins

Amino Acid Sequences and Three-Dimensional Structures

The fully processed human LHR and FSHR (http://www.ncbi.nlm.nih.gov/gene/3973, and http://www.ncbi.nlm.nih.gov/gene/2492) are 675 and 678 amino acid residues long, respectively. By convention, the amino acid residues of the hLHR and hFSHR have been numbered from the initiator methionine of their precursor sequences obtained by virtual translation of the open reading frames of the cognate DNA (cDNA).[211,212] Algorithms that predict the most likely site of cleavage of signal peptides predict these to be between residues 24 and 25 of the hLHR and residues 17 and 18 of the hFSHR. Therefore the N-terminus of the mature hLHR and hFSHR are predicted to be Leu25 and Cys18 respectively. An alignment of the amino acid sequences of the hLHR and hFSHR is presented in Figure 2.4.

When grouped with the TSH receptor, the gonadotropin receptors form the glycoprotein hormone receptor family. One can readily recognize three distinct domains in this family of receptors, a large N-terminal domain that contains about 300 residues and is predicted to be extracellular, a serpentine region containing seven transmembrane segments connected by three extracellular loops and three intracellular loops, and a C-terminal tail that is predicted to be located intracellularly (Fig. 2.4). The presence of seven transmembrane segments suggests that the gonadotropin receptors are members of the superfamily of G protein-coupled receptors (GPCRs). The GPCR superfamily can be readily divided into several major subfamilies and the glycoprotein hormone receptors belong to the rodhopsin/β_2-adrenergic receptor-like subfamily of GPCRs.[213]

The amino acid sequences of the ECD of the gonadotropin receptors are 46% identical and this region is of particular interest because it is responsible for the recognition and high affinity binding of the hormones to their cognate receptors.[211,214,215] It can be divided into three subregions: an N-terminal cysteine-rich region, a region composed of several copies of a structural motif rich in leucine and other hydrophobic residues (the leucine-rich repeat, LRR), and a C-terminal cysteine-rich domain also known as the hinge region (Fig. 2.4).

The crystal structures of a large portion of the ECD of the hFSHR (residues 1-268, Fig. 2.4) complexed with a single chain hFSH analog has provided much needed light into the three dimensional structure of this important receptor region and its involvement in ligand binding.[9] The nine LRRs predicted from the primary structure form nine parallel β sheets as expected but an additional β sheet is comprised of residues in the N-terminal cysteine rich region. The first seven β sheets form a fairly flat structure but the last three have a horseshoe-like curvature that gives the ECD the overall shape of a slightly curved tube (Fig. 2.5A). The individual LRRs are irregular in length and conformation but they each have a β strand that collectively form the concave surface and a coiled structure that collectively form the outer surface of the LRR domain (Fig. 2.5A). FSH is bound to the concave surface of the ECD of the FSHR like two hands clasping each other with the receptor wrapping itself around the middle section of the hormone (Fig. 2.5A). All 10 β strands (as well as additional structures) of the LRRs of the hFSHR are in contact with the hormone and many of these contacting amino acid residues of the hFSHR are also conserved in the other two glycoprotein hormone receptors (Fig. 2.5A and B). The contact surface area between the hormone and the receptor is large and highly charged.

As expected both hormone subunits participate in receptor binding. Important points of receptor contact for the hormone involve the C-terminal portions of the α and β subunits as well as the α and ββ L2 loops (Fig. 2.5C and D). A comparison of the crystal structure of free hFSH[8] with that of the receptor-bound hormone[9] revealed that the structures of the free and bound hFSH are quite similar but the hormone is more rigid when bound to the receptor. The most obvious change in the hormone is on the C-terminus of FSHα which becomes buried at the receptor interface where it forms contacts with receptor residues that are highly conserved among the three glycoprotein hormone receptors.[9]

The crystal structure of large region of the ECD of the TSHR in complex with a TSHR autoantibody has also been solved.[216] Although the number of LRRs is different between the TSHR and the FSHR the overall structure of the ECD of the TSHR is very similar to that of the ECD

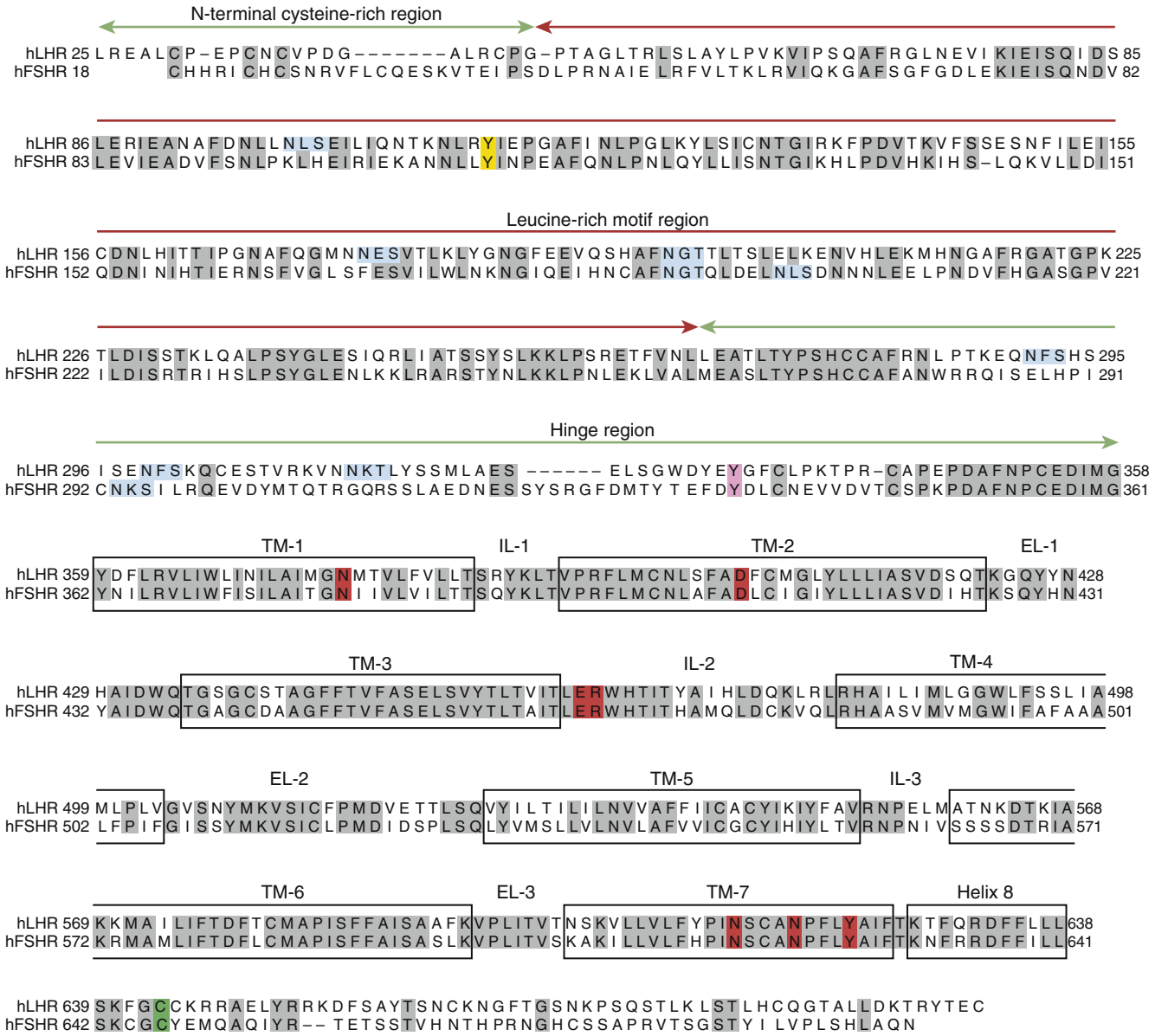


FIGURE 2.4 Amino acid sequence alignment of the human luteinizing hormone receptor (hLHR) and human follicle-stimulating hormone receptor (hFSHR). Amino acid sequences were obtained from a public Web site (www.ensembl.org/index.html). The boundaries of the three distinct regions of the extracellular domain discussed in the text (N-terminal cysteine-rich region, leucine-rich motif region, and hinge region) are marked with *green, red,* and *green arrows,* respectively. The seven transmembrane (TM) helices and the putative cytoplasmic helix 8 are delineated by *black boxes* and labeled TM-1 through TM-7 and helix 8, respectively. The three extracellular (EL) and four intracellular (IL) loops that connect the transmembrane regions are labeled EL-1 through EL-3 and IL-1 through IL-4, respectively. Identical residues between the two receptors are shown with *gray boxes*. The consensus sequences for N-linked glycosylation are shown with *blue boxes*. The tyrosine that participates in dimer formation for the FSHR and is conserved in the hLHR is shown in *yellow*. The conserved tyrosines that may be sulfated are shown in *pink*. The conserved cysteines that are believed to be palmitoylated are shown with the *green box*. Residues that are highly conserved among the rhodopsin/β2-adrenergic family of G-protein–coupled receptors are shown in *red. (Copyright © 1999–2008 The European Bioinformatics Institute and Genome Research Limited, and others. All rights reserved.)*

of the FSHR.[216] Interestingly the TSHR surface that binds the autoantibody is remarkably similar to the surface of the FSHR that binds FSH.[216]

The crystal structure also revealed that the FSH/FSHR complex forms a dimer using the outer surface of LRRs 2-4 in the hFSHR. Mutation of one of the residues predicted to be on this surface (Tyr110, highlighted in yellow in Figure 2.4) did not affect the dimerization of the hFSHR expressed in heterologous cell types, however.[217] The crystal structure of the TSHR in complex with a TSHR antibody did not show any dimers.[216]

Since the hinge region is missing from the two ECD crystal structures, nothing is known about its contribution to the overall conformation of the ECD or the receptors. The finding that residues 1-268 of the hFSHR (the fragment used for the crystal structure) binds hFSH with high affinity suggests that the hinge region of the hFSHR is not involved in binding. Likewise, a number of laboratory-designed and

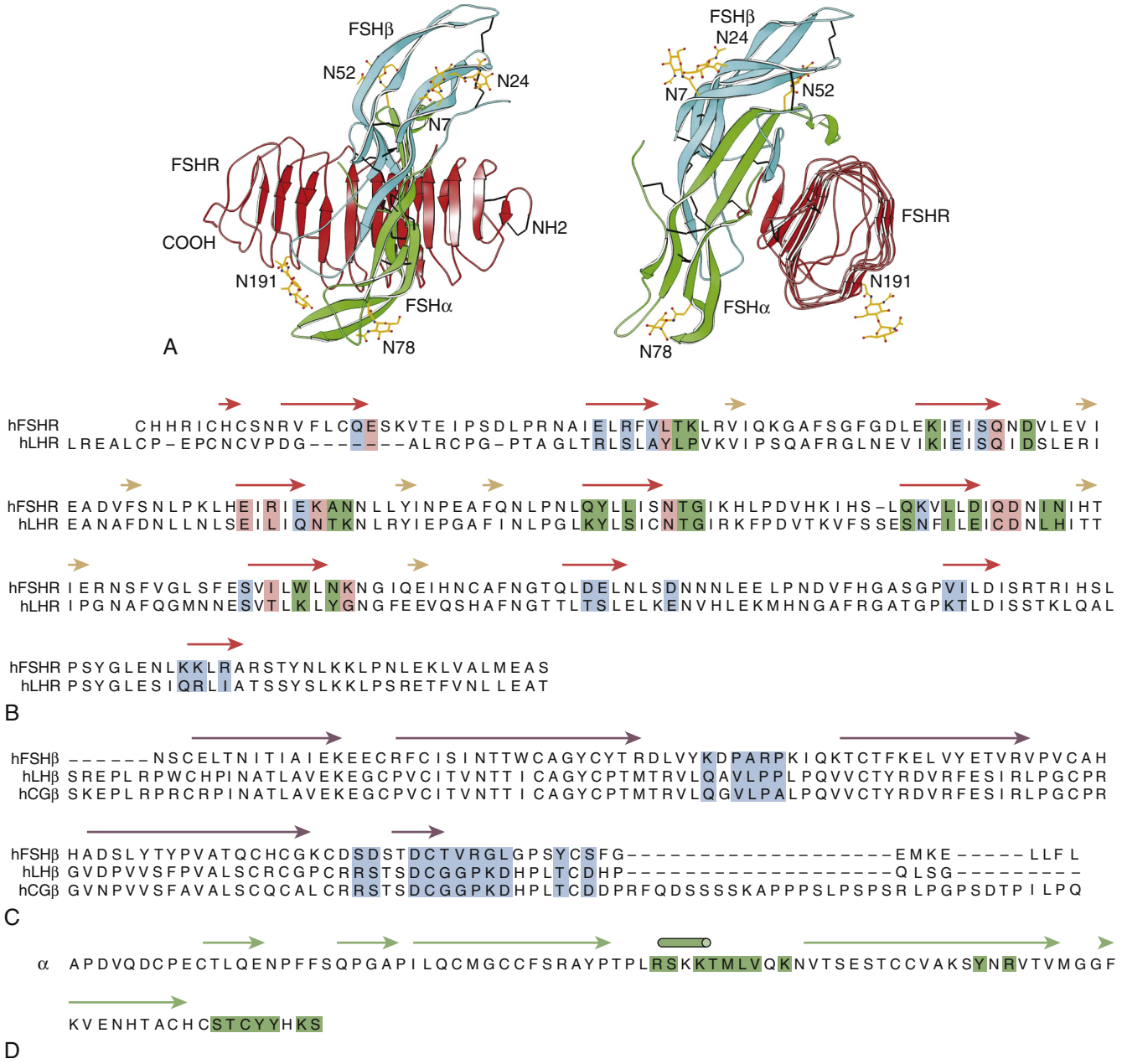


FIGURE 2.5 Interactions between the gonadotropin receptors and their cognate hormones. **A,** Ribbon diagrams of two views of the human follicle-stimulating hormone (hFSH)/follicle-stimulating hormone receptor (FSHR) ectodomain (ECD) rotated 90° around the vertical axis. The α and β chains of FSH are shown in *green* and *blue,* respectively, and the ECD of the FSHR is shown in *red*. Disulfides are shown in *black,* and the N-linked carbohydrates (of the hormone and the receptor) present in the crystal structure are shown in *yellow*. **B** to **D,** Sequence alignments and secondary structure of portions of the ECD of the two gonadotropin receptors **(B),** the β-subunits of the three gonadotropins **(C),** and the common α subunit **(D)**. *Arrows* designate β strands and *cylinders* designate α helices. For the receptor ectodomains **(B),** β strands located on the concave face of the FSHR ectodomain are shown in *red,* and those located in the convex face are shown in *beige*. The FSHR ectodomain residues shown in *green, blue,* or *pink* are buried at the receptor–ligand interface by the α subunit, the β subunit, or both, respectively. The residues in FSHβ shown in *blue* **(C)** and those shown in green on FSHα **(D)** are buried at the receptor interface. hCG, human chorionic gonadotropin; hLH, human luteinizing hormone; LHR, luteinizing hormone receptor. *(Modified from Fan QR, Hendrickson WA. Structure of human follicle-stimulating hormone in complex with its receptor.* Nature *433:269–277, 2005. © The Nature Publishing Group [2005].)*

naturally-occurring mutations of the LHR show that the hinge region of the hLHR is not necessary for the high-affinity binding of hLH or hCG.[211] Nevertheless, the high degree of conservation of some hinge region residues in the glycoprotein hormone receptor family (Fig. 2.4) suggests that this region plays an important role in other aspects of receptor function such as activation (addressed later in the text). A highly conserved Tyr present in this region (Fig. 2.4) was shown to be sulfated in the cell surface TSHR and mutation of this Tyr impairs TSH binding and activation.[218] Sulfation of the equivalent Tyr in the LHR or FSHR has not been demonstrated, but mutations of this residue in the

gonadotropin receptors also impair hormone binding and activation.*[218]

The serpentine domain of the gonadotropin receptors is characterized by the canonical GPCR structure containing seven transmembrane (TM) segments joined by three alternating intracellular and extracellular loops (Fig. 2.4). The amino acid sequences of this region of the hLHR and hFSHR are 72% identical (Fig. 2.4). A three dimensional structure of the transmembrane domain of the gonadotropin receptors is lacking but the three dimensional structure of several other GPCRs with short extracellular domains have now been solved[213] (also see http://gpcr.scripps.edu) and the transmembrane domain of the gonadotropin receptors is likely to be very similar. Transmembrane domain residues that are highly conserved among the rodhopsin/β_2-adrenergic receptor-like subfamily of GPCRs are also highlighted in Figure 2.4.

Surprisingly, the intracellular domains of the gonadotropin receptors are the most divergent of the three domains (~27% identity, Fig. 2.4). An intracellular cysteine residue present in the juxtamembrane region of the C-terminal tail of the rodhopsin/β_2-adrenergic receptor-like subfamily of GPCRs is, however, among the most highly conserved residues of this subfamily of GPCRs and all members of this subfamily examined to date have been shown to be palmitoylated at this site. This cysteine is towards the C-terminal end of a cytoplasmic helical segment of other GPCRs that is referred to as helix 8 (http://gpcr.scripps.edu) and the palmitate present at this highly conserved position is thought to be embedded in the membrane. The LHR is unusual in having two adjacent cysteines in this position (Fig. 2.4). Although the palmitoylation of the hLHR has not been studied, the mature form of the rLHR expressed in 293 cells, has been shown to be palmitoylated at both of these residues.[211] The equivalent cysteine in the hFSHR is also palmitoylated.[219]

Glycosylation

The ECDs of the hLHR and hFSHR have six and three consensus sites for N-linked glycosylation, respectively (Fig. 2.4). Although it is clear that the hLHR and hFSHR contain N-linked carbohydrates, studies determining whether all potential sites for carbohydrate attachment are utilized have not been performed with the human receptors. This question has only been addressed for both the recombinant rat rLHR and rFSHR expressed in heterologus cell lines as well as the endogenous porcine (p) LHR. In the LHR at least five, or perhaps all six of the glycosylation sites have been reported to be glycosylated.[211] A single study done with the recombinant rFSHR expressed in mammalian cells concluded that only two of the three glycosylation sites are glycosylated.[220]

The FSHR/FSHR complex used in the crystallography studies is partially deglycosylated and full carbohydrate structures are therefore not available. It is clear, however, that carbohydrate residues are present in all four potential glycosylation sites of the hormone (αAsn52, αAsn78, βAsn7, and βAsn24) as well at the first potential glycosylation site of the receptor (Asn191). It is also clear that these are not part of the hormone/receptor interface (Fig. 2.5). Similarly, the potential glycosylation sites of the TSHR are located on the surface of the TSHR that is not involved in binding autoantibodies.[216] These data are consistent with the findings that glycosylation of the gonadotropins or the receptors are not required for hormone binding.[211]

Folding, Maturation, and Transport to the Plasma Membrane

Mammalian cells expressing the recombinant hLHR display several distinct glycoprotein species with molecular masses (estimated from SDS SDS gels) ranging from 65 to 240 kDa.[211] The mature LHR present at the cell surface has been identified as an 85-95 kDa protein whereas a 65-75 kDa band has been identified as an immature, partially glycosylated precursor that is located in the endoplasmic reticulum.[211] The higher molecular weight bands ranging from 165-240 kDa are oligomers of the immature or mature receptors.[211,221] Precursor, mature, and oligomerized forms of the hFSHR can also be detected in transfected cells, but there is more variation on the reported molecular weights of these products. Estimates for the molecular mass of the mature cell surface FSHR range from 74 to 89 kDa whereas estimates for the molecular mass of the immature intracellular precursor range form range from 67 to 82 kDa.[220,222-224] Higher molecular weight forms of the FSHR (~170 kDa) have also been identified and appear to be oligomers of the immature intracellular precursor.[224]

Two different studies using the hLHR or rLHR and site directed mutagenesis, removal of carbohydrate moieties by glycosidase treatment or pharmacological inhibition of carbohydrate attachment, suggest that the nonglycosylated LHR can fold properly, transit to the cell surface (albeit at reduced levels), bind hormone and transduce signals.[211] A similar series of studies done with the rFSHR suggests that one of the two carbohydrate moieties present in this receptor (as stated earlier in the text) is required for the proper folding of the nascent receptor into the conformation that can bind FSH with high affinity.[220] Interestingly the intracellular precursor of the LHR can bind LH and hCG with high affinity,[211] but the intracellular precursor of the FSHR cannot bind FSH.[225]

Many of the studies on the sizes and nature of the different forms of gonadotropin receptors have been done by immunological detection of the epitope-tagged receptors expressed in heterologous cell lines. Immunological detection of the endogenous gonadotropin receptors expressed in

*Shortly after the submission of this Chapter the crystal structure of the entire ectodomain of the FSHR in complex with FSH was published (see paper by Jiang et.al. in the list of recommended readings). This important publication showed that the ectodomain of the FSHR is composed of a short N-terminal region followed by 12 leucine-rich repeats and a short C-terminal region. Importantly, there is a hairpin loop between the 11th and 12th repeats. This overall structure of the ECD hormone complex resembles a right hand holding an American football where the hairpin loop is the thumb of the hand. This structure now suggests that FSH first binds to a hormone binding subdomain composed of LRRs 1-8 and that this interaction induces a conformational change in the L2β loop of FSH that promotes the formation of a second binding site where this loop interacts with LRR 8 and 9 as well as the insertion of a sulfated tyrosine residue in the receptor (Y335, highlighted in yellow in Fig 2.4) between the α and β subunits of FSH. This interaction of the sulfated receptor tyrosine with the hormone is now thought to be essential to the activation of the receptor and the initiation of signal transduction.

the testes or ovaries has been much more difficult because they are expressed at low densities and because of the nature of the gonadotropin receptor antibodies available. Using monoclonal or polyclonal antibodies that have been rigorously validated, the mature and immature forms of the LHR and FSHR described above can indeed be immunologically detected in target tissues.[211]

Low Molecular Weight Gonadotropin Receptor Agonists and Antagonists

Since there is a physical separation between the region of the gonadotropin receptors that binds the hormones (ECD) and the region involved in receptor activation (TM domain, introduced earlier in the text) there must be a way by which binding of the hormones to the ECD transmits a signal that induces conformational changes in the TM domain. In contrast to what is known about conformational changes in the TM domain of other GPCRs, however,[213,226] little is known about these potential changes in the gonadotropin receptors. Nevertheless, the importance of the TM domain of the gonadotropin receptors in receptor activation is underscored by the discovery of a number of low molecular weight (LMW) compounds that interact with the TM domain of the gonadotropin receptors and either serve as allosteric agonists or antagonists.

The first LMW non-peptide gonadotropin receptor agonists and antagonists were identified using high-throughput screening approaches.[227,228] One of the compounds identified in these screens was Org 43553 (molecular weight ~500), a nanomolar potent, allosteric LHR agonist that interacts with the endodomain of LHR and selectively activates the adenylyl cyclase pathway.[229,230] This is in contrast to LH/CG, which bind to the extracellular domain of the LHR (introduced earlier in the text). Org 43553 exhibited a shorter circulatory half-life than hCG, stimulated testosterone production in primary Leydig cells, and was able to induce ovulation in female mice and rats after a single-dose oral administration.[231] Org 43533 also did not induce ovarian hyperstimulation syndrome (OHSS), in preclinical rodent models.[232] Dimeric ligands based on the parent, Org 43533, are also effective, allosteric LHR agonists.[233] Lastly, as discussed below, another allosteric, LMW LHR agonist, Org 42599, functionally rescued the cell surface expression of intracellularly retained LHR mutants that caused Leydig cell hypoplasia in men.[234]

LMW FSHR antagonists capable of inhibiting ovulation and the steroidogenic response *in vitro* have been developed and may have potential as non-steroidal contraceptives.[227,235] LMW agonists that specifically activate the FSHR through allosteric mechanisms have also been described.[236,237] Oral administration of two recently developed LMW FSHR agonists with a short circulatory half-life resulted in surge-like profiles and induced formation of luteinized unruptured antral follicles thereby preventing ovulation in several animal models without affecting cyclicity.[236] Inhibition of follicular rupture was shown to be reversible within one cycle. These studies present a novel approach for reversible female contraception using the short-acting LMW FSHR agonists.

These LMW compounds[238] appear to have great potential for future clinical applications in the treatment of infertility and perhaps as non-steroidal contraceptives. They offer advantages over current therapies utilizing gonadotropins in that they can be administered orally and the LMW compounds can be synthesized at reduced cost and without the variability seen among the preparations of recombinant hormones.

Other Receptors Related to the Gonadotropin Receptors

The LHR, FSHR, and TSHR are the founding members of a subfamily of GPCRs characterized by the presence of a large N-terminal ECD containing several LRRs (stated earlier in the text). This glycoprotein hormone receptor family, which has been renamed the leucine-rich repeat-containing G-protein-coupled receptor (LGR) family, has been expanded to include five additional LGRs designated LGR4-8.[239] Some of these are also now known as the relaxin family peptide receptors (RXFP).[240] Interestingly, LGR8 (RXFP2) is a receptor for insulin-like peptide 3, which is involved in testicular descent.[241]

The Gonadotropin Receptor Genes and Transcripts

Genes

The human lutropin and follitropin receptors are encoded by single genes located about 200 Kb apart in the short arm of chromosome 2 *LHCGR*; http://www.ncbi.nlm.nih.gov/gene/3973 and *FSHR*; http://www.ncbi.nlm.nih.gov/gene/2492.

The *LHCGR* is about 70 Kb in length and is composed of 11 exons whereas the *FSHR* is about 190 Kb long and is composed of 10 exons. The transmembrane and C-terminal domains of the receptors are encoded by exon 10 for the *FSHR* and by exon 11 of the *LHCGR*. These exons also code for the C-terminal end of the hinge region of the extracellular domain of each receptor. The N-terminal cysteine-rich region, all of the leucine rich repeats, and the N-terminal end of the hinge region of the extracellular domain of these receptors arise from the splicing of the remaining exons.

The 5′ flanking regions of the *LHCGR* and *FSHR* are rich in GC and the proximal promoters of both genes are devoid of TATA boxes.[242,243] The cis- and trans-acting elements that control the transcription of the mouse, rat and human *LHCGR* have been examined in some detail and have been reviewed elsewhere.[242] Initial studies with transgenic mice harboring reporter genes driven by ~7, ~, or ~0.17 kb of the 5′-flanking sequence of the mouse *Lhcgr* express the transgene in adult Leydig cells, but not in fetal Leydig cells or ovarian cells.[244] More recent studies have shown that transgenic mice harboring reporter genes driven by 2-4 kb of the 5′-flanking sequence of the rat *Lhcgr* express the transgene in the gonads, adrenal glands, kidneys, and several areas of the peripheral and central nervous systems.[245,246]

Studies on the transcriptional control of the *FSHR* have been done mostly with the rodent genes and reviewed recently as well.[243]

Transcripts

There is controversial evidence regarding the number and nature of multiple FSHR transcripts.[243] Most of the studies on this issue have been done in sheep and mouse models, and as many as four different transcripts that arise

from alternative splicing and are presumably translated into functional proteins with distinct signaling properties have been reported.[247] Multiple transcripts of the LHR have also been identified and they arise from alternative splicing or from the use of two different polyadenylation domains present in the 3′ flanking region of the *LHCGR*.[242] Although the presence of these transcripts raises intriguing questions regarding their possible physiological roles, it is important to point out that for the most part, data that demonstrate the presence of proteins arising from such transcripts is lacking.

Naturally Occurring Mutations of the Gonadotropin Receptor Genes

A number of naturally occurring mutations of the *LHCGR* and *FSHR* associated with human reproductive disorders have been reported. These are shown in Figures 2.6 and 2.7 and have been extensively reviewed.[116,211,215,242,248,249]

Naturally occurring loss of function or inactivating mutations of the hLHR occur throughout the polypeptide chain as shown in Figure 2.6. These are all germ-line mutations and a phenotype is obvious only in individuals who are homozygotes or compound heterozygotes. In women, inactivating mutations of the hLHR are associated with low estrogen production and annovulatory disorders.[116,211,215,248,249] In men, the most severe phenotype is a complete absence of Leydig cells and pseudohermaphroditism. Other milder phenotypes in 46XY individuals include hypospadias and micropenis.[116,211,215,248,249]

The phenotype associated with the deletion of exon 10 deserves special mention. This mutant was initially found in a 46XY individual who failed to undergo puberty in spite of normal sex differentiation.[250] When expressed in a heterologous cell line a hLHR construct lacking the amino acids encoded by exon 10 (residues 290-316 in the hinge region of the ECD; Fig. 2.4) is transported properly to the cell surface and binds hLH and hCG with high affinity.[251] Interestingly, cells expressing this mutant respond to hCG normally but their sensitivity to hLH was reduced about 40-fold.[251] Therefore, the residues encoded by exon 10 of the hLHR do not participate in hormone binding, but appear to be involved in receptor activation by hLH but not by hCG. The LHR of the marmoset monkey provides an interesting evolutionary parallel to the hLHR variant lacking exon 10. The marmoset pituitary does not express LHβ[252] and exon 10 of the marmoset monkey *LHCGR* is spliced out of the

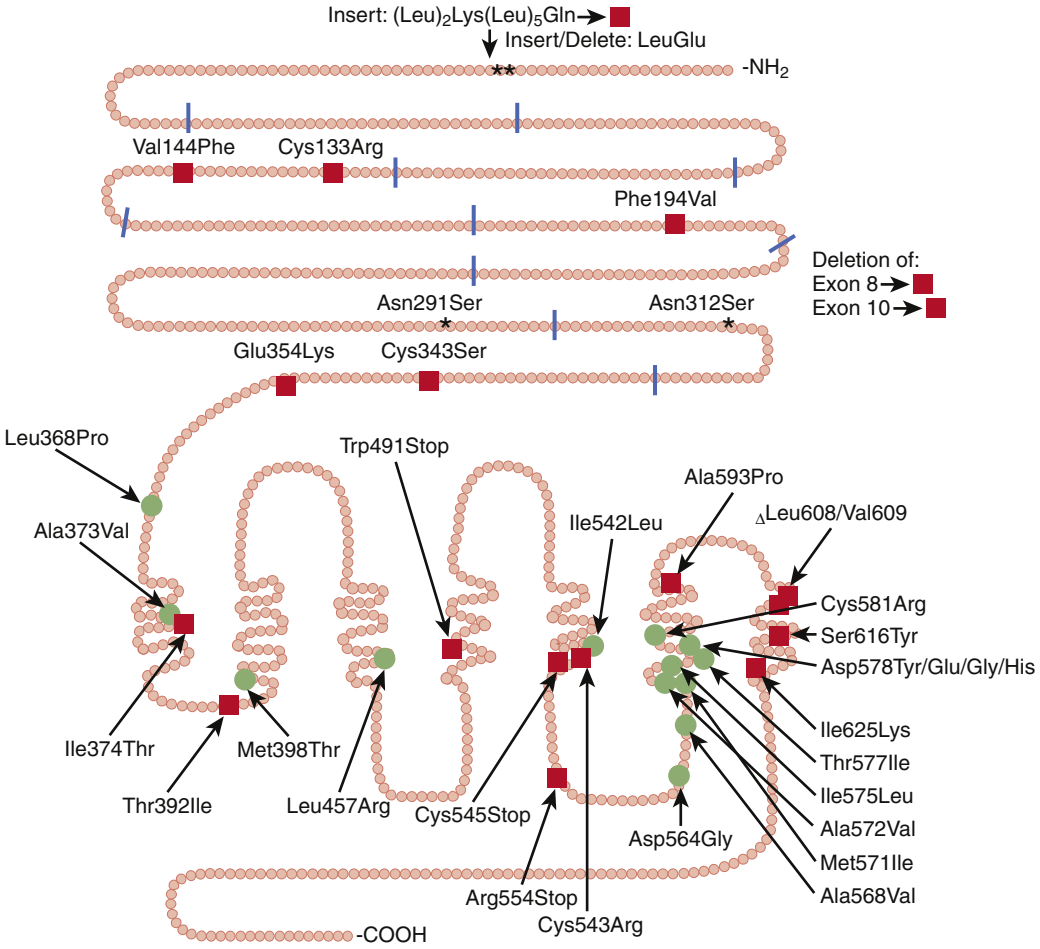


FIGURE 2.6 Location of naturally occurring polymorphisms, loss-of-function mutations, and gain-of-function mutations of the human luteinizing hormone receptor (hLHR). Polymorphisms are shown with *asterisks,* loss-of-function mutations are shown with *red squares,* and gain-of-function mutations are shown with *green dots*. The sequences coded for by the different exons are demarcated by vertical *blue bars. (From Themmen APN. An update of the pathophysiology of human gonadotrophin subunit and receptor gene mutations and polymorphisms.* Reproduction *130:263–274, 2005. © Society for Reproduction and Fertility [2005]. Reproduced by permission.)*

mature messenger ribonucleic acid mRNA[253] yet this receptor can bind hCG with a high affinity and respond to the bound hCG.[253] A recent study has begun to identify regulatory elements of the *LHCGR* that influence the usage of exon 10.[254]

As shown in Figure 2.6, the inactivating mutations of the hLHR can take many forms, ranging from single amino acid mutations, nonsense mutations that result in truncated receptors, small insertions or deletions as well as large insertions or deletions. Although some of these mutations prevent hormone binding or activation (as stated earlier in the text), many of them impair the maturation and/or transport of the hLHR precursor so that the expression of the mature hLHR at the cell surface is completely lost or a least partially reduced.[249] Therefore the phenotype of individuals harboring inactivating mutations of the hLHR is mostly due to a net loss of cell surface hLHR rather than to the expression of a receptor that binds hormone but cannot become activated.

In contrast to the heterogeneous location of the naturally occurring loss-of-function mutations of the hLHR, all naturally occurring gain-of-function mutations reported to date are localized in exon 11 which codes for C-terminal end of the hinge region of the extracellular domain as well as the transmembrane and intracellular regions of the hLHR (Fig. 2.6).[116,211,215,242,248,249] All of these are single point mutations and many of them cluster to transmembrane helix

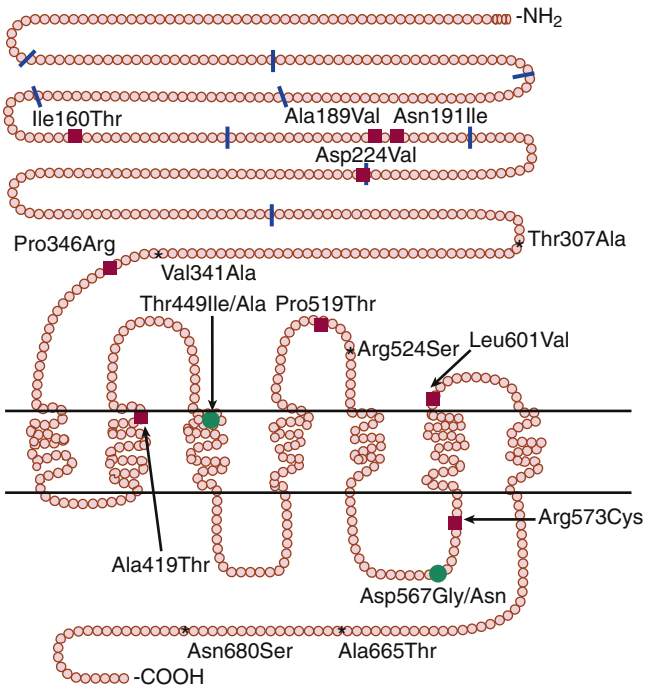


FIGURE 2.7 Location of the naturally occurring polymorphisms, loss-of-function mutations, and gain-of-function mutations of the human follicle-stimulating hormone receptor (hFSHR). Polymorphisms are shown with asterisks, loss-of-function mutations are shown with *red squares,* and gain-of-function mutations are shown with *green dots*. The sequences coded for by the different exons are shown with *vertical blue bars. (From Themmen APN. An update of the pathophysiology of human gonadotrophin subunit and receptor gene mutations and polymorphisms.* Reproduction *130:263–274, 2005. © Society for Reproduction and Fertility [2005]. Reproduced by permission.)*

6 and intracellular loop 3 (Fig. 2.6). Four of these mutations are associated with a single residue (Asp578), which is found mutated to Gly, Glu, Tyr, and His (Fig. 2.6). Gain of function mutations of the hLHR are found in heterozygous boys with familial male limited precocious purberty, but there is no phenotype in female carriers.[116,211,215,242,248,249] All of these mutations are also germ-line, with the exception of the Asp578His mutation, which has only been found as a somatic mutation in the Leydig cells of several unrelated boys with precocious puberty and Leydig cell adenomas.[255-258]

When expressed in heterologous cell types these gain of function hLHR mutants display variable levels of constitutive (i.e., hormone-independent) activity, and addition of LH or hCG to cells expressing these mutants may or may not result in additional activation.[116,248,249,258-262]

Naturally occurring mutations of the *FSHR* are fewer in number but can be similarly classified (Fig. 2.7). The most severe phenotype in homozygous females harboring inactivating mutations such as Ala189Val and Ala419Thr is hypergonadotropic hypogonadism, arrest of follicular maturation beyond the primary stage, and complete lack of responsiveness to hFSH. Less severe phenotypes are observed in women who are homozygous or compound heterozygous for other mutations and they include secondary amenorrhea, gonadotropin resistance, and follicular development up to the antral stage.[116,263] The phenotype in homozygous males is not nearly as obvious. Sperm quality is reduced but fertility is maintained.[116,263] The functional properties of many of these hFSHR mutants have been studied by expressing the mutants in heterologous cell lines. As in the case of the hLHR many of these mutations impair the transport of the hFSHR to the plasma membrane thus inducing a complete lack of hFSH binding and therefore responsiveness.[116] At least one of them, however, (Ala419Thr in transmembrane helix 2) impairs signal transduction while having little effect or no effect on hFSH binding or the proper trafficking of the hFSHR to the plasma membrane.[264]

Only three activating mutations of the *FSHR* have been described and they are all located in exon 10 which codes for the transmembrane and intracellular domains (Fig. 2.7). The Asp567Gly in intracellular loop 3 was identified in a hypophysectomized male with undetectable gonadotropins who responded to testosterone treatment with normal spermatogenesis.[265] The activating nature of this mutation, however, is not readily apparent when the mutant receptor is expressed in heterologous cells.[265] The other two activating mutations of the *FSHR* (Fig. 2.7) were initially discovered in women with recurrent spontaneous ovarian hyperstimulation syndrome.[266-268] When these mutants are expressed in heterologous cell types, they display a weak but detectable level of constitutive activity; they also display increased sensitivity to hCG and to TSH while maintaining normal sensitivity to hFSH.[215] This latter change in binding specificity is responsible for the ovarian hyperstimulation experienced by the women harboring these mutations. In this context, it is worth mentioning that one naturally occurring mutation of the extracellular domain of the TSHR that renders it more sensitive to hCG was found in two women who experienced hyperthyroidism during pregnancy.[269]

Polymorphisms of the Gonadotropin Receptor Genes

Many single nucleotide polymorphisms (SNPs) of the *LHCGR* and *FSHR* have been identified: (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=3973; http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=2492).

Only three SNPs of the *LHCGR* occur in exons. A six nucleotide in-frame insertion/deletion between codons 18 and 19 of exon 1 results in the expression of two hLHR variants that differ by the presence or absence of a Leu-Gln pair near the C-terminus of the signal peptide (Fig. 2.6)[270,271] The functional implications of the presence or absence of this insertion are unclear, however.[270,272,273] Two additional frequent SNPs in exon 10 of the hLHR gene code for either Asn or Ser in codons 291 and 312 in the hinge region of its extracellular domain. The presence of Asn or Ser at either of these positions does not appear to change the expression or functions of the hLHR.[274] This is interesting because codon 291 is a glycosylation site when Asn is present in this position (Fig. 2.4).

Only five of the *FSHR* SNPs are exonic. They are all located in exon 10 but only four cause a coding change (Ala307Thr, Arg524Ser, Ala665Thr, and Ser680Asn). The most common and best studied polymorphisms are Ala307Thr and Ser680Asn (Fig. 2.7). These two polymorphisms are in linkage disequilibrium and the most common allelic variants, Thr307/Asn680 and Ala307/Ser680, are almost equally distributed among Caucasians.[116] The Ala307Thr and Ser680Asn polymorphism appears to have predictive value for determining the optimal dose of FSH to use in controlled ovarian hyperstimulation protocols.[263,275-277] Women harboring the Ala307/Ser680 variant require higher doses of FSH. These variants may also be predictive of the responsiveness of PCOS patients to clomiphene.[275-277] Studies of the Ser680 or Asn680 hFSHR expressed in heterologous cells types have failed to reveal any differences in the functional properties of these receptors, however.[278,279] A polymorphism in the 5′ flanking region of the *FSHR* (-29 G/A) has also been reported to affect responsiveness to FSH stimulation because of its effect on the expression of the FSHR but there is no agreement on this finding yet.[275-277]

A more complete and up-to-date list of mutations and polymorphisms of the gonadotropin receptors can be found at: http://gris.ulb.ac.be.

Regulation of the Expression of the Gonadotropin Receptors

Transcriptional Regulation

In addition to directing the basal expression of the *LHCGR* and *FSHR*, transcriptional regulation of these genes is involved in the hormonal regulation of receptor expression in the ovary and perhaps the testes as well. For example, the increase in LHR that occurs during the growth and differentiation of rodent granulosa cells is accompanied by an increase in LHR mRNA that is mediated, at least in part, by an increase in the transcription of the *LHCGR*.[211] An exhaustive review of the hormonal regulation of the transcription of the *LHCGR* and *FSHR* is, however, beyond the scope of this chapter and the reader is referred to recent reviews of this important but complex area.[242,243,280] A summary of the regulatory elements that modulate basal expression of the *LHCGR* and *FSHR* has already been presented above.

Post-Transcriptional Regulation

Post-transcriptional regulation is also an important aspect of the regulation of the LHR mRNA and the LHR during the preovulatory LH surge.[280] This important level of regulation seems to be mediated by mevalonate kinase, an enzyme that is involved in cholesterol metabolism that is also an LHR mRNA binding protein.[280] The LH-induced activation of steroidogenesis and subsequent depletion of cholesterol triggers an increase in the transcription of genes that participate in cholesterol biosynthesis including mevalonate kinase. The increased levels of this protein may thus serve the dual role of enhancing cholesterol synthesis (an enzymatic function) and decreasing the levels of LHR mRNA by virtue of its ability to bind the LHR mRNA and enhance its degradation.[280]

Post-Translational Regulation

The processing and transport of the immature LHR from the endoplasmic reticulum to the cell surface have important implications to human disease because the majority of the naturally occurring mutations of the hLHR that cause gonadotropin resistance (as stated earlier in the text) are due to the intracellular retention of the misfolded hLHR.[281-283] Interestingly, when added to cells expressing some of the misfolded hLHR mutants, one of the low molecular weight agonists of the LHR (Org 42599, mentioned earlier in the text) partially rescues these naturally occurring mutants and enhances gonadotropin responsiveness.[284]

The gonadotropin receptors form oligomers in the endoplasmic reticulum and at the plasma membrane, in a process that is unrelated to receptor activation.[217,285] Importantly, co-expression of a misfolded gonadotropin receptor with its wild-type counterpart impairs the cell surface expression of the wild-type receptor and attenuates signaling.[217,221,285,286] Coexpression of splice variants of the LHR may also regulate the expression of the LHR and FSHR by forming intracellular oligomers that prevent the proper processing of the intracellular LHR precursor[287,288] For example, LHR transcripts lacking exon 9 are prevalent in normal human ovaries but the resulting protein is not able to bind hCG or to be adequately processed to be expressed at the cell surface.[289,290] When coexpressed with the wild-type hLHR or hFSHR the mutant lacking exon 9 associated with the immature forms of these receptors and decreased their cell surface expression.[289,290] Other studies have suggested that coexpression of a signaling inactive LHR with a binding inactive LHR leads to their dimerization and functional complementation such that, together, they can partially restore LH signaling,[291-294] but the interpretation of these results has been challenged.[295]

Some reports indicate that during development several rat tissues such as the gonads, adrenal glands, and kidneys express the immature form of the LHR whereas nervous tissues express both the mature and immature forms.[245,246] Interestingly, rodent gonads appear to express the mature LHR only after birth,[246] suggesting that the developing rat gonads are insensitive to the actions of LH. This finding is consistent with the phenotype of the LHR knockout mice which display arrested gonadal development only after

birth.[296,297] The expression of the mature LHR in rat tissues also seems to be under hormonal control as indicated by the finding that the mature LHR becomes detectable in the female adrenal glands and kidney during pregnancy.[246] Direct actions of gonadotropins on the kidneys are controversial, but expression of functional LHR that mediate direct actions on LH/CG in the pregnant adrenal gland have been well documented (discussed later in the text).

Another level of posttranslational regulation involves phosphorylation and regulated degradation of the mature gonadotropin receptors. This has been demonstrated for both, the LHR and the FSHR but most of these experiments have been done in heterologous cell types and their physiological significance is still debated.[260,298-307]

Phosphorylation of GPCRs is an important event for binding arrestins, a family of GPCR binding proteins that are involved in signaling, internalization, and desensitization.[308-310] The phosphorylation of the hLHR appears to be largely dispensable for arrestin binding and internalization,[259,260,311,312] whereas the phosphorylation of the rFSHR appears to be needed for arrestin binding and internalization.[305,311,313-315] It is also now accepted that some GPCRs can display "biased" signaling, either using arrestins or G proteins,[310,316] and recent reports suggests that partially deglycosylated equine LH and one of the inactivating mutants of the FSHR (Ala189Val, Fig 2.7) may signal through arrestins rather than G proteins.[310,317]

Hormone-induced internalization of the gonadotropin receptors is also dependent on arrestin binding and is an important process, because, depending on the fate of the internalized receptor, it can serve the function of preserving or preventing hormonal responsiveness. Interestingly, after hormone-induced internalization, the complex formed by hCG and the rodent or porcine LHR is largely routed to an intracellular degradation pathway[211] that is ultimately responsible for the degradation of hCG and a net loss of cell surface LHR and hormonal responsiveness.[211] The fate of the internalized hLHR is different, however, in that a substantial portion of the hCG-hLHR complex is routed to a recycling rather than a degradation pathway.[211] Cells expressing the hLHR recycle most of the internalized hormone and receptor and thus, the loss of hormonal responsiveness induced by gonadotropins is not as pronounced in cells expressing the hLHR as in those expressing the rLHR.[318] In contrast to the difference in the fates of the internalized hLHR and rFSHR, the internalized hFSHR and rFSHR are both routed mostly to a recycling pathway.[319] Cells expressing these receptors recycle most of the internalized FSH and FSHR and thus, the loss of hormonal responsiveness induced by FSH is relatively minor.[318]

Several amino acids present in the extreme C-terminus of the hLHR and one cellular protein that is important for recycling of the hLHR have been identified.[320-322] Amino acids of the FSHR that are important for recycling are also located in the extreme C-terminal tail.[319]

Expression of the Gonadotropin Receptors in Extragonadal Tissues

For many years, the LHR and FSHR were thought to localize strictly to gonadal cells. In the testes, the LHR and FSHR are thought to be restricted to Leydig and Sertoli cells, respectively. In the ovary, expression of the LHR occurs in theca, interstitial and, granulosa cells whereas the FSHR is localized to granulosa cells. Certainly, the main physiological roles of the gonadotropin receptors can be attributed to their actions in the ovaries and the testes as demonstrated by the phenotypes of individuals harboring activating or inactivating mutations of these genes (as stated earlier in the text) and by the phenotypes of mice with targeted inactivation of the *LHCGR*[296,297] or *FSHR*.[323,324]

There is a large but still controversial body of literature suggesting that functional LHR, may be present in a number of extragonadal tissues.[325,326] The suggestion of extragonadal LHR expression has been based in many cases upon the detection of fragments of the LHR mRNA. Attempts to detect immunoreactive LHR protein often result in the identification of a protein (or proteins) that do not match the expected molecular weight of the authentic gonadal LHR (as stated earlier in the text). A comparison of the actions of LH/CG in isolated cells or tissues from wild-type and LHR null mice would go a long way in shedding light on this controversy but curiously this has not been done often. In a recent study on the potential angiogenic activity of hCG it was shown that the proangiogenic effect of hCG was clearly ameliorated in blood vessels isolated from the LHR null mice.[327] This finding clearly shows that a functional, classical LHR is expressed in blood vessels.

Some of the clearest evidence for the extragonadal expression of a functional LHR comes from studies done on a woman who developed pregnancy-dependent Cushing syndrome and later developed it again after menopause.[328] After menopause there is clear evidence that the cortisol levels in this woman were controlled by LH because suppression of the hypothalamic/pituitary axis with leuprolide controlled the hypercortisolism.[328] Clinical findings in a number of other cases also seems to be explained by the inappropriate expression of the LHR in the adrenal cortex or in adrenocortical tumors.[329,330] Lastly, ectopic expression of functional LHR in the adrenal cortex seems to be a common finding in transgenic or knock-out mouse models with elevated levels of gonadotropins,[330] and expression of the mature LHR can be readily documented in the adrenal glands of pregnant rats.[246]

Studies on the potential extragonadal expression of the FSHR are more limited in nature but equally controversial. A recent study suggesting that FSH has direct actions in bone because of the expression of the FSHR in osteoclasts[331] has been challenged.[332-334] There is also some evidence for the expression of the FSHR in the prostate,[335] as well as tumor blood vessels.[334]

In spite of all this information, the phenotypes of genetically engineered mice with exaggerated or absent gonadotropin actions, as well as the phenotypes of men or women harboring activating or inactivating mutations of these gonadotropin receptors, it (as stated earlier in the text) appears to be explained entirely by the classical actions of LH and FSH in gonadal tissues.[205,326,336]

Signaling Pathways Activated by the Gonadotropin Receptors

Although most investigators agree that the effects of the gonadotropins on the differentiated function of their target

cells are mediated mostly (if not entirely) by the activation of the Gs/adenylyl cyclase/cAMP/PKA pathway, it is abundantly clear now that this is not the only pathway activated by these receptors. Additional pathways are activated as detailed below and these may be involved in other gonadotropin-dependent events such as the proliferation and/or differentiation of target cells.

The LHR was one of the first GPCRs shown to independently activate two G protein-dependent signaling pathways, adenylyl cyclase and phospholipase C.[337,338] This observation was initially made in heterologous cells expressing the recombinant mouse LHR but it has now been extensively reproduced by a number of investigators using a variety of cell lines transfected with either the rodent or human LHR.[211] The FSHR also activates both of these cascades when expressed in non-target[302-304] or target cells.[339,340] In general, however, the gonadotropin receptor-mediated activation of phospholipase C is detectable only when cells expressing a high density of receptors are exposed to high concentrations of gonadotropins[262,340] and there is no consensus on the physiological consequences of this activation.[339-342] In addition, the LHR has been reported to activate members of the other three families of G proteins[261,343,344] but again, little is known about the functional consequences of the activation of these G proteins.

The ovarian ERK1/2 cascade is also activated by the FSHR and LHR but the mechanisms involved in its activation are complex and poorly understood.[339,345-349] The importance of this cascade on the actions of LH is underscored by the finding that a granulosa cell specific deletion of ERK1/2 in mice results in infertility due to an ovulatory defect.[350,351] This study also showed that most of the ovarian genes that are up- or down-regulated by LH are targets of ERK1/2.[350,351]

More recently, the two gonadotropin receptors (but specially the LHR) have been shown to activate small GTPases such as Ras,[346,349,352] and tyrosine kinases such as members of the Src and/or ErB receptor family.[346,347,350] The molecular basis of the activation of these pathways are still actively being investigated, but the LHR-induced transactivation of the EGF network, which involves several EGF-like growth factors and several ErB receptors is now known to be an essential mediator of the LHR-provoked ovulatory response.[347]

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 3

Prolactin in Human Reproduction

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Prolactin (PRL), a hormone with roles in reproduction, lactation and metabolism, is made by the pituitary lactotrophs which, in the normal human pituitary, comprise about 15% to 25% of the total number of cells. Lactotrophs are similar in number in both sexes and do not change significantly with age.[1] During gestation and subsequent lactation there is considerable lactotroph hyperplasia due to the stimulatory effect of the hormonal milieu of pregnancy.[2,3] The hyperplastic process involutes within several months after delivery, although breast-feeding retards this process.[3] This stimulatory effect of pregnancy on the lactotrophs also holds true for prolactinomas, explaining why very significant tumor enlargement may occur during pregnancy. As described later in this chapter, the proliferation of lactotrophs and increase in PRL secretion is due, at least in part, to autocrine and paracrine mechanisms playing out within the pituitary gland. This chapter reviews the physiology of PRL and the pathophysiology of hyperprolactinemic states, their diagnosis, and management.

Anatomy and Physiology

Lactotroph Morphology and Ontogeny

During embryologic development, the ectodermal primordial cells of the anterior and intermediate lobes of the pituitary make contact with the neuroectoderm of the floor of the diencephalon. Inductive interactions between these tissues occur that are necessary for their subsequent interdependent development.[4,5] There are a number of transcription factors (Six-3, Hesx1, Lhx3, Lhx4, Sox2, Sox3, Pitx2, Otx2, Bmp2, Bmp4, and GLi2) that are sequentially expressed in the developing hypothalamus and pituitary that lead to the final determination of the five mature pituitary cell types and the functional integration of the hypothalamic-pituitary system.[4-6]

The POU homeodomain transcription factor, Pou1f1 (Pit-1), is necessary for the activation of the *PRL*, growth hormone *(GH)*, GH releasing hormone *(GHRH)* and thyroid-stimulating hormone β *(TSHβ)* genes as well as being necessary for the differentiation and proliferation of these cell lineages. Point mutations in the POU homeodomain of *Pou1f1* have been found to be the cause of the syndrome of combined GH, PRL, and TSH deficiencies found in humans, with absence of somatotroph, lactotroph, and thyrotroph cells.[4-6] A second paired-like homeodomain factor, known as Prophet of Pit-1 (Prop-1) has also been found to be necessary for the expression of Pit-1 and mutations of the *Prop-1* gene cause variable deficiencies of GH, PRL, TSH, luteinizing hormone (LH) and follicle-stimulating hormone (FSH).[4-6] Mutations in the *Lhx4* and *Hesx1* genes caused combined pituitary hormone deficiencies as well as other brain developmental defects.[5,6] Lactotrophs and somatorophs are thought to arise from a common progenitor, the mammosomatotroph, under the regulation of these and other transcription factors during embryologic development, but it is possible that some lactotrophs arise directly from a common pituitary progenitor cell without passing through this mammosomatotroph step.[7]

Prolactin Gene Structure and Regulation

The *PRL* gene in the human is located on chromosome 6.[8] The human *PRL* gene is approximately 10 kb long and consists of five coding exons, one non-coding exon, and four large introns.[9,10] In the 5′ flanking region there are specific regions responsible for tissue-specific Pou1f1 transcription activation. The transcription enhancement of the *PRL* gene by Pou1f1 can be modified by other factors, such as thyrotropin-releasing hormone (TRH), epidermal growth factor (EGF), cyclic adenosine monophosphate (cAMP),[11-13] glucocorticoids,[14] and estrogens.[15] Other areas of the 5′ flanking region have also been found to involved in the suppression of *PRL* gene transcription by thyroid hormone and stimulation by estrogen.[15-19] Estrogen also increases PRL by modulating the inhibitory effect of dopamine (DA) on *PRL* gene transcription.[20,21]

A number of factors affecting PRL secretion, including TRH and dopamine (DA), act through the phosphoinositide-signaling pathways with resultant release of calcium from the endoplasmic reticulum and an activation of membrane calcium channels.[22] Increases in intracellular calcium result in activation of protein kinase C, which phosphorylates downstream effector proteins.

A second transduction pathway involving membrane phospholipids is the arachidonate pathway. In vitro stimulation of membrane-bound phospholipase A_2 by TRH, angiotensin II, neurotensin and other direct stimulators releases PRL and arachidonic acid from pituitary cells.[23,24] This stimulated release of arachidonic acid release is blocked by inhibitors of phospholipase A_2[23] and by DA.[25] Arachidonic acid increases PRL release primarily by an increase in calcium influx, not through mobilization of intracellular calcium stores.[26]

In addition to effects on protein kinase C, calcium also binds to topoisomerase II, which then interacts with specific DNA binding sites and to calmodulin. In turn, calmodulin then binds to a number of different intracellular enzymes.[27] TRH appears to cause rapid release of PRL via activation of

protein kinase C and calcium store mobilization but not calcium channel-mediated transport, and its effects on *PRL* gene transcription and PRL synthesis involve all three processes.[27]

Dopamine, the primary PRL inhibitory factor (PIF), acts through the D_2 DA receptor, inhibiting adenyl cyclase, the cAMP/protein kinase A pathway and possibly other mechanisms.[28] DA inhibits the PRL release induced by activation of calcium channels and by mobilization of intracellular calcium stores.[29] Conversely, the calcium channel blockers nimodipine, verapamil, and diltiazem antagonize the inhibitory effect of DA *in vitro*.[30] However, administration of the calcium channel blocker verapamil to humans causes an *increase* in serum PRL levels[31,32] and does *not* inhibit the TRH-induced PRL rise.[32] This was shown to be due to a decrease in tuberoinfundibular generation of DA.[33] Dihydropyridine and benzothiazepine calcium channel blockers have no effect on PRL levels in humans,[33] and it is likely that the effect of verapamil is via action at N-type calcium channels present in neuronal tissues.[33]

Vasoactive intestinal peptide (VIP) stimulates PRL release primarily through stimulation of adenyl cyclase and the production of intracellular cAMP.[34,35]

Hormone Biosynthesis

The mature PRL mRNA formed after nuclear processing of the original heteronuclear RNA is only about 1 kb long and codes for a 227 amino acid sequence which includes an initial 28 amino acid signal peptide and the 199 amino acid structural peptide.[9,10]

There is considerable heterogeneity in the final PRL product, depending upon variations in the extent of post-translational modifications (which include cleavage, polymerization, glycosylation, phosphorylation, and degradation). About 80% to 90% of PRL extractable from pituitaries and in serum is monomeric, 8% to 20% is dimeric (molecular mass 45 to 50 kDa), and 1% to 5% is polymeric.[36,37] These larger molecular weight polymers have decreased binding to receptors and display decreased bioactivity in a variety of receptor assays[36,37] but have normal bioactivity in the Nb_2 lymphoma cell bioassay.[37] Some of the cleavage products of PRL have been found to have anti-angiogenic function but their physiologic significance is not clear.[38]

Several patients described as having elevated basal serum PRL levels but normal reproductive function were found to have elevated proportions of polymeric PRL. This "macroprolactinemia" presumably resulted in less PRL bioactivity.[39-42] In other cases, the larger molecular weight moieties consist of monomeric PRL bound to immunoglobulin[43]; however, in many such reported cases the proportion of monomeric PRL in the blood is still elevated (discussed later in the chapter).

Decidual Prolactin

PRL levels in maternal blood rise throughout gestation and are of pituitary origin.[44,45] However, PRL concentrations in amniotic fluid are 10- to 100-fold higher than either maternal or fetal blood levels.[44] Cultured human chorion and decidual cells synthesize and release a PRL into the amniotic fluid that is identical to pituitary PRL in structure and bioactivity.[46-49] Decidual PRL mRNA is indistinguishable from pituitary PRL mRNA except for four silent nucleotide differences and the decidual *PRL* gene is about 150 nucleotides longer in the 5′ untranslated region.[50-52]

The regulation of decidual PRL production differs from that of pituitary PRL. DA agonists[53] and antagonists[54] given to the mother respectively decrease and increase maternal serum PRL levels, but have no effect on amniotic fluid PRL levels. Decidual PRL production is increased by progesterone and progesterone plus estrogen (but not estrogen alone),[51,55] insulin, insulin-like growth factor 1 (IGF-1), and relaxin.[51,56,57] The function of decidual PRL in humans remains obscure, although there is some evidence from animal studies that it may contribute to the osmoregulation of the amniotic fluid, fetal lung maturation, uterine contractility, and modulation of the uterine immune system.[58] Recent evidence suggests that decidual PRL may silence the expression of genes detrimental to pregnancy.[59] Interestingly, in some animal species, a large number of PRL-related genes in trophoblastic tissue have evolved whose products involve the coordination of maternal, extraembryonic and fetal tissues including immunologic activity.[60]

Measurement Of Prolactin

With conventional assays there is considerable variation in different laboratories in the measurement of PRL levels and normal values need to be established for each laboratory.[61] Over the past decade, two-site immunoradiometric assays (IRMA) and chemiluminometric assays (ICMA) have come into wide use because of improved sensitivity and precision and short incubation periods.

Hook Effect

In patients with large prolactinomas, the very high PRL levels may saturate the antibodies in two-site assays, preventing the formation of the PRL-antibody sandwich. As a result, labeled antibody is lost and a falsely low PRL value may be reported.[62,63] St. Jean and colleagues noted this high end "hook effect" in 5.6% of 69 patients thought to have clinically nonfunctioning adenomas.[62] Therefore, in patients with large macroadenomas, PRL assessments should always be performed in both undiluted and 1:100 diluted serum to exclude the "hook effect" when two-site assays are used.

Macroprolactin

Normally, over 90% of PRL is present in serum as the 23 kDa monomer and less than 10% is in larger molecular weight forms (discussed earlier in the chapter). These large molecular forms are referred to as macroprolactin and are usually comprised of PRL bound to immunoglobulin (IgG), but sometimes it is in the form of oligomers.[64-66] The gold standard method of measurement is to put a serum sample on a gel chromatography sizing column.[65-67] However, another more easily done method is to add polyethylene glycol (PEG) to the serum; the larger molecular weight forms will precipitate and the monomeric form is left in the supernatant.[64-68] However, this method is very assay-dependent and not technically feasible on all commercial assays.[69]

Some investigators have stated that in a patient with hyperprolactinemia, if the amount of PRL left after PEG precipitation is less than 50% or 40%, then the hyperprolactinemia is due to macroprolactin.[68] However, others

have suggested that since some macroprolactin is present in normal serum and/or some monomer is precipitated with PEG, that the "normal" range should be recalculated from normal samples after PEG treatment; therefore, hyperprolactinemia can be attributed to macroprolactin only when the level of nonprecipitated PRL, i.e., monomeric PRL, is within the normal range.[70] It is clear that in many studies even when the amount of nonprecipitated PRL is less than 40% of the total, the level is still above the normal range.

An important issue is the bioactivity of the macroprolactin, as it is implied that it is of no biological significance. In most series looking at patients with macroprolactin, there tend to be fewer symptoms in those whose hyperprolactinemia has been attributed to macroprolactin than in those with true hyperprolactinemia.[67-71] When macroprolactin is put into the rat Nb2 lymphoma cell culture system, a standard *in vitro* prolactin bioassay system, macroprolactin tends to have the same bioassayable activity as monomeric PRL.[72] However, when bioassays are used in which the human PRL receptor was stably transfected and expressed in murine Ba/F-3 cells or human embryonic kidney-derived 293 (HEK-293) cells, the bioactivity of macroprolactin was shown to be decreased.[73,74]

In studies in which patients with hyperprolactinemia, thought to be due (often in retrospect) to macroprolactin, have been treated with dopamine agonists, galactorrhea, when present, has generally disappeared, but oligo-amenorrhea has been variably responsive.[67,68,70,71] Long-term follow-up studies of patients diagnosed with macroprolactinemia show considerable instability of levels (up to fivefold).[67]

In clinical practice, if a patient has relatively typical symptoms, such as galactorrhea, amenorrhea, or impotence and is found to have mild hyperprolactinemia, the usual exclusions should be looked for (medication history, hypothyroidism, elevated creatinine, pregnancy—discussed later in the chapter), and then the patient should have a Magnetic Resonance Imaging (MRI) scan, primarily to exclude a large lesion such as a craniopharyngioma or clinically nonfunctioning adenoma. If the patient has equivocal symptoms (such as headaches or decreased libido), but has normal menses and no galactorrhea and is found to have mild hyperprolactinemia, assessment for macroprolactin would seem reasonable using PEG precipitation. The decision that the hyperprolactinemia is due to macroprolactin would then depend on demonstrating an abnormal amount of PEG-precipitable PRL, and an absolute level of monomer that is within the normal range. Only in such a patient would an MRI scan not be indicated, but such patients should continue to be followed clinically and biochemically on a periodic basis.

Hormone Secretion Patterns

Episodic Secretion

Prolactin is secreted episodically. There are 13 to 14 peaks per day in young subjects, with a peak duration lasting 67 to 76 minutes, a mean peak amplitude of 3 to 4 ng/mL, and an interpulse interval of 93 to 95 minutes.[75,76] Disinhibition seen with hypothalamic tumors causes an increase in basal PRL levels due to an increase in pulse amplitude, not pulse frequency.[77] There is an increase in the amplitude of the PRL secretory pulses that begins about 60 to 90 minutes after the onset of sleep; the secretory pulses increase with non-rapid eye movement (REM) sleep and fall prior to the next period of REM sleep.[78] An increase in circulating PRL levels of 50% to 100% occurs within 30 minutes of meals[79] due to the amino acids generated from the protein component of the meals, with phenylalanine, tyrosine, and glutamic acid being the most potent.[80]

Changes in Prolactin with Age

Prolactin levels are elevated almost 10-fold in infants following delivery due to the stimulatory effect of maternal estrogen, but then gradually decrease so that levels are normal by 3 months of age.[81] PRL levels then rise modestly during puberty to adult levels.[81] PRL levels in women gradually fall by about 50% over the first 18 months after menopause, but this decrease is considerably less in women treated with estrogen replacement therapy[82] (although some studies have shown no change in PRL levels with hormone replacement therapy[83]). In hyperprolactinemic women, estrogen replacement therapy causes no change in PRL levels.[84] In men, mean serum PRL concentrations are about 50% lower in older men compared with younger men.[85]

Changes in Prolactin Levels during the Menstrual Cycle

Some, but not all, women have higher PRL levels at midcycle and lower levels in the follicular phase.[44,61,86-89] Some studies have shown that PRL and LH secretion are often synchronous in the luteal phase and that very small doses of gonadotropin-releasing hormone (GnRH) can cause the secretion of both PRL and LH at this time.[90]

Changes in Prolactin Levels during Pregnancy

Basal PRL levels gradually increase throughout the course of pregnancy.[44,45] This has generally been attributed to the stimulatory effect of the hormonal milieu of pregnancy (primarily estrogenic) causing lactotroph hyperplasia.[2,3] By term, PRL levels may be increased 10-fold to levels over 200 ng/mL,[44,45,81] preparing the breast for lactation.

Changes in Prolactin Levels with Postpartum Lactation

Within the first 4 to 6 weeks postpartum, basal PRL levels remain elevated in lactating women and each suckling episode triggers a rapid release of pituitary PRL.[44,91] Over the next 4 to 12 weeks, basal PRL levels gradually decrease to normal, while the PRL increase which occurs with each suckling episode is gradually extinguished.[44,91] The decreases in basal and stimulated PRL levels between 3 and 6 months postpartum are largely the result of decreased breastfeeding as formula is introduced into the baby's diet. If intense nursing behavior is maintained, basal PRL levels remain elevated and postpartum amenorrhea persists.[92] High intensity lactation-induced failure to ovulate and menstruate has long been used as a method of contraception in a number of developing countries.[92]

Breast stimulation may cause an increase in PRL levels in some healthy women who are not breastfeeding; such increases have not been found in men.[91] Chronic nipple stimulation with nipple rings has been reported to cause sustained galactorrhea.[93]

Changes in Prolactin Secretion with Stress

Prolactin is one of the pituitary hormones released by stress, along with adrenocorticotropic hormone (ACTH) and GH. The stress-induced rise in PRL in humans generally results in a doubling or tripling of PRL levels and lasts less than 1 hour.[94] However, prolonged critical illness does *not* cause a sustained elevation of PRL; rather there is a reduction in the pulsatile secretion with an overall lowering of levels.[95] Acute exercise has also been regarded as a form of stress and results in an acute, transient increase in PRL levels.[94] Although chronic, high-level exercise, such as occurs in runners training for a marathon, is often associated with menstrual disturbances, it is not associated with sustained hyperprolactinemia.[96]

Neuroendocrine Regulation

The hypothalamus exerts a predominantly inhibitory influence on PRL secretion through one or more PRL inhibitory factors (PIF) that reach the pituitary via the hypothalamic-pituitary portal vessels (Fig. 3.1). There are PRL-releasing factors (PRF) as well. Disruption of the pituitary stalk leads to a moderate increase in PRL secretion as well as to decreased secretion of the other pituitary hormones.

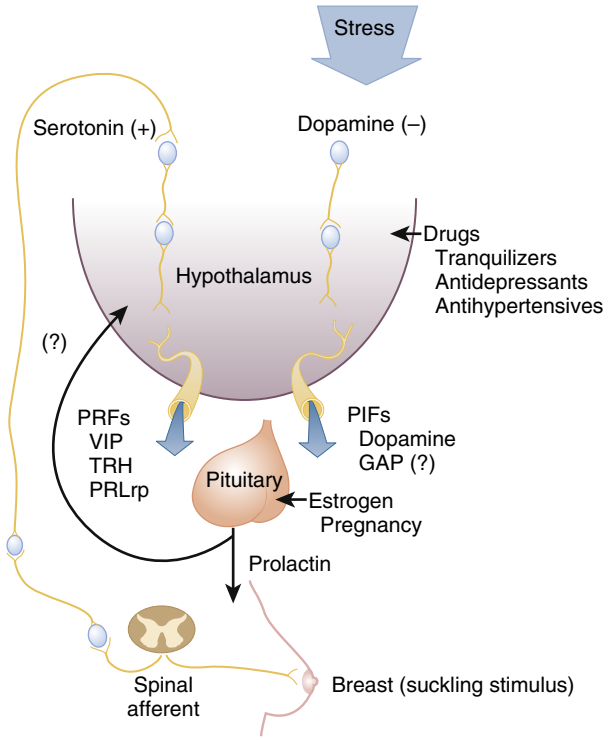


FIGURE 3.1 Regulation of prolactin secretion. Prolactin release is stimulated by a number of Prolactin Releasing Factors (PRF's), including Vasoactive Intestinal Peptide (VIP), Thyrotropin Releasing Hormone (TRH), and PRL releasing peptide (PRLrp), and is inhibited by Prolactin Inhibiting Factors (PIF's), predominantly dopamine but possibly also by the 56 amino acid peptide portion of the precursor to GnRH, known as GnRH Associated Peptide (GAP). Estrogens and the hormonal milieu of pregnancy are also stimulatory to prolactin production. The primary target organ for prolactin is the breast. Suckling activates neural afferent pathways to the hypothalamus where appropriate increases in PRF's and/or decreases in PIF's effect prolactin release in the puerperium. Within the hypothalamus serotoninergic pathways are stimulatory and dopaminergic pathways are inhibitory to prolactin release. *(Reproduced from Molitch ME. Disorders of prolactin secretion.* Endocrinol Metab Clin N Amer *30: 585–610, 2001, with permission.)*

Prolactin Inhibitory Factors

Dopamine

Dopamine is the predominant, physiologic PIF. The concentration of DA found in the pituitary stalk plasma (about 6 ng/mL) is sufficient to decrease PRL levels and is 5- to 10-fold higher than levels found in peripheral plasma.[97] Stimuli that result in an acute release of PRL usually also result in an acute decline in portal vessel DA levels.[98-100] In most physiologic circumstances that cause a rise in PRL, such as lactation, there is likely to be a fall in DA along with a simultaneous rise in PRFs (such as VIP).[101]

Experiments in mice in which the DA D_2 receptor or DA transporter have been "knocked out" (KO) with inactivating mutations have confirmed the results of earlier studies that employed pharmacologic methods or lesioning. DA D_2 receptor KO mice develop lactotroph hyperplasia with scattered multifocal prolactinomas and sustained hyperprolactinemia.[102-104] DA action within the synapse terminates as a result of DA reuptake by the DA-secreting neurons via the DA transporter. In contrast to the findings with the DA receptor KO mice, DA transporter KO mice have increased dopaminergic tone and lactotroph hypoplasia.[105] Although such mice have normal circulating levels of PRL, these levels cannot increase in response to various stimuli and the mice are therefore unable to lactate.[105]

In humans, infusion of DA causes a rapid suppression of basal and stimulated PRL levels.[106,107] Studies with low-dose DA infusions in humans have shown that DA blood concentrations similar to those found in rat and monkey hypothalamic-pituitary portal blood[97,108] are able to suppress PRL secretion.[109,110] Blockade of endogenous DA receptors by a variety of drugs causes a rise in PRL.[111,112]

The axons responsible for the release of DA into the median eminence originate in dorsal portion of the arcuate nucleus and inferior portion of the ventromedial nucleus of the hypothalamus. These axons terminate in the median eminence, a pathway known as the *tuberoinfundibular DA (TIDA) pathway*.[113,114] The DA that traverses the TIDA pathway binds to the D_2 DA receptors on the lactotroph cell membrane.[114]

The inhibitory action of DA on PRL secretion is partially blocked by estrogen administration. This is largely due to the direct action of estrogen on the estrogen response element of the PRL gene, as previously mentioned. Estrogen is able to partially block the inhibitory action of DA on PRL secretion; infusions of DA into women during the early follicular phase (when estrogen levels are low) result in a greater suppression of PRL than infusions during the late follicular or periovulatory phases (when estrogen levels are higher).[115]

Other Inhibitory Factors

Whether DA alone can account for all of the PIF activity of the hypothalamus has long been a question. Inhibitory

activity has been shown by GnRH-associated protein (GAP), a peptide in the carboxyterminal region of the precursor to GnRH[116] and γ-aminobutyric acid (GABA)[117,118] in various experimental studies but their physiologic importance in the human remains unknown.

Prolactin Releasing Factors

Thyrotropin-releasing hormone (TRH) causes a rapid release of PRL from pituitary cell cultures[119] and humans after intravenous injection.[120] The smallest dose of TRH that releases TSH also releases PRL in humans.[121] However, immunoneutralization of endogenous TRH with TRH antisera only causes suppression of basal PRL levels in rats in some studies,[122] but not in other studies[123,124]; mice with targeted disruption of the TRH gene (TRH KO) become hypothyroid but have normal PRL levels.[125] Suckling causes a rise in PRL but not TSH.[126] On the other hand, in hypothyroidism, basal TSH and PRL levels are elevated[127] with both being normalized by thyroxine treatment.[127,128] Conversely, in hyperthyroidism, PRL levels are not low basally but the PRL response to TRH is markedly blunted and returns to normal with correction of the hyperthyroidism.[127,128] These conflicting data support a role for TRH as a physiologic PRF, albeit not the primary one, or even one of major physiologic importance.

Vasoactive Intestinal Peptide and Peptide Histidine Methionine

Vasoactive intestinal peptide stimulates PRL synthesis and release from pituitary cell preparations, and humans in vivo.[129-133] VIP neuronal perikarya are present in the paraventricular nucleus with axons terminating in the external zone of the median eminence.[134] Passive immunoneutralization with anti-VIP antisera in rats partially inhibits the PRL responses to suckling, ether-induced stress[135,136] and estrogen.[137] This same type of VIP immunoneutralization in DA receptor-blocked rats suppresses PRL pulsatile secretion.[138] Similar results for suckling have been obtained with a VIP antagonist.[139]

Within the VIP precursor is another similarly sized peptide known as peptide histidine methionine (PHM).[140] PHM and VIP colocalize in the hypothalamus and median eminence.[141] PHM given to humans has caused a PRL increment in some experiments,[141] but not others.[133]

Complicating the role of VIP as a PRF is the finding that VIP is actually synthesized by anterior pituitary tissue.[142] Antisera to VIP inhibit basal PRL secretion from dispersed pituitary cells *in vitro*,[143,144] suggesting a local "autocrine" role for VIP in PRL regulation within the pituitary. The precise physiologic roles of VIP versus PHM and hypothalamic VIP versus pituitary VIP are still not clear.

Serotonin

Serotonin and its precursor, 5-hydroxytryptophan, cause a release of PRL in rats whether injected systemically[145] or into the third ventricle.[146] A variety of experiments using blockers of serotonin synthesis, receptors, or reuptake by nerve terminals, have shown that serotonin mediates, in part, the PRL elevations associated with suckling and proestrous.[147-149] In humans, infusion of the serotonin precursor, 5-hydroxytryptophan, results in a prompt increase in PRL levels.[150,151] Nocturnal PRL secretion is inhibited by cyproheptadine.[152] Fenfluramine, a serotonin releasing agent, caused a fourfold rise in PRL in humans that could be partially blocked by cyproheptadine.[153] Fluoxetine, a serotonin reuptake inhibitor, also increases PRL levels modestly (with levels remaining within the normal range).[154] Although it is possible that serotonin is a direct secretagogue for PRL, either through transport from the hypothalamus by the portal vessels or through an autocrine action within the pituitary, its role in this regard is still uncertain. Serotonin likely mediates, in part, the nocturnal surge of PRL; it may well participate in the suckling-induced rise in PRL through the ascending serotoninergic pathways from the dorsal raphe nucleus by causing VIP release.

Other Neuroactive Peptides and Neurotransmitters

Opioid Peptides

In rats, various opioid peptides cause release of PRL.[155,156] Studies employing specific agonists and antagonists have shown that the μ receptor is the predominant one involved in PRL release.[157,158] Most evidence suggests that the opioid peptides do not have a direct effect on the pituitary[156] and stimulate PRL release by inhibiting DA turnover and release by the TIDA pathway.[159,160] In humans, morphine and morphine analogs increase PRL release acutely[161] and chronically.[162] However, blockade of the μ receptor with naloxone has minimal to no effect on PRL levels either basally or with stimulation by hypoglycemia, exercise, sleep, TRH, or physical stress.[163,164]

In contrast to these findings, an increase in PRL levels in response to naloxone given in the late follicular and midluteal phases of the menstrual cycle has been reported.[165] The interpretation of these changes is not straightforward, but overall, it appears that the endogenous opioid pathways play, at most, only a minor role in the regulation of PRL secretion.[166]

Growth Hormone Releasing Hormone

A number of studies have found GH releasing hormone (GHRH) to have PRL releasing properties. The initial clue to this action of GHRH came when patients with acromegaly due to GHRH secreting tumors were found to be hyperprolactinemic and their PRL levels fell in parallel with GH after tumor excision.[167] GHRH has also been reported to release PRL *in vivo* in healthy humans[168] and chronic therapy with GHRH in children with GH neurosecretory dysfunction results in a sustained elevation of PRL levels.[169] Although large amounts of GHRH clearly can release PRL, the physiologic significance of these observations remains unknown.

Posterior Pituitary, Oxytocin, and Vasopressin

Studies in animals have shown that oxytocin, in levels found in the hypothalamic-pituitary portal vessels, can stimulate PRL release when added to the incubation medium of pituitary cells or when given intravenously, but it lowers PRL levels when directly injected into the third ventricle.[170] Studies in which endogenous oxytocin was eliminated by passive immunization with oxytocin antisera or by oxytocin antagonists show a reduction and a delay in the suckling induced PRL surges.[170] Very limited studies in humans suggest that oxytocin administered intravenously has no effect

on basal PRL levels and causes only a minimal increase in VIP-stimulated PRL levels.[171]

To date, there are no studies of the effects of vasopressin on PRL secretion in humans. Whether there are other PRF's in the posterior pituitary in addition to oxytocin, vasopressin, and their respective neurophysins has been a matter of controversy.

Gonadotropin-Releasing Hormone

Gonadotropin-releasing hormone releases PRL from rat pituitary cells *in vitro*.[172] GnRH has been found to cause a release of PRL in anovulatory women[173,174] and in women with infertility being treated with menopausal gonadotropins.[175] In 24% to 78% of normal women there is a PRL response to GnRH; this response is dependent upon the phase of the menstrual cycle, with the highest number of subjects responding in the periovulatory phase.[176]

Postmenopausal women also have a PRL response to GnRH that is augmented with estrogen supplementation.[177] There is no PRL release in response to GnRH in healthy, eugonadal males, but such a release does occur in transsexual men who receive high doses of estrogen.[178] Analysis of PRL and LH secretory pulses in women shows a high degree of concordance.[90] This cosecretion of LH and PRL suggests that the response to GnRH is physiological and is evidence against a physiological role for the inhibitory cosecreted GAP.

Prolactin-Releasing Peptide

Hinuma and colleagues discovered a 31-amino acid peptide capable of releasing PRL, that has been termed prolactin releasing peptide (PrRP); the discovery came while looking for endogenous ligands for an orphan receptor (termed HGR3) present in the human pituitary.[179] In pituitary cell preparations, PrRP released PRL with a potency equal to that of TRH.[179] However, although PrRP is found in neuronal perikarya in the paraventricular and supraoptic nuclei, PrRP-immunoreactive nerve fibers are found only in the internal zone of the median eminence and not the external zone,[180] casting uncertainty on the physiological significance of this peptide with respect to PRL secretion. Although PrRP has been shown to affect the hypothalamic-pituitary-adrenal axis stress response and feeding behavior, at present it is not thought that PrRP plays a significant role in PRL regulation in humans or any other species.[181]

Other Neuroactive Peptides and Neurotransmitters

There is evidence that several other neuropeptides and neurotransmitters affect PRL secretion in a variety of experimental paradigms in animals, including angiotensin, neurotensin, substance P, cholecystokinin, bombesin, secretin, gastrin, galanin, endothelin, somatostatin, relaxin, melatonin, basic fibroblast-growth factor, bradykinin, calcitonin, calcitonin gene-related peptide, histamine, norepinephrine, and acetylcholine. However, their physiological significance, especially in humans, is unknown.

Prolactin Short Loop Feedback

Experiments conducted in rats suggest that PRL is able to feed back negatively on its own secretion (short-loop feedback or autofeedback).[182] Most evidence suggests that such feedback occurs via augmentation of hypothalamic TIDA turnover.[183] Direct confirmation of the importance of short-loop feedback in rodents comes from recent studies using mice with targeted disruption of the PRL gene (*PRL* gene KO). Mice with the *PRL*-KO have no pituitary PRL and have markedly decreased DA in TIDA neurons along with hyperplasia of lactotrophs that do not make PRL.[184-186] Direct evidence for such PRL short loop feedback in the human has not been demonstrated. However, it has been suggested that altered regulation of gonadotropin and TSH secretion in hyperprolactinemic patients may constitute indirect evidence of PRL-induced augmentation of TIDA activity.[187]

Prolactin Action

PRL has a great diversity of actions in many species of animals, including osmoregulation, growth and developmental effects, metabolic effects, actions on ectodermal and integumentary structures and actions related to reproduction.[188] Its primary physiological action in humans, however, is the preparation of the breast for lactation in the postpartum period.[189] Elevated levels of PRL affect many tissues and PRL receptors are found in many tissues.

Prolactin Receptors

The PRL receptor is a member of the class 1 cytokine receptor superfamily. The human PRL receptor gene is located on chromosome 5p14-p13.2 and has 10 exons with exons 3 to 10 encoding the full length activating long form of the receptor.[190] Two isoforms of the PRL receptor result from alternative splicing and differ in the length and composition of the cytoplasmic tail, being referred to as the long and intermediate forms; the short form found in the mouse is not present in humans.[191,192]

PRL binds to its receptor with high affinity, the dissociation constant (K_d) being 10^{-10} mol/L.[192] Half-saturation of the receptor occurs at a hormone concentration of 7 ng/mL.[192] PRL binding causes dimerization of the receptor, a necessary step for signaling.[192]

PRL receptors are widely distributed throughout the body, being present in the breast, pituitary, liver, kidney tubules, adrenal cortex, prostate, ovary, testes, seminal vesicles, epididymis, intestine, skin, pancreatic islets, lymphocytes, lung, myocardium, and brain.[192] PRL release caused by suckling increases PRL receptor levels in the breast and liver, resulting in a much greater PRL binding activity in lactating animals than in those not lactating.[192] Signal transduction of the activated, dimerized receptor involves the JAK-STAT pathway.[192] Activation of the MAP kinase cascade has also been reported after receptor activation but whether this involves JAK 2 is unknown.[192]

The physiologic roles of PRL in most of the tissues in which receptors are present are not known. Although some have suggested that PRL may play a role in the development of breast and other cancers, this has not been firmly established. Future research with PRL receptor antagonists acting at the tissue level may allow a more clear delineation of additional physiological and pathological functions of PRL.[193]

Prolactin Effects on the Breast

Prolactin, GH, cortisol, insulin, estrogen, progesterone, and thyroxine all contribute to breast development. The high

concentrations of estrogen and progesterone produced by the placenta, coupled with the estrogen-induced high concentration of circulating PRL, and the high concentrations of placental lactogen cause development of the lobular, alveolar tissue during pregnancy.[189] Once the breast is fully developed and hormonally primed, PRL stimulates the production of milk proteins and other components.[189] Prior to term, the high estrogen levels suppress the effects of the high PRL levels on milk production, but the rapid fall off of estrogen levels following delivery allows milk production to proceed.[194] Bromocriptine-induced suppression of this physiological hyperprolactinemia in the puerperium causes a rapid cessation of milk production.[195] The key role of PRL in milk formation has been demonstrated by the finding that mice with either the *PRL* gene KO[184] or the PRL receptor KO[196] are unable to lactate.

Galactorrhea

The persistence of galactorrhea for more than 1 year after normal delivery and cessation of breastfeeding or its occurrence in the absence of pregnancy generally is taken as a definition of inappropriate lactation. The incidence of galactorrhea in healthy women has been reported as ranging from 1% to 45% of subjects tested.[197,198] This variability is probably the result of differences in the techniques used to express milk from the breast and the way in which nonmilky secretions are classified.

Inappropriate lactation may be an important clue to the presence of pituitary-hypothalamic disease, especially if accompanied by amenorrhea. Combined data from 14 published series suggest that 27.9% of galactorrheic women with normal menses have elevated PRL levels.[199] More recent experience, however, suggests that galactorrhea may be present in about 5% to 10% of normally menstruating women and basal PRL levels are normal in more than 90% of these women. Lowering of PRL levels will almost always lead to a marked decrease or abolition of lactation regardless of whether PRL levels are initially elevated or not.

Prolactin Effects on Gonadotropin Secretion

The effects of PRL levels in the normal range on gonadotropin secretion are not known. However, *PRL* gene-KO and PRL receptor-KO female mice are sterile and have disordered estrous cycles.[184,196] Females with the PRL receptor KO had fewer primary follicles, fewer eggs ovulated, fewer eggs fertilized, poorer progression of those eggs that were fertilized to the blastocyst stage, and uteruses that were refractory to implantation by the blastocyst.[196] Furthermore, they had decreased estradiol and progesterone levels.[200]

In healthy women treated with short-term bromocriptine to lower PRL levels to about 5 ng/mL, there was no change in the pulsatile secretion of LH and FSH but estradiol levels were higher the last 3 days of the follicular cycle. Progesterone levels were lower during the luteal phase.[201] Data are not available on reproductive function and prolactin levels for the new short-acting bromocriptine agent (Cycloset®) used for the treatment of diabetes.

Hyperprolactinemia has a number of effects on various steps in the reproductive axis. Hyperprolactinemia has been found in most studies to suppress LH pulsatile secretion (Fig. 3.2) by decreasing pulse amplitude and

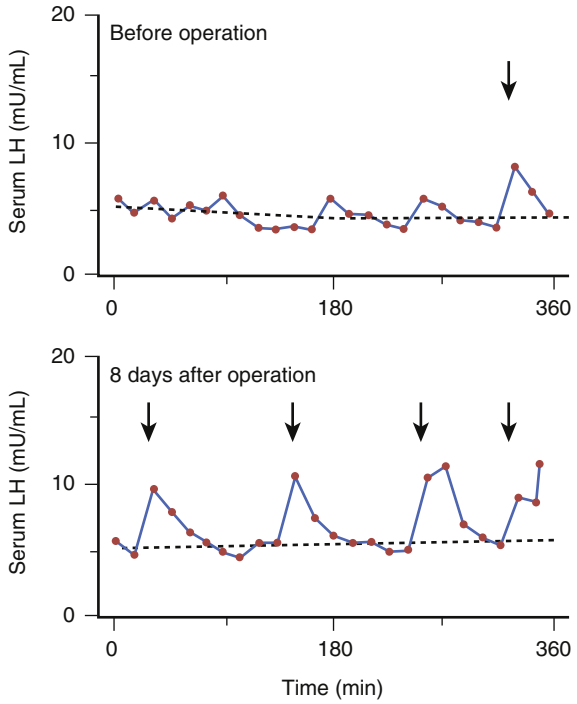


FIGURE 3.2 Pre- and postoperative serum luteinizing hormone (LH) concentrations following selective resection of a prolactinoma. The start of an LH pulse is indicated by an *arrow*. *(Adapted from Stevenaert et al., early normalization of luteinizing hormone pulsatility in women with microprolactinomas.* J Clin Endocrinol Metab *62:1044–1047, 1986.)*

frequency.[202-204] With menopause in humans, hyperprolactinemia can prevent the expected rise in gonadotropins[205]; normalization of PRL levels with bromocriptine results in a rise in gonadotropin levels and hot flashes.[205]

Hyperprolactinemia inhibits pulsatile gonadotropin secretion by a number of mechanisms. It had been postulated that the pulsatile gonadotropin secretion was directed by the hypothalamic GnRH pulse generator and that alteration of pulsatile secretion necessarily means a direct hypothalamic action of PRL. Consistent with that notion, PRL inhibits GnRH release from hypothalamic neuron cell lines through an action on PRL receptors expressed by these cells.[206] Measurement of portal vessel GnRH levels in rats showed a marked inhibitory effect of hyperprolactinemia in one study,[207] but not in another.[208]

The pituitary gonadotroph response to GnRH in hyperprolactinemia has generally been found to be decreased in rats[208,209]; in contrast, the response may be normal, increased or decreased in humans.[210,211] The number of GnRH receptors on gonadotroph cells in hyperprolactinemic rats is reduced[212] even when endogenous GnRH is replaced with intraarterial pulses of GnRH.[205,213] In addition to these effects, hyperprolactinemia in women has been associated with loss of positive estrogen feedback on gonadotropin secretion.[214]

Prolactin Effects on the Ovary

Prolactin is trophic to corpus luteum function in rats, giving rise to the name luteotrophic hormone.[215] The role of PRL in normal ovarian function in humans is not as

well established, however. McNatty and colleagues demonstrated that low physiologic concentrations of PRL are necessary for progesterone synthesis by human granulosa cells, but that high concentrations are inhibitory in vitro.[216] Other studies suggest that PRL can activate the expression of Type 2 3β-hydroxysteroid dehydrogenase, the final enzymatic step in progesterone biosynthesis.[217] Del Pozo and colleagues found no effect on luteal function in women treated with bromocriptine to lower normal PRL levels[218]; others, however, have found that such a decrease in PRL resulted in lowered progesterone levels and short luteal phases.[203,219,220] On the other hand, short luteal phases have also been reported in hyperprolactinemic women.[221]

In humans, plasma PRL levels greater than 100 ng/mL have been found to cause an increase of antral fluid PRL levels and reductions in antral fluid FSH and estradiol levels and a decrease in granulosa cell number.[222] Perfusion studies of human ovaries *in vitro* reveal a direct suppressive effect of PRL on progesterone and estrogen secretion.[223] PRL can inhibit estrogen formation by antagonizing the stimulatory effects of FSH on aromatase activity[224] and direct inhibition of aromatase synthesis has also been shown.[225]

In some but not all studies, PRL levels have been found to be elevated in women with polycystic ovaries (PCO).[226,227] Bromocriptine treatment of hyperprolactinemic patients with PCO usually resulted in a reduction of testosterone and LH levels, and often a resumption of ovulatory cycles. One hypothesis suggests that the increased estrogen levels found in PCO stimulate increased PRL secretion,[226] but the association between PCO and hyperprolactinemia has been questioned.[227,228]

When amenorrhea or oligo-amenorrhea is associated with galactorrhea, it usually is a manifestation of hyperprolactinemia. In combined series totaling 471 patients with galactorrhea-amenorrhea, 75.4% were found to have hyperprolactinemia.[199] Although the amenorrhea caused by hyperprolactinemia usually is secondary, it can also be primary if the disorder begins before puberty.[229,230] In two studies of 33 patients evaluated for primary amenorrhea and low gonadotropin levels, 9 (27%) were found to have hyperprolactinemia.[229,230] In patients with primary amenorrhea due to hyperprolactinemia, failure to develop normal secondary sexual characteristics may be the presenting problem. Galactorrhea is variable in this setting because the breast may not have been exposed to appropriate priming with estrogen and progesterone. Young women with hyperprolactinemia and primary amenorrhea tend to have macroadenomas more commonly than those with secondary amenorrhea; the reason for this difference remains uncertain.

Hyperprolactinemia has been found in many women with a short luteal phase, as noted previously. A short luteal phase is likely to be the first evidence of interference in the normal cycle by hyperprolactinemia.[231] In the initial period of shortened luteal phase, progesterone levels are subnormal, suggesting deficient corpus luteum function.

Infertility also may be a presenting symptom of patients with hyperprolactinemia and is invariable when gonadotropin levels are suppressed with anovulation. In women (367 women combined from multiple studies) studied for infertility, one-third were found to have hyperprolactinemia.[199] Most of the women had amenorrhea and galactorrhea as well, but in one series of 113 cases of infertility, 5 of the 22 hyperprolactinemic women had neither amenorrhea nor galactorrhea.[232] PRL excess may be important in this type of patient, as suggested by the finding that treatment of similar patients with bromocriptine restored fertility.[232] In some of these women, transient hyperprolactinemia lasting for 1 to 2 days during the cycle can be documented; this subset usually responded to bromocriptine, experiencing increased progesterone during the luteal phase and improved fertility.[233]

Reduced libido and orgasmic dysfunction are found in most hyperprolactinemic amenorrheic women when such complaints are specifically elicited.[210] Reduction of PRL levels to normal restores normal libido and sexual function in most of these women.[234]

Prolactin Effects on the Testes

The role of PRL in normal testicular function is not well understood. Half of the male rats with PRL receptor-KO mutations were fully fertile, but the remainder were either completely or partially infertile despite normal sexual behavior and normal spermatogenesis (as determined by histological evaluation of the testes).[196] Male mice with the *PRL* gene-KO were fully fertile and had normal plasma testosterone levels and normal testosterone release from the testes, despite decreased plasma LH levels and LH and FSH secretion from the pituitary *in vitro* and decreased weights of the seminal vesicles and ventral prostate.[235]

In studies of healthy men, bromocriptine-induced suppression of normal PRL levels for 8 weeks resulted in suppression of basal and hCG-stimulated testosterone levels.[236] This finding implies a physiologic role for PRL in normal testosterone production in humans. PRL is present in human semen in very high concentrations[237] and PRL has been shown to stimulate adenyl cyclase, fructose utilization, glycolysis, and glucose oxidation in human spermatozoa.[238]

Chronic hyperprolactinemia results in impotence and decreased libido in over 90% of cases.[239-247] Galactorrhea in men has been reported in 10% to 20% of cases and is virtually pathognomonic of a prolactinoma.[239-245,248] As previously noted, there is a decrease in the pulsatile secretion of LH and FSH in hyperprolactinemic men, and testosterone levels are low or are in the lower part of the normal range.[239-248] With normalization of PRL levels with cabergoline, testosterone levels normalize in about two-thirds of men and erectile function normalizes in 60%.[246,249]

The testosterone response to stimulation with hCG has been reported to be both decreased[243,250] and normal[239,251]; in those with decreased responses there is improvement in the response when PRL levels are lowered with bromocriptine.[243] If there is sufficient normal pituitary tissue, reduction of elevated PRL levels to normal usually results in a return of normal testosterone levels.[244-246,252,253] Although some studies in rats have suggested that drug-induced elevated PRL levels cause a partial block in the enzyme 5-α reductase, resulting in a decrease in dihydrotestosterone levels, this has not been

found in studies of men with prolactinomas.[250] Carter and colleagues noted that testosterone therapy of hyperprolactinemic men does not always correct the impotence until PRL levels are brought down to normal.[239] Whether or not this is due to a decrease in dihydrotestosterone levels has not been verified directly.

Sperm counts and motility are decreased with an increase in abnormal forms in hyperprolactinemic men.[246,249,251] Histological studies reveal abnormal seminiferous tubule walls and altered Sertoli cell ultrastructure.[254] The semen analysis does not always return to normal despite normalization of testosterone and PRL levels.[249,252]

A number of surveys have attempted to assess the frequency of hyperprolactinemia among men with complaints of impotence or infertility. Between 2% and 25% of males with impotence have been found to be hyperprolactinemic in various series.[232,243,245,255-258] However, only 1% to 5% of infertile men have been found to be hyperprolactinemic.[259-261] Although these frequencies are relatively low, the modest cost of measuring PRL is justified given that hyperprolactinemia is, in general, easily treatable.

Prolactin Effects on the Adrenal Cortex

Although PRL receptors are found on cells of the adrenal cortex, the physiological role of PRL in adrenal steroidogenesis is unknown. Plasma dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS) levels have been found to be mildly elevated in about 50% of women with hyperprolactinemia in some,[262] but not all, studies.[263] In most of these studies, however, the investigators did not try to correlate the androgen levels with the presence of hirsutism or other indices of hyperandrogenism. The abnormal androgen levels return to normal with correction of the hyperprolactinemia by bromocriptine.[232]

Prolactin Effects on Bones

PRL may have a physiological role in calcium and bone metabolism. The PRL receptor-KO mouse has a decrease in bone formation rate and bone mineral density in association with increased parathyroid hormone levels, but also had decreased estradiol and progesterone levels.[200] Thus, it is difficult to know how much of the abnormal bone metabolism is attributable to the inability to respond to PRL as opposed to estrogen deficiency.

Hyperprolactinemic women have decreased bone mineral density,[264-267] but whether this effect is mediated by estrogen deficiency[264] or is a direct effect of the hyperprolactinemia[265,266] has been debated. The decreased bone mass is accompanied by an increase in bone turnover makers[267] and fractures.[268] Correction of the hyperprolactinemia results in an increase in bone mass.[269,270] Studies of hyperprolactinemic women who were not amenorrheic and hypoestrogenemic have shown that their bone mineral density is normal,[271,272] confirming the initial hypothesis that estrogen deficiency mediates the bone mineral loss. A similar, androgen-dependent loss of bone mineral is found in hyperprolactinemic men,[273,274] is accompanied by an increase in vertebral fractures[275] and is reversible with reversal of the hypoandrogenic state.[273]

Prolactin Effects on the Immune System

Prolactin is produced by T- and B-lymphocytes but its synthesis is under control of an alternative upstream promoter.[276] In animal studies, lowering of normal PRL levels by bromocriptine or anti-PRL antibodies results in impaired lymphocyte proliferation and macrophage activating factor production[277] but in *PRL* gene-KO and PRL receptor-KO mice, PRL has not been found to be essential for normal immune function.[278,279] Conversely, in rat models of systemic lupus erythematosus, PRL levels are elevated and bromocriptine causes improvement in a variety of autoimmune parameters.[280-282]

Studies in humans have been conflicting. Some studies of patients with hyperprolactinemia have shown increased rates of autoantibodies (including antithyroid, anti-double-stranded DNA, anti-Ro, anticardiolipin, and antinuclear antibodies) without clinical evidence of disease.[283-285] Conversely, elevated levels of PRL have been found in patients with systemic lupus erythematosus, rheumatoid arthritis, psoriatic arthritis, multiple sclerosis, Reiter's syndrome, Sjögren's syndrome, and uveitis.[285-288] In some studies, conventional immunosuppressive treatment of these disorders resulted in a lowering of PRL levels and conversely treatment with bromocriptine resulted in clinical improvement in the autoimmune condition (see Chuang and Molitch for detailed review).[289] Although PRL appears to have an immunomodulatory function, at present, the relationship of pituitary vs. lymphocytic PRL to these autoimmune conditions in humans is still uncertain and treatment aspects remain to be established.

Pathologic States of Prolactin Secretion

Hypoprolactinemia

PRL deficiency may occur in the setting of panhypopituitarism, generally as a result of pituitary infarction or following pituitary surgery. When the cause of the hypopituitarism is hypothalamic or stalk dysfunction, PRL levels generally rise due to disinhibition of PRL secretion (which will be explained later in this chapter). However, when the pituitary tissue is actually destroyed, as in Sheehan's peripartum necrosis, PRL levels are usually low.[290] The finding of low PRL levels following surgery for a pituitary tumor usually is indicative of very severe hypopituitarism.[291] Idiopathic PRL deficiency has been described in only a single human.[292] Clinically, hypoprolactinemia manifests as an inability to lactate postpartum.

Hyperprolactinemia

The differential diagnosis of sustained hyperprolactinemia encompasses a spectrum of pharmacological and pathological entities (Box 3.1). This section discusses those causes of hyperprolactinemia other than prolactinomas.

Medications

Psychoactive medications

The antipsychotic agents (phenothiazines and butyrophenones) are dopamine receptor blockers and uniformly result in elevated PRL levels, generally no higher than 100 ng/mL, but some patients have been reported with levels as

Box 3.1 Etiologies of Hyperprolactinemia

PITUITARY DISEASE
Prolactinomas
Acromegaly
Cushing's disease
"Empty Sella syndrome"
Lymphocytic hypophysitis

HYPOTHALAMIC DISEASE
Nonsecreting pituitary adenomas
Meningiomas
Craniopharyngiomas
Sarcoidosis
Langerhans cell histiocytosis
Other tumors
Neuraxis irradiation
Vascular
Pituitary stalk section

MEDICATIONS
Antipsychotics
- Phenothiazines
- Butyrophenones
- Atypicals

Antidepressants
- Tricyclics
- SSRI's (rare)
- MAO inhibitors

MEDICATIONS—cont'd
Antihypertensives
- Reserpine
- Methyldopa
- Verapamil

Other medications
- Metoclopramide
- Cocaine
- Opiates

NEUROGENIC
Spinal cord lesions
Chest wall lesions
Breast stimulation

OTHER
Pregnancy
Hypothyroidism
Chronic renal failure
Cirrhosis
Pseudocyesis
Adrenal insufficiency
Ectopic

MACROPROLACTINEMIA

IDIOPATHIC

high as 365 ng/mL.[111,112] PRL levels usually fall to normal within 48 to 96 hours of discontinuation of antipsychotic drug therapy.[293] Atypical antipsychotic agents that are combined serotonin-dopamine receptor antagonists, such as risperidone and molindone, cause similar elevations of PRL.[294] However, many of the other atypical antipsychotic agents, such as quetiapine, olanzapine, and aripiprazole do not cause hyperprolactinemia, and when feasible, hyperprolactinemic patients on risperidone or one of the phenothiazines may be switched to these drugs.[294-297] The lack of hyperprolactinemic effect of these other atypical antipsychotic agents is thought to be due to their being only transiently and weakly bound to the D_2 receptor and to their having agonist as well as antagonist activity at the D_2 receptor.[294]

Tricyclic antidepressants cause modest hyperprolactinemia in about 25% of patients.[298] Monoamine oxidase (MAO) inhibitors, when used chronically, may also cause a minimal elevation of PRL levels.[299] The mechanisms by which these drugs cause increased PRL levels are not certain and they likely facilitate several possible stimulatory pathways. Serotonin reuptake inhibitors, by increasing synaptic serotonin levels, very rarely cause hyperprolactinemia.[154,294,300,301] Other antidepressants including nefazodone, bupropion, venlafaxine, trazodone, and lithium do not cause hyperprolactinemia.[294] Chronic opiate abuse is associated with mild hyperprolactinemia and menstrual dysfunction.[294] Cocaine abuse has also been associated with chronic, mild hyperprolactinemia.[302]

Antihypertensive Drugs

Alpha-methyldopa causes moderate hyperprolactinemia by inhibiting the enzyme L-aromatic amino acid decarboxylase (which is responsible for converting L-dopa to dopamine) and by acting as a false neurotransmitter to decrease dopamine synthesis. Short-term and chronic verapamil therapy has been found to increase basal PRL secretion and the PRL response to TRH[31-33,303-305]; patients have been described with galactorrhea associated with sustained hyperprolactinemia from verapamil therapy.[303,304] In a survey of patients taking verapamil in an outpatient clinic, PRL levels were found to be elevated in 8.5% of patients.[305] Verapamil blocks the hypothalamic generation of dopamine.[33] Other calcium channel blockers such as the dihydropyridines and benzothiazepines have no action on PRL secretion, implying that the action of the phenylakylamine, verapamil, likely is acting on the neuronal N-type calcium channel.[33]

Protease Inhibitors

Although one report showed that some HIV positive patients treated with protease inhibitors developed galactorrhea and hyperprolactinemia,[306] a second report implied that the elevations of PRL seen in some patients can largely be attributed to other medications received and stress.[307] The mechanism and frequency of this are unknown.

Other Medications

Metoclopramide and domperidone, two drugs commonly used to increase gastrointestinal motility and help stomach emptying in patients with gastroparesis diabetocorum, are D_2 receptor blockers. These drugs cause hyperprolactinemia in over 50% of patients and commonly cause symptoms of amenorrhea and galactorrhea in women and erectile dysfunction in men.[294] Although there are isolated case reports of hyperprolactinemia being associated with H_2 blockers and protease inhibitors, more systematic studies of these classes of medications have failed to support an etiological role for them.[294]

Stress

As previously mentioned, physical stress (such as physical discomfort, exercise, and hypoglycemia) causes an acute, transient rise in PRL levels (discussed earlier in the chapter). Chronic hyperprolactinemia, due to prolonged physical stress, has not been reported and chronic illness generally suppresses PRL levels. Psychological stress may cause minimal increases of PRL but chronic hyperprolactinemia has not been reported with any chronic psychiatric state except in pseudocyesis; in this condition, PRL levels fall with psychotherapy.[308]

Kidney disease

Hyperprolactinemia occurs in 73% to 91% of women and 25% to 57% of men with end-stage renal disease.[309,310] This is due to decreased PRL clearance as well as autonomous production and bromocriptine results in suppression of PRL levels.[309] About one-quarter of individuals with renal insufficiency not requiring dialysis (serum creatinine ranging from 2.0 to 12.0 ng/mL) have PRL levels in the 25 to 100 ng/mL range.[310] When such patients take medications known to alter hypothalamic regulation of PRL (such as methyldopa or metoclopramide) PRL levels may rise to over 2000 ng/mL.[310] Correction of the renal failure with transplantation causes a return of PRL levels to normal.[310] Hyperprolactinemia plays a role in the hypogonadism of

chronic renal failure, but probably does not explain all of the abnormalities in every case.

Cirrhosis

Basal PRL levels are elevated in patients with alcoholic cirrhosis in frequencies varying from 16% to 100%[311-313] and in patients with nonalcoholic cirrhosis from 5% to 13%.[311] In one study, 50% of patients with hepatic encephalopathy were found to be hyperprolactinemic.[314] An underlying defect in hypothalamic dopamine generation has been hypothesized as the cause of the hyperprolactinemia in these encephalopathic patients.

Hypothyroidism

A modest increase in the level of PRL in 8% to 36% of patients with overt hypothyroidism and a smaller percentage with subclinical hypothyroidism; levels greater than 25 ng/mL are uncommon.[315] The mechanisms involved probably include increased TRH production, increased sensitivity of lactotrophs to TRH and possibly increased pituitary VIP generation. Therapy with L-thyroxine will cause the PRL levels to return to normal and can even result in a regression of pituitary size (which was due to thyrotroph hyperplasia).[315,316]

Adrenal insufficiency

Glucocorticoids have a suppressive effect on *PRL* gene transcription and PRL release. Rare cases of hyperprolactinemia have been reported in patients with adrenal insufficiency whose PRL levels return to normal with glucocorticoid replacement.[317,318]

Neurogenic Stimulation

Sexual breast stimulation and suckling cause a reflex release of PRL that is mediated, by afferent neural pathways going through the spinal cord. Chest wall and cervical cord lesions have been reported to result in elevated PRL levels and galactorrhea through stimulation of these afferent neural pathways.[319] Similar chronic elevations of PRL have been reported after mastectomy, nipple piercing rings, thoracotomy, and chronic spinal cord injuries.[320,321]

Ectopic Prolactin Secretion

Ectopic production of PRL is exceedingly rare[322]; however, there have been reported cases of symptomatic hyperprolactinemia due to well-documented PRL production from a renal cell carcinoma,[323] a gonadoblastoma,[324] and ectopic pituitary tissue in ovarian teratomas.[325,326] Given the great frequency of prolactinomas, "idiopathic hyperprolactinemia" and other causes of hyperprolactinemia, a search for an ectopic source of PRL secretion is not warranted unless some other tumor shows up coincidentally.

Hypothalamic-Pituitary Stalk Disease

Hyperprolactinemia caused by lesions of the hypothalamus and of the pituitary stalk is due to disturbance of the neuroendocrine mechanisms that control PRL secretion.[327] From hypothalamic lesion work in animals, it has been assumed that this PRL elevation is due to disinhibition of the tonic PIF (DA) acting at the level of the pituitary lactotrophs. However, many of these patients have normal TSH and ACTH function, implying that there still is significant transmission of hypothalamic releasing factors to the pituitary.

Generally, patients with total stalk section (in whom the PRL rise is due solely to dopamine deficiency), have lower PRL levels than those with partial stalk-hypothalamic dysfunction who have DA plus continued PRF activity. In a recent series of 226 patients with clinically non-functioning pituitary adenomas, 99% had serum PRL levels less than 84 ng/mL.[328] However, a number of cases have been reported in the literature by others with PRL levels between 104 and 219 ng/mL.[327-329] Irradiation of pituitary and non-pituitary brain tumors may also cause hyperprolactinemia along with hypopituitarism, presumably due to hypothalamic damage and dysregulation.[330]

Idiopathic Hyperprolactinemia

When no specific cause for hyperprolactinemia is found, it is designated as idiopathic. In many such cases, small prolactinomas may be present that are too small to be detected by current radiological techniques. In other cases, the hyperprolactinemia may be attributed to presumed hypothalamic regulatory dysfunction, but no dysfunction specific to idiopathic hyperprolactinemia has been definitively elucidated. Long-term follow-up of such patients has found that in about one-third, PRL levels return to normal; in 10% to 15% there is a rise in PRL levels to greater than 50% over baseline; and in the remaining patients, prolactin levels remain stable.[331,332] Over a 2 to 6 year follow-up of 199 patients, only 23 were found to have evidence of microadenomas and none developed macroadenomas.[331-335] In one study, about 25% of patients with idiopathic hyperprolactinemia are found to be anti-pituitary antibody positive, suggesting that there may be some element of subclinical hypophysitis present.[336]

Clinical Testing of Hyperprolactinemic Patients

Because PRL is secreted episodically and some PRL levels during the day may be above the upper limit of normal established for a given laboratory, the finding of minimally elevated levels in blood requires confirmation in several samples. As indicated in the previous section, there are a number of conditions that may cause moderate PRL elevations of less than 250 ng/mL. A careful history and physical examination, screening blood chemistries, thyroid function tests and a pregnancy test will exclude virtually all causes except for hypothalamic-pituitary disease.[337,338] In cases with less certain symptoms, macroprolactinemia should be looked for (discussed earlier in the chapter).

When there is no obvious cause of the hyperprolactinemia from the routine screening, a radiologic evaluation of the hypothalamic-pituitary area is mandatory to evaluate for a mass lesion.[337-339] This includes patients with even mild PRL elevations. At present, such an evaluation is generally done using magnetic resonance imaging (MRI) with gadolinium enhancement or computed tomography (CT) with intravenous contrast enhancement. For prolactinomas, there is a fairly good correlation between PRL levels and the size of the tumor.[339] Patients with microprolactinomas rarely have PRL levels of more than

250 mg/dL.[337,338] It should be emphasized here that it is very important to distinguish between a large nonsecreting tumor causing modest PRL elevations (usually <250 ng/mL) from a PRL secreting macroadenoma (PRL levels usually >>250 ng/mL), as the therapy may be quite different. It is important to have serum run at 1:100 dilution in patients with large macroadenomas to exclude the "hook effect." Stimulation and suppression tests give nonspecific results with regard to the differential diagnosis of hyperprolactinemia and reveal no more information than just measurement of basal PRL levels.[337,338]

Prolactinoma

Classification and Epidemiology

Prolactinomas are generally classified clinically by size: microadenomas are less than 10 mm in diameter; macroadenomas are greater than 10 mm in diameter; and macroadenomas with extrasellar extension. The direction and degree of extrasellar extension are of obvious clinical importance. Serum PRL levels usually parallel the size of the tumors. Malignant prolactinomas are exceedingly rare.

Prolactinomas are the most common of the secreting pituitary adenomas, older studies reporting an incidence of 6 to 10 cases per million and a prevalence of 60 to 100 cases per million.[340] However, recent series from Belgium, Switzerland, Finland, and the United Kingdom report incidences and prevalences about five times higher.[341-344] Prolactinomas occur more commonly in women, in whom they are generally microadenomas, whereas those in men are generally macroadenomas (discussed later in the chapter).

Natural History of Prolactinomas

Autopsy Studies

Pituitary adenomas have been found at autopsy in about 11% of subjects not suspected of having pituitary disease while alive.[345] In the studies in which PRL immunohistochemistry was performed, 40% stained positively for PRL.[345] In these postmortem studies, all but seven of the tumors (99.97%) were less than 10 mm in diameter; however, there are a number of clinical reports of incidentally found macroadenomas.[345]

Natural History of Untreated Prolactinomas

Six studies have been published in which patients with microadenomas were observed for long periods without treatment. In these studies, women with prolactinomas documented by sella computed tomography or CT who refused surgery or medical treatment were followed for a period of up to 8 years.[346-351] Of the 139 women observed, there was evidence of tumor growth by these methods in only 9 (6.5%). In retrospect, we now know the high false-positive and false-negative rates of polytomography.

One potential stimulus to growth of a microadenoma was hypothesized to be the use of oral contraceptives. However, careful case-control studies using normal or amenorrheic control women and long-term epidemiologic surveys have not shown such a relationship.[352] In other studies, estrogens have been administered for 2 to 4 years to women with microadenomas and idiopathic hyperprolactinemia, resulting in no tumor enlargement in any patients.[353-355] However, individual cases with enlargement of tumors during estrogen therapy have been reported,[356] so hyperprolactinemic patients treated with estrogens should be followed carefully with periodic monitoring of PRL levels.

Many other potential factors have been investigated to determine why some tumors grow to become macroadenomas and why some macroadenomas are extremely large and invasive. Histologically, invasive prolactinomas have a high Ki-67 labeling index, which reflects increased cell proliferation.[357] Altered expression of adhesion molecules, matrix metaloproteinases, and extracellular matrix components have also been found.[357] An increase in angiogenesis has also been found in these large tumors.[358]

Pathogenesis of Prolactinomas

A primary defect in hypothalamic regulation of PRL secretion, such as a defect in dopaminergic tone had earlier been hypothesized to either cause or facilitate the growth of prolactinomas.[359] However, most information now favors the hypothesis that prolactinomas arise *de novo* as intrinsic disorders of the pituitary due to a single cell mutation with monoclonal cell proliferation and that most changes in hypothalamic function in patients with tumors are secondary to the tumors.[360,361]

A large number of potential mutations that could be pathogenetic for prolactinomas in humans have been investigated. Mutations causing loss of function in the dopamine D_2 receptor or gain of function of the TRH receptor have been looked for without success.[362-364] Evaluation of prolactinomas for mutations in the G proteins coupling the D_2 receptor to adenyl cyclase and the TRH receptor to its intracellular activating pathways have also been unsuccessful,[365,366] although prolactinomas resistant to bromocriptine may have decreased levels of the $G_{i2\alpha}$ protein that couples the D_2 receptor to adenyl cyclase.[365] As noted below, bromocriptine-resistant prolactinomas also have a decrease in the short D_2 receptor isoform, resulting in decreased inhibition of adenyl cyclase.[367] Thus, these alterations in D_2 isoforms and $G_{i2\alpha}$ may play a role in the pathogenesis of dopamine resistant prolactinomas, but those tumors comprise only 8% to 15% of prolactinomas.[365]

Many other studies have examined prolactinomas for putative oncogenes and tumor suppressor genes. No evidence for amplification or rearrangements have been found for the putative oncogenes *Pit-1*, *Prop-1*, *N-ras*, *H-ras*, *K-ras*, *myc II*, *N-myc*, *c-myc*, *myb*, *blc1*, *h-SF1*, *p16*, *p27*, *p53*, *sea*, *nm23*, or *c-fos* or the *menin* tumor suppressor gene.[360,361,368] Investigations are ongoing regarding abnormalities of cell cycle regulation, various signaling pathways and transcriptional regulators,[360] but their roles in the pathogenesis of prolactinomas and the stimulation of their growth still remain unclear.

Prolactinomas in Multiple Endocrine Neoplasia Type I

Prolactinomas occur in about 20% of patients with Multiple Endocrine Neoplasia Type I (MEN-I).[369] The MEN-I

(MENIN) gene is thought to function as a constitutive tumor suppressor gene, so an inactivating mutation results in tumor development. As noted previously, similar mutations have not been found in sporadic prolactinomas. The fact that prolactinomas develop in only a subset of MEN-I patients suggests that there may be a secondary modifying gene at a different locus that acts with the *MENIN* gene to produce prolactinomas.[369] There is evidence that the prolactinomas in patients with MEN-I may be larger, more aggressive and more resistant to treatment than sporadic prolactinomas.[370,371]

When patients with apparently sporadic prolactinomas were screened for hypercalcemia, 14.3% in one series were found to have hyperparathyroidism, of whom one-third were found to have gastrinomas upon screening for pancreatic tumors.[372] This figure is higher than the 2% to 3% reported previously.[373,374] However, even a figure of 2% suggests that obtaining a careful family history and measuring calcium levels are useful in the evaluation of all prolactinoma cases.

Prolactinomas in Familial Isolated Pituitary Adenomas (FIPA)

Germline mutations in the aryl hydrocarbon receptor interacting protein *(AIP)* gene were identified in a Finnish population of patients with familial isolate pituitary adenoma (FIPA) associated with somatotropinomas and prolactinomas.[375] In the initial report, 8 out of 54 patients from several families with a predisposition to prolactinomas and somatotropinomas harbored one of three different *AIP* germline mutations.[375] AIP appears to be a classic tumor suppressor gene with tumors in affected patients showing somatic loss of the wild-type allele. The genetics are autosomal dominant with variable penetrance and within families different types of tumors may be present, i.e., prolactinomas, somatotropinomas, and somatolactotopinomas.[376] In FIPA families, prolactinomas tend to present during childhood, be aggressive, have high rates of extrasellar expansion, and are resistant to dopamine agonists.[374, 376]

Pathology

Prolactinomas can invade local tissues and may have varying histologic features, but they cannot be termed truly malignant unless metastases distant from the original tumor can be demonstrated. Fortunately, true malignant prolactinomas are exceedingly rare and just over 40 have been reported.[377,378]

PRL secreting tumors may also secrete other hormones. The most common combination is PRL plus GH, about 25% to 40% of GH secreting tumors being found to make PRL. One particular variant of these PRL-GH-secreting tumors is the *acidophil stem cell adenoma*. These tumors have irregular, elongated cells with irregular nuclei and may have oncytic changes with very large mitochondria. In contrast to the other tumors secreting both hormones, patients with these tumors have more marked elevations of PRL than GH and usually present with menstrual abnormalities, galactorrhea, decreased libido, or impotence. They may have little in the way of acromegalic features.[379] These tumors are usually macroadenomas at the time of presentation and generally have had a relatively short course.[379] Other hormone combinations include PRL and ACTH, PRL and TSH, and PRL and FSH.[380] Interestingly, these plurihormonal tumors are generally monoclonal in origin,[381] as are most pure prolactinomas (discussed earlier in the chapter).

Local Mass Effects

Local mass effects may well cause symptoms in patients with macroadenomas, depending upon size and extent of extrasellar extension. The frequency of such symptoms is much lower than in patients with nonsecreting tumors because prolactinoma patients usually present with symptoms of reproductive or sexual dysfunction (discussed later in the chapter). Visual field defects due to chiasmal compression depend upon the amount of suprasellar extension. Because of the great variation in how these tumors grow superiorly with respect to the location of the chiasm, visual field defects can range from the classical, complete bitemporal hemianopsia to small, partial quadrantic defects, to scotomas.[382] There are no specific types of visual field defects peculiar to prolactinomas compared to other types of tumors.

Ophthalmoplegias are relatively uncommon, due to invasion of the cavernous sinus with entrapment of cranial nerves III, IV, V_1, V_2, and VI. In some patients a cavernous sinus syndrome may develop, consisting of ophthalmoplegia and pain or hyperesthesia in the distribution of V_1.[383] The carotid artery may be encased within the tumor but narrowing of the artery is not seen.[384] Shrinkage of these very large, invasive tumors with dopamine agonists can be quite dramatic and satisfying,[383,385,386] as surgery rarely is curative and is potentially fraught with complications.[387-389]

Extensive invasion of the floor of the skull, with massive destruction of bone, may occur, but this rarely causes problems of entrapping cranial nerves or compressing vital brain structures.[385-389] Extrasellar extension in other directions may rarely cause temporal lobe epilepsy and hydrocephalus.[385,388] These large, invasive tumors are uncommon but not rare and should be differentiated from true carcinomas; a demonstration of metastases distant from the primary tumor is necessary for the latter diagnosis. Histologically, these invasive tumors have no specific features to differentiate them from noninvasive prolactinomas.[388] Many of these patients respond quite well to dopamine agonists, occasionally shrinking to the point where no tumor is even detectable on scan, and PRL levels are normal.[383,385,386] Rarely, these tumors function as a "cork" at the base of the skull so that cerebrospinal fluid leaks may occur with substantial tumor shrinkage; surgery is necessary to repair such leaks to avoid the risk of meningitis.[386] For some tumors that do not respond to medication, surgical debulking and irradiation may be necessary (discussed later in the chapter).[385]

Local mass effects may also cause hypopituitarism because of direct pituitary compression or hypothalamic/stalk dysfunction. The larger the tumor, the more likely there is to be one or more hormonal deficits.[253,385-389] All patients with macroadenomas need to be evaluated for possible deficits in pituitary function.[337]

Clinical Manifestations

The clinical manifestations due to hyperprolactinemia are discussed above. The frequencies with which various

clinical manifestations occur in patients with prolactinomas vary depending upon referral patterns.

Women

In older series, almost all premenopausal women presented because of symptoms of galactorrhea, amenorrhea, or infertility. In a summary of 21 series of 1621 women with prolactinomas undergoing transsphenoidal surgery, the frequency of oligo-amenorrhea was 92.9% and of galactorrhea was 84.7%.[390]

Although secondary amenorrhea is more common, primary amenorrhea may also occur. Presentation because of severe headaches or visual field disturbance due to large tumors is uncommon in women because they usually initially seek medical attention for menstrual dysfunction or galactorrhea (which generally occur even with minimal PRL elevations and long before the tumors have grown large).[391] Postmenopausal women with prolactinomas usually present with mass effects from large tumors,[383] although others are found simply because of a history of "premature" menopause.[392]

Men

Men with prolactinomas often seek medical attention because of symptoms related to the size of the tumor, rather than because of impotence, loss of libido or infertility.[393] Prostate volume is reduced when men are hyperprolactinemic, presumably due to their low testosterone levels; prostate size returns to normal when PRL levels are normalized.[394] In a summary of 16 series comprising 444 men with prolactinomas (not all of whom went to surgery) 77.9% were impotent, 36.6% had visual field defects, 33.8% had partial or complete hypopituitarism, 29.1% complained of headaches, and 10.9% had galactorrhea.[390] Thus, about one-third of men had symptoms due to tumor size. Radiologic investigation demonstrates a macroadenoma in 80% to 90% of cases in most studies.[390,393]

There has been much speculation about the possible reason why a considerably greater proportion of men have macroadenomas compared with women. One hypothesis suggests that men tend to ignore symptoms of sexual dysfunction longer than do women, writing off impotence and decrease in libido to aging.[395] Thus, the prolonged course without therapy permits tumors to grow large. This line of reasoning ignores the data from women regarding the rather uncommon occurrence of progression of size of microadenomas. This observation would point to a more fundamental biological difference in the growth of prolactinomas between the sexes. The difference is unlikely to be due to the differences in target organ sex hormones, (i.e., estrogens and testosterone), because estrogens tend to be strongly growth-promoting. Whether or not there are tumor growth factors that might have a differential effect on prolactinoma growth in the different sexes is unknown. Immunohistochemical studies of tumors using the Ki-67 (MIB-1) antibody, which correlates with tumor growth, showed no difference between the sexes when corrected for tumor size.[396] Further examination of these issues may yield important information regarding the pathophysiology of these tumors.

Children and Adolescents

Children and adolescents may present with growth arrest, pubertal delay, or primary amenorrhea, in addition to the more standard presentations of galactorrhea, oligo-amenorrhea, and mass effects such as headaches or visual disturbances.[397-401] Low bone mineral density is also commonly seen.[402] In contrast to adults, there is a disproportionately large number of patients who have macroadenomas.[397,399-401] Hypopituitarism may be present in those with macroadenomas.[399] Furthermore, the percentage of patients resistant to dopamine agonists may be higher than in adults; Colao and colleagues reported that PRL levels were normalized in only 10 of 26 children and adolescents taking bromocriptine, 5 of 15 taking quinagolide, and 15 of 20 taking cabergoline.[401] The reasons for the high percentage of large macroadenomas and the relative resistance to dopamine agonists are not known, but it is tempting to speculate that these peculiarities may be linked.

Treatment

The indications for therapy in patients with prolactinomas may be divided into two categories: effects of tumor size and effects of hyperprolactinemia. As noted above, in 93% of patients, microprolactinomas do not enlarge over a 4 to 6 year period of observation. Thus, the simple argument that therapy is indicated for a microadenoma to prevent it from growing is fallacious. On the other hand, if a documented adenoma exists, it needs to be followed closely to determine if it is growing. It is very unlikely for a prolactinoma to grow significantly with no increase in serum PRL levels, although this phenomenon has been reported.[403] Therefore, after an initial scan showing a microadenoma, most patients can just be followed with serial PRL measurements. If PRL levels rise or the patient develops symptoms of mass effects such as headaches, then repeat scanning is indicated. Certainly a microadenoma that is documented to be growing demands therapy, for the size change alone, as it may be one of the 7% that will grow to be a macroadenoma.

Macroadenomas have already demonstrated their propensity for growth. Therefore, observation alone is inappropriate unless there are specific contraindications to therapy. Local or diffuse invasion or compression of adjacent structures, such as the stalk or optic chiasm, are additional indications for therapy.

Other indications for therapy are relative, being due to the hyperprolactinemia itself. These include decreased libido, menstrual dysfunction, galactorrhea, infertility, hirsutism, impotence, and premature osteoporosis. If a woman with a microadenoma has normal menses and libido and she is not bothered by the galactorrhea, there is no specific reason for therapy. On the other hand, therapy is indicated for a woman with amenorrhea and anovulation who wishes to become pregnant. However, if such a woman did *not* wish to become pregnant, then therapy to prevent osteoporosis or to improve libido is only relatively indicated and there are potential alternative therapies besides lowering PRL levels.

The ability to follow a patient closely with PRL measurements, CT or MRI scans, and estimations of bone mineral density, coupled with rather precise estimates of the efficacy of various modes of therapy allow a highly individualized way of following patients and choosing the proper timing and mode of therapy.

Surgery

Transsphenoidal surgical success rates are highly dependent upon the experience and skill of the surgeon as well as the size of the tumor. An analysis of the surgical results from 50 published series shows that normalization of PRL levels was achieved in 1596 of 2137 (74.7%) microadenomas and 755 of 2226 (33.9%) macroadenomas.[393] Clearly, for the macroadenomas the lower success rate in large part was dependent on the size of tumors chosen for surgery. In many series, the object was, appropriately, debulking of a very large tumor rather than cure and in other series very large tumors were not operated upon.

From the series compiled, recurrence rates for microadenomas (147 of 809 = 18.2%) and macroadenomas (106 of 465 = 22.8%) were similar.[393] It should be stressed here, that for virtually all of these recurrences, the recurrence is that of hyperprolactinemia and not documented radiological evidence of tumor regrowth. With recurrence of the hyperprolactinemia there usually also is a recurrence of sexual/reproductive dysfunction that usually is an indication for medical therapy to reduce PRL levels. Based on the cure and recurrence rates cited earlier in the text, the ultimate, long-term surgical cure rates using a normal PRL level as the criterion, is 61.1% for microadenomas and 26.2% for macroadenomas.

In an analysis of 84 patients with macroadenomas (36 were prolactinomas), Nelson and colleagues found that of those with normal preoperative pituitary function, only 78% retained normal function postoperatively.[404] One-third with some pituitary deficits prior to surgery improved and one-third with such deficits had worsened pituitary function after surgery; none of the panhypopituitary patients improved following surgery.[404]

Complications from transsphenoidal surgery for microadenomas are quite infrequent, the mortality rate being at most 0.6%, the major morbidity rate being about 3.4% (visual loss, stroke/vascular injury, meningitis/abscess, oculomotor palsy) and cerebrospinal fluid (CSF) rhinorrhea occurring in 1.9%.[393,405-407] The mortality rate for transsphenoidal surgery for all types of secreting and nonsecreting macroadenomas ranges from 0.25% to 1.2%, the major morbidity rate is about 6.5% (visual loss, stroke/vascular injury, meningitis/abscess, and oculomotor palsy), and the rate of CSF rhinorrhea is about 3%.[393,405-407] Transient diabetes insipidus is quite common with transsphenoidal surgery for both micro- and macroadenomas and permanent diabetes insipidus (DI) occurs in about 1% of surgeries on macroadenomas.[405-407] Although visual field defects and reduction in visual acuity can be improved in 74% of patients whose macroadenomas abut the optic chiasm,[408] a small number of patients with normal visual fields may have a reduction of vision after surgery due to herniation of the chiasm into an empty sella, direct injury or devascularization of the optic apparatus, fracture of the orbit, postoperative hematoma, or cerebral vasospasm.[409] In general, complication rates fall with increasing experience of the neurosurgeon.[406,407]

In recent years, endoscopic, endonasal transsphenoidal surgery has evolved into a commonly used technique. This method gives a superior panoramic view with a shorter operating time and local complication rate. However, surgical remission rates are no better with this newer approach.[410,411]

Radiotherapy

Because of the excellent therapeutic responses to transsphenoidal surgery and medical therapy (described later in the chapter), radiotherapy is generally not considered to be a primary mode of treatment for prolactinomas. There are reports of just over 250 patients who have been treated with conventional radiotherapy alone, or in combination with bromocriptine, or after failure of surgical cure.[393] About 35% of patients can achieve normal PRL levels following surgery plus irradiation, usually between 5 and 15 years after irradiation.[393]

The major adverse effect of radiotherapy is hypopituitarism. This complication occurs with frequencies as high as 93%.[393,412] Additional complications that occur months to years after radiotherapy of pituitary adenomas include second malignancies, cerebrovascular accidents, optic nerve damage, radiation brain necrosis, neurologic dysfunction, and soft tissue reactions. Second malignancies have been reported to be significantly increased 15 to 20 years following the primary irradiation.[413-415] Radiation-induced optic atrophy occurs in 2% to 5% of patients and is due to ischemic damage to the optic apparatus.[416] Radiotherapy-induced encephalopathy is rare but can be devastating and occurs only with high doses.[417]

A new form of radiotherapy used increasingly in recent years, which allows the precise delivery of a single, necrotizing dose to the tumor with little radiation to surrounding tissue is referred to as "stereotactic" radiotherapy ("gamma knife" and linear accelerator).[393] Cranial nerves in the cavernous sinus are relatively radioresistant but the optic nerves, chiasm, and tracts are radiosensitive,[418] so that this type of treatment appears to be advantageous for postoperative tumor residing in the cavernous sinus. Data extending out to only 2 to 3 years on almost 300 patients suggests that this technique may be more effective at reducing hormone levels and tumor size in a shorter period of time with fewer complications than conventional radiotherapy.[393] However, only one study reported using stereotactic radiotherapy as primary therapy without dopamine agonist, finding that only 16 of 77 (21%) patients achieved normal PRL levels.[419] Although it is hoped that many of the complications from brain irradiation will be lower with focused radiotherapy compared to conventional radiotherapy, data thus far suggest that the frequency of hypopituitarism will likely be the same.[420]

Thus, with radiotherapy only small numbers of patients reach normal levels of PRL and then only after many years. Radiotherapy seems best reserved as adjunctive therapy for those patients with enlarging lesions who have not responded to either medical or surgical treatment, and the newer techniques of focused radiotherapy would appear to offer advantages of efficacy, rapidity of effect, and possibly less adverse effects, especially for residual tumor in the cavernous sinus. However, in patients who were intolerant or unresponsive to dopamine and who were still hyperprolactinemic following transsphenoidal surgery, normoprolactinemia was achieved in only 18% following stereotactic radiotherapy.[421]

Medical Therapy

Bromocriptine

Bromocriptine was the first D_2 DA receptor agonist to be used for the treatment of hyperprolactinemia. Because of its

short half-life, it usually must be taken two or three times daily. Bromocriptine is successful in normalizing PRL levels or causing return of ovulatory menses in 80% to 90% of patients.[422,423] When PRL levels and return of menses were studied in the same patients, it was found that substantially reducing PRL levels to slightly elevated levels was often enough to restore ovulation and menses despite the fact that normal PRL levels were achieved in only 70% to 80% of treated patients.

There is little intraindividual variability in the absorption and peak blood levels achieved but there is considerable interindividual variability.[424] There is also considerable variability in the PRL lowering effects of a given dose of bromocriptine that does not correlate with serum bromocriptine levels, implying differences in sensitivity to the drug.[425] Decreased response to bromocriptine *in vivo* has been shown to correspond to decreased numbers of DA receptors on lactotroph cell membranes and decreased inhibition of adenyl cyclase when the same tumors are studied *in vitro* following surgery.[426]

In vitro studies have shown that bromocriptine decreases not only PRL synthesis but also DNA synthesis, cell multiplication, and tumor growth.[427] The initial report that bromocriptine was able to reduce tumor size in humans was by Corenblum and colleagues.[428] An analysis of tumor size responses to bromocriptine from 24 different series of patients (totaling 302 patients) with macroadenomas showed that 76.8% had some tumor size decrease in response to bromocriptine with periods of observation ranging from 6 weeks to over 10 years (Fig. 3.3).[390] In 10 studies with 112 patients in whom the size change was quantified, 45 (40.2%) had a greater than 50% reduction in tumor size, 32 (28.6%) had a 25% to 50% reduction in tumor size, 14 (12.5%) had a less than 25% reduction, and 21 (18.7%) had no evidence of any reduction in tumor size.[390] In 15 patients with microadenomas, Bonneville and colleagues found that 6 tumors disappeared completely, 5 decreased approximately 50% in volume and 4 remained unchanged with treatment of 3 to 12 months.[429]

The time course of tumor size reduction is variable. Some patients may experience an extremely rapid decrease in tumor size, with significant changes in visual fields being noted with 24 to 72 hours and significant changes noted on scan within 2 weeks.[430] In others, little change may be noted at 6 weeks but scanning again at 6 months may show significant changes.[390,431] In many patients, progressive tumor size reduction can be noted over several years.[390,431,432]

Visual field improvement occurs in 80% to 90% of patients with defects.[253,390,431,433] The visual field improvement generally parallels and often precedes the changes seen on scan.[253,433] It is often difficult to determine before treatment whether visual defects are temporary or permanent and only the response to therapy provides a final answer. These studies with medical therapy are reassuring in that a relatively slow chiasmal decompression over several weeks provides excellent restoration of visual fields and that immediate surgical decompression is not necessary. Usually, when there is no significant change in visual fields despite significant evidence of tumor reduction on scan, subsequent surgery also does not improve these fields.[434] Reduction in tumor size may also be accompanied by improved pituitary function.[253,435] When the prolactinoma is present prepubertally, improved pituitary function allows resumption of normal growth and pubertal development.[436]

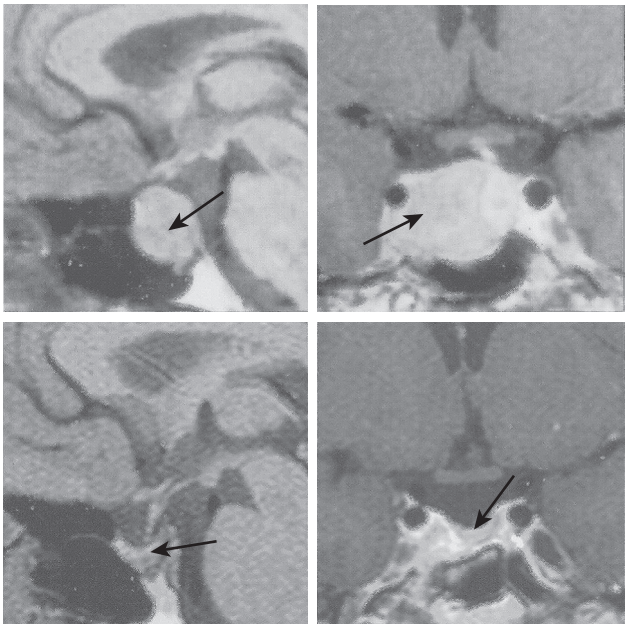


FIGURE 3.3 MRI scans of a patient with a macroadenoma before *(above)* and during *(below)* bromocriptine treatment. *Left:* sagittal view, *right:* coronal view. Note the marked decrease in tumor size.

The extent of tumor size reduction does not correlate with basal PRL levels, nadir PRL levels achieved, the percentage of PRL decrease, or whether PRL levels reached normal. Some patients have excellent reduction in PRL levels into the normal range but only modest changes in tumor size; others may have persistent, mild hyperprolactinemia (although suppressed greater than 88% from basal values) with almost complete disappearance of tumor.[253] A reduction in PRL levels always precedes any detectable change in tumor size, however; therefore patients whose PRL levels do not respond to therapy have no reduction in tumor size. Once maximum size reduction is achieved, the dose of bromocriptine can often be substantially reduced, gradually.[437]

Rarely, the prolactinoma serves as a "cork" and tumor size reduction with bromocriptine may cause CSF rhinorrhea.[387,438] Fibrosis has been reported in some tumors with marked shrinkage which may hinder later surgical cure of macroadenomas.[439,440] For most patients, however, continued dopamine agonist treatment is preferable to trying late surgery. Prolonged bromocriptine treatment for up to 10 years appears to be well tolerated[435,441-446] and the dose can often be reduced considerably.[437]

With discontinuation of short-term (several weeks to months) treatment, macroadenomas can re-expand within 2 weeks.[447] However, over 90% of tumors treated for several years with good size reduction, remain reduced in size when the drug is stopped, although PRL levels may increase to above normal levels and require treatment.[393,441,443,445] In a study of 69 patients with macroadenomas and 62 with microadenomas, Passos and colleagues found that 16% of patients with macroadenomas and 21% with microadenomas maintained normal PRL levels after stopping a median of 47 months of therapy.[448] In two more recent studies,

about 50% of patients with microadenomas maintained normal PRL levels after stopping therapy.[446,449]

Therapy discontinuation must be done very cautiously, if at all, in patients with very large tumors who have excellent tumor size reduction. The best approach is probably to reduce the dose gradually, following PRL levels, and only discontinue the drug if there are no increases in PRL levels or tumor size on low doses.

The most common adverse effects are nausea and sometimes vomiting; these are usually transient but may recur with each dose increase. Orthostatic hypotension usually is only a problem when initiating therapy and rarely recurs with dose increases. Limiting nausea and vomiting occurs in 5% to 10% of patients and digital vasospasm, nasal congestion, and depression occur rarely in patients when doses less than 7.5 mg/day are used.[422]

Side effects can be minimized by starting with one daily 1.25 mg dose taken with a snack at bedtime. The dose can then be gradually increased to 2.5 mg twice daily with meals over 7 to 10 days. PRL levels should be checked after 1 to 2 months; most patients who respond to this therapy will do so within this period. Dose higher than 7.5 mg per day are usually not necessary, except in some patients with very large tumors.

One other notable side effect is a psychotic reaction. Turner and colleagues noted psychotic reactions in 8 of 600 patients receiving either bromocriptine or lisuride for hyperprolactinemia or acromegaly.[450] Symptoms included auditory hallucinations, delusional ideas, and changes in mood. Rare reports of exacerbation of preexisting schizophrenia also exist; therefore the drug should be given cautiously to such patients.[451] Psychotic reactions usually resolve within 72 hours of stopping the drug. It should be noted that phenothiazines given to such patients may also blunt the action of bromocriptine on prolactinomas.[253]

One concerning problem is the tumor that initially shrinks in response to a dopamine agonist but then enlarges. This is usually due to noncompliance, which is further worsened by the tendency for the patient and physician to resume the full dose instead of gradually restarting. This tends to make side effects worse, further exacerbating the noncompliance. However, several case reports of tumor enlargement in compliant patients have been reported.[452-455] Pelligrini and colleagues have shown markedly fewer DA binding sites in tumors that grow during bromocriptine treatment compared with those that shrunk or remained unchanged in response to therapy.[455]

In other studies, Caccavelli and colleagues have shown that resistant cells express a decreased proportion of the shorter DA D2 receptor isoform which is coupled to phospholipase C more efficiently than the long DA D2 receptor isoform.[456] Recently, Peverelli and colleagues have found that this decreased D2 receptor expression is associated with a decrease in filamin, a cytoskeleton protein that associates with the receptor.[457] Prolactinomas that are resistant to bromocriptine are often sensitive to cabergoline.[458]

Although extremely rare, tumors that continue to enlarge while being treated with dopamine agonists may turn out to be carcinomas. A rare case of an adenoma transforming to a sarcoma during bromocriptine therapy has also been reported.[459]

An alternative method of giving bromocriptine has been found to be successful in some cases. Vermesh and colleagues reported that similar reductions in PRL levels are achieved with oral and intravaginal administration of oral bromocriptine tablets.[460] However, the drug effect lasts for up to 24 hours with a single dose and gastrointestinal side effects were much less with the intravaginal route. Katz and colleagues reported that a macroadenoma in a woman who was unable to tolerate oral bromocriptine responded well with tumor shrinkage to intravaginal bromocriptine.[461] Many women have now been treated with intravaginal bromocriptine with similar results, although some develop local irritation at the site of tablet placement. Thus, the gastrointestinal side effects appear to be caused by local effects rather than being mediated centrally.

Pergolide

Another dopamine agonist that has been demonstrated to have efficacy in the treatment of prolactinomas is pergolide, which had been approved by the U.S. Food and Drug Administration for the treatment of Parkinson's disease. Pergolide is comparable to bromocriptine with respect to tolerance and efficacy, including tumor size reduction.[462] However, pergolide was withdrawn from the U.S. market in 2007 because of an association with cardiac valvular lesions similar to those seen in patients with Carcinoid syndrome. These associations were seen only when it was used in the high doses needed for Parkinson's disease.[463,464] No such lesions were seen in patients treated with bromocriptine.[463,464] No cardiac valvular lesions have been reported in patients treated with the lower doses used for prolactinomas.

Quinagolide

Quinagolide (CV 205-502) is a non-ergot dopamine agonist that can be given once daily with similar tolerance and efficacy to bromocriptine and pergolide.[390,465] About 50% of patients who are resistant to bromocriptine respond to quinagolide.[390,465-467] Although side effects are similar, some patients appear to tolerate quinagolide better than bromocriptine.[390,465-467] In studies in which 105 patients were assessed for tumor size reduction in a semiquantitative way, 50 (48.1%) experienced greater than 50% tumor size reduction, 21 (20.2%) experienced a 25% to 50% size reduction, 18 (17.3%) experienced less than 25% reduction, and 15 (14.4%) had no change in tumor size.[390] Quinagolide is not approved for use in the U.S.

Cabergoline

Cabergoline is different from the other dopamine agonists in that it has a very long half-life and can be given orally once or twice weekly. The long duration of action stems from its slow elimination from pituitary tissue,[468] its high affinity binding to pituitary DA receptors,[469] and extensive enterohepatic recycling.[470] After oral administration, PRL lowering effects are initially detectable at 3 hours, then gradually increase so that there is a plateau of effect between 48 and 120 hours[470,471]; with weekly doses there is a sustained reduction of PRL.[472]

Several studies have shown that cabergoline is generally more effective than bromocriptine in lowering PRL levels and has substantially fewer side effects.[471-476] In a

prospective, double-blind comparison study of 459 women (279 microadenomas, 3 macroadenomas, 167 idiopathic hyperprolactinemia, 10 other), of women treated with cabergoline, 83% achieved normoprolactinemia, 72% achieved ovulatory cycles and 3% discontinued the medication because of adverse effects. Of women treated with bromocriptine, 59% achieved normoprolactinemia, 52% achieved ovulatory cycles, and 12% stopped the drug because of adverse effects.[475] In other studies, cabergoline treatment of men caused a rapid improvement of sperm number and quality.[476,477] Rare patients experience limiting nausea and vomiting with cabergoline, and they may be treated with intravaginal cabergoline as well.[478]

Several studies have assessed the effect of cabergoline on macroadenoma size.[472-474,479-485] A total of 130 patients in these series had their tumor size assessed in a semiquantitative way in studies ranging from 3 to 24 months duration of treatment. Of these 130, 33 (25.4%) experienced a greater than 50% tumor size reduction, 61 (46.9%) had a 25% to 50% reduction, 8 (6.9%) had a less than 25% reduction, and 28 (21.5%) had no change in tumor size. In many of these studies, many of the patients had been previously treated with other DA agonists, changing therapy because of intolerance or resistance, and this may color the findings. In a comparison of groups of patients, Colao and colleagues found that 25 of 26 (96%) patients who had never received prior dopamine agonists had tumor shrinkage of greater than 50%, whereas 13 of 19 (68%) who had been intolerant of prior bromocriptine, 21 of 33 (64%) who had been resistant to bromocriptine, and 14 of 20 (70%) who had been responsive to bromocriptine had similar reductions.[486] Thus, in the drug-naive patient, cabergoline clearly seems to be the most efficacious in reducing PRL levels to normal and in decreasing tumor size and has the least adverse effects. Visual defects tend to improve with the decreases in tumor size with cabergoline, similar to what has been seen with bromocriptine.[487]

In patients who have had normalization of their PRL levels for several years, cabergoline may be withdrawn gradually to see if hyperprolactinemia recurs. Colao and colleagues reported that after cabergoline withdrawal, of 105 normoprolactinemic patients with microadenomas, hyperprolactinemia recurred in 40% of those with tumors visible on MRI scan but in only 24% of those without visible tumors and of 70 normoprolactinemic patients with macroadenomas, hyperprolactinemia recurred in 58 of those with tumors still visible on MRI scan but in only 26% of those without visible tumors.[488] In a similar study of 67 patients with microadenomas, Biswas and colleagues found that only 31% remained free of recurrence of hyperprolactinemia.[447] More recently, Kharlip and colleagues found rates of recurrence of 52% in 31 patients with microadenomas and 55% in 11 patients with macroadenomas,[489] and Barber and colleagues found rates of recurrence of 64% in 45 patients with microadenomas and 93% in 15 patients with macroadenomas.[490] Overall, the rates of recurrence are lower for cabergoline compared to bromocriptine.[491] Interestingly, however, the amount of drug-induced tumor fibrosis is greater with bromocriptine use compared to cabergoline use.[492]

Some patients with macroprolactinomas are very resistant to even cabergoline and may require very high doses.[493,494] The dose of cabergoline can be gradually increased, as long as there is a stepwise fall in PRL with each stepwise increase in cabergoline dose and the patient is not experiencing side effects.[493-495] An additional approach involves adding an aromatase inhibitor to reduce testosterone-to-estrogen conversion in men.[495] In rare cases, the alkylating agent, temozolomide, has been found to be effective in reducing tumor size and PRL levels in invasive, giant prolactinomas that are not malignant.[496-498] Temozolomide has also been used successfully in some cases of the very rare PRL-secreting pituitary carcinomas.[499]

Cardiac valvular lesions similar to those seen in patients treated with pergolide have also been found in patients treated with cabergoline. As with pergolide, these associations were seen only when it was used in the high doses needed for Parkinson's disease.[463,464] Clinically significant cardiac valvular lesions have not been generally reported in patients treated with the lower doses generally used for treatment of prolactinomas, i.e., doses 2 mg per week or less.[494,500-503] However, in resistant patients who are receiving much greater than usual doses, it may be prudent to monitor them with echocardiograms. Given that mild, clinically insignificant valvular regurgitation is very common in the normal population, it may be difficult in a given individual without a prior echocardiogram to determine whether such a finding is of clinical significance.[494]

Conclusions Regarding Treatment

Microadenomas

The risk of progression of microadenoma to macroadenoma is under 7%, so the patient unconcerned with fertility has no pressing need for therapy. On the other hand, long-term hypogonadism due to hyperprolactinemia may be associated with premature osteoporosis in both sexes; treatment reverses the increased rate of bone loss. For the woman with continued menses and no hypoestrogenemia, the risk of osteoporosis is not increased. For the correction of gonadal function with prevention of osteoporosis and restoration of libido, most patients should be treated unless they have normal estrogen and testosterone function.

If fertility is not an issue, then estrogen replacement therapy or a DA agonist could be tried. Because of its efficacy in reducing PRL levels, its favorable adverse effect profile, and once- or twice-weekly dosing, cabergoline appears to be the initial drug of choice for most patients with prolactinomas. If fertility is the primary reason to restore ovulation, then some consider bromocriptine to be the better choice because of its more established safety profile (discussed later in the chapter).

The cost of treatment and the necessity of taking medications for many years make some patients choose transsphenoidal surgery as their primary option. Surgery may also be indicated for the 5% of patients who either cannot tolerate or do not respond to DA agonists. Initial surgical cure rates for microadenomas appear to be in the 65% to 85% range, with a later recurrence for hyperprolactinemia of about 20% (thus the ultimate cure rate is in the 60% range). Radiation therapy has a very restricted role in patients with microadenomas, being limited to those who do not respond to or are intolerant of DA agonists and who are not cured by surgery.

Macroadenomas

Because of their excellent results and the rather poor results of surgery in most patients, DA agonists are recommended as the initial therapy for patients with PRL-secreting macroadenomas. Surgery can be performed later in patients whose tumor responses to such medications are not optimal. Even if this subsequent surgery is necessary for tumor debulking, it rarely is curative and a DA agonist is usually necessary for treatment of the hyperprolactinemia. Because of its better tolerability and generally better efficacy, cabergoline is the DA agonist of choice. Radiation therapy again has a very limited role here, being used for those who have no response to or are intolerant to dopamine agonists, or whose tumor was documented to actually grow while on dopamine agonists and after incomplete surgical removal. Stereotactic radiotherapy appears to be the best form of radiotherapy at this point, although long-term complications have not yet been assessed fully.

When dopamine agonist therapy is stopped, the prolactinoma may return to its original size, often within days to weeks. However, most studies with longer-term treatment have found re-expansion in less than 10% of patients in whom dopamine agonists were withdrawn, despite recurrence of hyperprolactinemia in 80% to 85% of those treated with bromocriptine, but a considerably lower percentage with cabergoline. This potential return to pretherapy size dictates extreme caution when withdrawing dopamine agonist therapy, because rapid tumor expansion may produce far more clinical symptoms than slow tumor enlargement. Often, however, the dose can be gradually tapered once maximal size reduction has occurred and, in suitable cases, stopped entirely if no re-expansion occurs.

The anatomical response of tumors to dopamine agonist treatment must be monitored carefully by CT or MRI and by visual field examinations to detect tumors that do not respond, including the very rare carcinomas and cases of tumor re-enlargement.

Pregnancy in Women with Prolactinomas

Hyperprolactinemia is usually associated with anovulation and infertility; correction of the hyperprolactinemia with dopamine agonists restores ovulation in about 90% of cases. When a woman harbors a prolactinoma as the cause of the hyperprolactinemia, two major issues arise when ovulation and fertility are restored: the effects of the DA agonist on early fetal development, and the effect of the pregnancy itself on the prolactinoma.

Effects of Dopamine Agonists on the Developing Fetus

As a general principal, it is advised that fetal exposure to medications be limited to as short a period as possible. Most advise that mechanical contraception be used after institution of DA agonist therapy until the first two to three cycles have occurred, so that an intermenstrual interval can be established. In this way, a woman will know when she has missed a menstrual period, a pregnancy test can be performed quickly, and the dopamine agonist stopped. Thus, the drug will have been given for only about 3 to 4 weeks of the gestation.

When used in this fashion, compared to what would be expected in the normal population,[504-506] bromocriptine has not been found to cause any increase in spontaneous abortions, ectopic pregnancies, trophoblastic disease, multiple pregnancies, or congenital malformations (Table 3.1).[507,508] Long-term follow-up studies of 64 children between the ages of 6 months and 9 years whose mothers took bromocriptine in this fashion have shown no ill effects.[509] Experience with the use of bromocriptine throughout the gestation, however, is limited to only just over 100 women; no abnormalities were noted in the infants except for one with an undescended testicle and one with a talipes deformity.[507,510-512]

Pergolide has been shown to cross the placenta in mice[513] and limited data suggest that there is an unacceptable risk of congenital malformations.[514,515] Initially no detrimental effects on pregnancy or fetal development in women who became pregnant during treatment with quinagolide were found.[516] However, a review of 176 pregnancies in women treated with quinagolide reported 24 spontaneous abortions, 1 ectopic pregnancy, 1 stillbirth, and 9 fetal malformations.[517] Therefore, neither pergolide nor quinagolide can be recommended if pregnancy is desired.

Cabergoline has been shown to cross the placenta in animal studies, but such data are lacking in humans. Data on exposure of the fetus during the first several weeks of pregnancy have been reported in almost 800 cases and such use has not shown an increased percentage of spontaneous abortion, premature delivery, multiple pregnancy, or congenital abnormalities.[518] Follow-up studies of up to 12 years following exposure to cabergoline during gestation showed no physical or developmental abnormalities in the 83 children followed by Ono and colleagues,[519] a slight retardation in verbal fluency in 2 and difficulty in achieving complete continence in 1 by age 4 among the 88 children followed by Lebbe and colleagues,[520] and seizures in 2 and "pervasive developmental disorder", an autism spectrum disorder, in 2 of the 61 children followed by Stalldecker and colleagues.[521]

In conclusion, with respect to using a dopamine agonist to facilitate ovulation and fertility, bromocriptine has the largest safety database and has a proven safety record for pregnancy. Although the database for cabergoline use in pregnancy is much smaller, it does not appear that cabergoline exerts any deleterious effects on pregnant women and the incidence of malformations in their offspring is not greater than in the general population. The safety databases for pergolide and quinagolide are quite limited, but they raise concerns, and so these drugs should not be used when fertility is desired. The effects of transsphenoidal surgery during gestation are not known specifically, but would not be expected to be significantly different from the effects of other types of surgery,[522] unless hypopituitarism should ensue.

Effect of Pregnancy on Prolactinoma Size

Estrogens have a marked stimulatory effect on PRL synthesis and secretion and the hormonal milieu of pregnancy can stimulate lactotroph cell hyperplasia.[2,3] Those autopsy studies showing lactotroph cell hyperplasia during pregnancy have now been corroborated *in vivo*; MRI scans showing a

Table 3.1 Pregnancy Outcomes Summarized for Women Who Became Pregnant While Taking Bromocriptine or Cabergoline

	Bromocriptine (N)	Bromocriptine (%)	Cabergoline (N)	Cabergoline (%)	Normal (%)
Pregnancies	***6.239***	***100***	***789***	***100***	***100***
- Spontaneous abortions	620	9.9	60	7.6	10-15
- Terminations	75	1.2	59*	7.5	20
- Ectopic	31	0.5	3	0.4	1.0-1.5
- Hydatidiform moles	11	0.2	1	0.1	0.1-0.15
Deliveries (known duration)	***4139***	***100***	***543***	***100***	***100***
- At term (>37 weeks)	3620	87.5	480†	88.4	87.3
- Preterm (<37 weeks)	519	12.5	67	11.6	12.7
Deliveries (known outcome)	***5120***	***100***	***471***	***100***	***100***
- Single births	5031	98.3	463	98.3	96.8
- Multiple births	89	1.7	12	1.7	3.2
Babies (known details)	***5,213***	***100***	***664***	***100***	***100***
- Normal	5,030	98.2	633	96.8	97
- With malformations	93	1.8	21	3.2	3.0

*Eleven of these terminations were for malformations.
†Five of these births were stillbirths.
From Molitch ME. Prolactinoma in pregnancy. *Best Prac Res Clin Endocrinol Metab* 25:885–896, 2011.

gradual increase in pituitary volume over the course of gestation, beginning by the second month and peaking the first week postpartum. In some cases, a final height of almost 12 mm is reached.[523]

The stimulatory effect of the hormonal milieu of pregnancy may also result in significant prolactinoma enlargement during gestation (Fig. 3.4). Tumor enlargement may also occur because the dopamine agonist that had caused the tumor to shrink has now been discontinued. Data have been compiled from many studies that analyzed the risk of symptomatic tumor enlargement in pregnant women with prolactinomas, divided according to tumor size (Table 3.2).[518] For women with microadenomas, only 18 of 658 pregnancies (2.7%) were complicated by symptoms of tumor enlargement (headaches and/or visual disturbances). Surgical intervention was not required in a single case and medical therapy with reinstitution of bromocriptine resolved the symptoms in the five in whom it was tried. In 49 of 214 pregnancies (22.9%) in women who had not undergone prior surgery or radiotherapy for their macroprolactinomas, there were similar symptoms of tumor enlargement. 148 women with macroadenomas were identified who had undergone surgery or radiation prior to pregnancy; their risk of tumor enlargement was lower (4.8%).

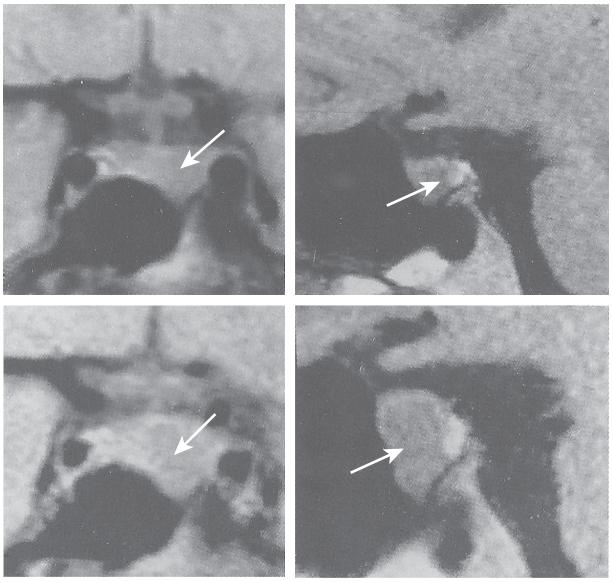


FIGURE 3.4 Coronal and sagittal MRI scans of an intrasellar prolactin secreting macroadenoma in a woman prior to conception *(above)* and at 7 months of gestation *(below)*. Note the marked tumor enlargement at the latter point, at which time the patient was complaining of headaches. *(From Molitch ME. Medical treatment of prolactinomas.* Endocrinol Metab Clin N Amer *1999;28:143–169, with permission.)*

Recommendations for Management During Pregnancy

Because of its well-established safety record and the smaller amount of data with cabergoline, bromocriptine is favored by some clinicians for women wishing to become pregnant. However, there really are no data to show that cabergoline is not safe and it is also commonly used in this setting. A patient with a microadenoma or a small, intrasellar macroadenoma treated only with a DA agonist should be carefully followed throughout gestation. PRL levels do not always rise during pregnancy in women with prolactinomas, as they do in healthy women, and PRL levels may also not rise with tumor enlargement.[524] Therefore, periodic checking of PRL levels is of no benefit. Because of the low incidence of tumor enlargement, routine, periodic visual field testing is not cost effective. Visual field testing and scanning are performed only in patients who become symptomatic. In the patient with tumor enlargement who does not respond to reinstitution of a DA agonist, surgery or early delivery may be required.

In a woman with a larger macroadenoma that may have suprasellar extension, there is approximately a 23% risk of clinically significant tumor enlargement during pregnancy when only a DA agonist is used. There is no clear-cut answer as to the best therapeutic approach, so this must be a highly individualized decision that the patient has to make after a clear, documented discussion of the various therapeutic alternatives.

Table 3.2 Effect of Pregnancy on Prolactinomas

Tumor Type	Prior Therapy	Number Of Patients	Symptomatic Enlargement
Microadenomas	None	658	18 (2.7%)
Macroadenomas	None	214	49 (22.9%)
Macroadenomas	Yes	148	7 (4.8%)

Data from Molitch ME. Prolactinoma in pregnancy. *Best Prac Res Clin Endocrinol Metab* 25:885–896, 2011.

One approach is to perform a prepregnancy transsphenoidal surgical debulking of the tumor. This should greatly reduce the risk of serious tumor enlargement, but cases with massive tumor expansion during pregnancy after such surgery have been reported.[525] After surgical debulking, bromocriptine or cabergoline would be required to restore normal PRL levels and allow ovulation.

A second approach would be to treat with bromocriptine or cabergoline to allow ovulation and then stop the drugs once pregnancy has been achieved, as in a woman with a microadenoma. This is the approach that is generally used.

A third approach, that of giving bromocriptine continuously throughout gestation, has been advocated.[512] At this point, however, data regarding the effects of continuous bromocriptine therapy on the developing fetus are still quite meager, and such therapy cannot be recommended without reservation. Data documenting safety of giving cabergoline throughout pregnancy are even fewer.[526-528] Should pregnancy at an advanced stage be discovered in a woman taking bromocriptine or cabergoline, the data that exist are reassuring and would not justify therapeutic abortion.

For patients with macroadenomas treated with DA agonists alone or after surgery, careful follow-up with one to three monthly visual field testing is warranted. Repeat scanning (without gadolinium) is reserved for patients with symptoms of tumor enlargement, evidence of a developing visual field defect, or both. Repeat scanning after delivery to detect asymptomatic tumor enlargement may be useful as well.

Should symptomatic tumor enlargement occur with any of these approaches, reinstitution of a DA agonist is probably less harmful to the mother and child than surgery. There have been a number of cases reported where such reinstitution of bromocriptine has worked quite satisfactorily, causing rapid tumor size reduction with no adverse effects on the infant.[518] Similarly, one case has been reported with the successful reinstitution of cabergoline.[526] Any type of surgery during pregnancy results in a 1.5-fold increase in fetal loss in the first trimester and a fivefold increase in fetal loss in the second trimester, although there is no risk of congenital malformations from such surgery.[522,529] Thus, DA agonist reinstitution would appear to be preferable to surgical decompression. However, such medical therapy must be very closely monitored, and transsphenoidal surgery or delivery (if the pregnancy is far enough advanced) should be performed is there is no response to the DA agonist and vision progressively worsens.

There is no evidence that breastfeeding stimulates tumor growth.[530,531] For women who wish to breastfeed, DA agonists must be withheld until such time as the woman wishes to stop, unless pregnancy-induced tumor growth required treatment.

Interestingly, postpartum PRL levels are often lower than they were prepartum with some patients achieving normalization of PRL without any therapy; the mechanism for this not known.[532] However, in many patients it may be reasonable to observe patients for a few months to determine their PRL and ovulatory status following delivery and cessation of breastfeeding, rather than automatically putting them back on their dopamine agonists.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 4

The Synthesis and Metabolism of Steroid Hormones

Jerome F. Strauss III

Steroid hormones and the secosteroid, vitamin D, belong to an ancient family of signaling molecules with diverse functions, including central roles in the regulation of female and male reproductive processes. The human steroid hormones are derived from cholesterol, an abundant plasma lipid and a structural component of plasma membranes and other organelles. Seemingly subtle modifications of the four fused rings of the sterol skeleton and side chain result in molecules with different and diverse activities. This chapter reviews the general features of the synthesis and metabolism of steroid hormones, the ways in which these processes are controlled physiologically, the ways in which these processes can be modified by pharmacological intervention, and some genetic disorders that interfere with normal steroid synthesis and metabolism.

Steroid Hormone Structure and Nomenclature

Steroid hormones share a cyclopentanoperhydrophenanthrene backbone. Each carbon in this fused-ring structure is assigned a number identifier, and each ring, a letter (Fig. 4.1). The naturally occurring steroid hormones are named according to the saturated ring structures of the parent compound: *cholestanes*, of which cholesterol (5-cholesten-3β-ol) is a representative, have 27 carbons; *pregnanes* have 21 carbons (e.g., 4-pregnen-3-20-dione, also known by its trivial name, *progesterone*); *androstanes* have 19 carbons (e.g., 17β-hydroxy-4-androsten-3-one, or *testosterone*); and *estranes* have 18 carbons (e.g., 1,2,5[10]-estratriene-17β-ol, or *estradiol*). *Gonanes* contain 17 carbons (the cyclopentanoperhydrophenanthrene backbone), represented by synthetic progestins (e.g., desogestrel, norgestimate, gestodene). The backbone name is **not** synonymous with biological activity, which is determined mainly by which members of a family of nuclear transcription factors (steroid hormone receptors) the molecules activate. For example, cortisol and progesterone are members of the pregnane family, yet they have different biological activities and act through different receptors. Synthetic molecules that activate the progesterone receptor are derived from androgens containing 18 carbons (19-nortestosterone derivatives) and are therefore members of the estrane family. 27-Hydroxycholesterol, a member of the cholestane family, is a selective estrogen receptor modulator.[1]

The locations of substituents in the steroid backbone are indicated by the carbon number to which they are attached. Substituents at several positions have a significant effect on metabolism and biological activity of steroid hormones, including carbons 3, 7, 11, and 17. Atoms attached to asymmetric centers are, by convention, given the designation α if they project below the plane of the ring structure (in figures of structures, a dashed line or hatched triangle indicates the α configuration). The designation β (a solid line or filled triangle) is given to atoms that project above the plane. Hormone receptors and steroid binding proteins (e.g., sex hormone binding globulin, SHBG) distinguish between stereoisomers. In the case of estrogen receptors, 17β-estradiol is active, but 17α-estradiol is essentially inert. In the case of androgen receptors, testosterone with a 17β-hydroxyl configuration is active, but epitestosterone with a 17α-hydroxyl configuration has little activity.

Different enzymes catalyze the oxidation or reduction of α and β hydroxyl groups and reduce the Δ4 double bond in the steroid A ring to form 5α or 5β molecules. Such 5α-reduced steroids can be active (e.g., 5α-dihydrotestosterone, which activates androgen receptors), or inactive (e.g., 5α-dihydroprogesterone) with respect to classical steroid hormone receptor function. However, the notion that pregnane 5α-reduced compounds are inactive has been challenged by the recent revelation that 5α-reduced glucocorticoids may have anti-inflammatory activity acting through glucocortcoid receptors.[2] Although 5β-reduced steroids are not capable of activating classical steroid hormone receptors, these steroids and some 5α-reduced molecules, exert biological effects outside of the family of steroid hormone receptors, including modulation of neurotransmitter receptor function.

The naturally occurring steroid hormones are rarely referred to in the medical literature by their systematic names, which designate the parent structure and the number, location, and (if appropriate) orientation of substituents; instead, the trivial names are preferred.

Steroid hormones used in clinical practice are either: derived from biological sources (e.g., conjugated equine urinary estrogens); semi-synthetic, produced usually from a plant sterol starting material which can be chemically modified to yield a structure that is identical to the naturally occurring molecules; totally synthetic, which may result in the production of hormone isomers with different biological activity; or steroid drugs which are molecules not

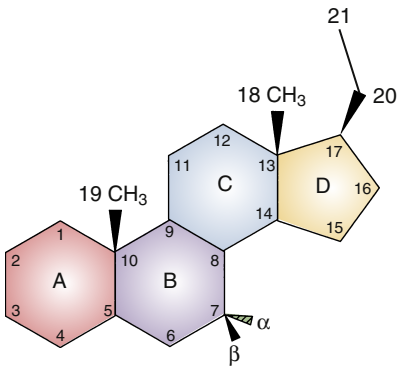


FIGURE 4.1 The steroid nucleus. Rings are identified with capital letters and carbon atoms are numbered. Substituents and hydrogens are shown projecting above *(β)* or below *(α)* the plane of the steroid nucleus.

found in humans and animals (e.g., medroxyprogesterone acetate and norgestrel). "Bioidentical" steroid hormones produced by total chemical synthesis may contain isomers that are not natural, which may result in biological activities that differ from the steroid hormones produced in humans. Because of this concern, it has been argued that the term "bioidentical" steroid hormones is misleading.

Organization of Steroidogenic Organs and Cells

The steroidogenic machinery is compartmentalized at the organ, cellular, and subcellular levels, which has important implications for the control of steroid hormone production.[3-6] Steroid synthesis involves a series of sequential modifications of cholesterol that result in the clipping of the side chain; alterations in olefinic bonds; and the addition of hydroxyl functions, proceeding invariably (although some have argued that shortcuts do exist) from cholesterol through the pregnane, androstane, and finally, estrane families.

Specific cell types can accomplish several of these sequential steps, but rarely can they generate an estrogen from cholesterol. Indeed, the requirement for cooperative efforts by two different tissues or cell types is a characteristic of estrogen biosynthesis. This joint effort enables the modulation of both androgen and estrogen production by factors that independently influence the cells involved in precursor synthesis, in addition to the cell type in which the final step, aromatization, occurs.[7] This cooperation is exemplified by estradiol synthesis in the ovarian follicle, where luteinizing hormone (LH) acts on the theca cells to stimulate production of androgen precursors and follicle-stimulating hormone (FSH) acts on granulosa cells to stimulate aromatization of these androgens into estrogens. Placental estrogen synthesis, likewise, requires precursors from another tissue, the fetal adrenal gland that is under the control of fetal pituitary adrenocorticotropic hormone (ACTH). The sulfated dehydroepiandrosterone secreted from the fetal zone of the adrenal cortex has negligible androgenic activity in the fetus and increased solubility in plasma, so it can be efficiently transported to the placenta, where cleavage of the sulfate group, followed by aromatization, takes place in the syncytiotrophoblast. Cooperative interaction among different cell types is also important in regulating the production of steroid hormones in the brain and hormone production in breast and endometrial cancers.

Another example of compartmentalization of the steroidogenic machinery at the organ level is the adrenal cortex, which has histologically and functionally distinct zones that determine the relative production rates of mineralocorticoids, glucocorticoids, and adrenal androgens.[4,8] The zona glomerulosa synthesizes mineralocorticoids; the zona fasciculata, glucocorticoids; and the zona reticularis and fetal zone of the fetal adrenal cortex produce androgens. One major functional distinction between the zona glomerulosa and the zonae fasciculata and reticularis is that aldosterone synthase is exclusively expressed in the zona glomerulosa, but 17α-hydroxylase is not. However, 17α-hydroxylase is abundant in the zonae fasciculata and reticularis. The zona reticularis expresses lower levels of type 2 3β-hydroxysteroid dehydrogenase (HSD3B2). Because the lyase of human 17α-hydroxylase does not efficiently act on progesterone, reduced conversion of pregnenolone to progesterone resulting from the low 3β-hydroxysteroid dehydrogenase activity facilitates the conversion of pregnenolone into dehydroepiandrosterone in the zona reticularis. Higher levels of cytochrome b_5 in the zona reticularis, which increase the lyase activity of human 17α-hydroxylase by an allosteric mechanism, further augments the capacity to produce dehydroepiandrosterone. The zona reticularis also has high levels of sulfotransferase. This constellation of enzymatic activities favors synthesis of dehydroepiandrosterone sulfate.

Acquisition, Storage, and Trafficking of Cholesterol

Steroidogenic cells have ultrastructural features that enhance their ability to obtain and store cholesterol for use in steroidogenesis (Fig. 4.2).[4,5] Unlike protein hormone–producing cells, steroid-producing cells do not store prefabricated hormone; they synthesize the hormones on demand from cholesterol that has been acquired from the plasma, synthesized de novo, or stored in membranes, or as sterol esters in lipid droplets. Because of cholesterol's limited solubility in water (it forms aggregates at 25 to 40 nM [the critical micelle concentration]), multiple proteins are required to move the steroid hormone precursor efficiently from one cellular compartment to another.

The plasma membrane has the highest content of free cholesterol, which is derived from plasma lipoproteins and de novo sterol synthesis. This sterol pool is not static, it exchanges with plasma free cholesterol and regularly cycles through the cell and back to the plasma membrane. During this cycling process, sterols can be diverted for use in steroid hormone synthesis or esterified and deposited in lipid droplets.

Numerous microvilli project from the plasma membrane on which lipoprotein-gathering receptors of the low-density lipoprotein (LDL) receptor family are located (e.g., LDL receptors; LDL receptor–related protein, very–low-density [VLDL] lipoprotein receptors). These receptors mediate lipoprotein uptake by an endocytic mechanism that delivers the lipoproteins to the lysosomes where the apolipoproteins are degraded. The lipoprotein cholesterol esters are then hydrolyzed by acid lipase (LIPA) to release free cholesterol.

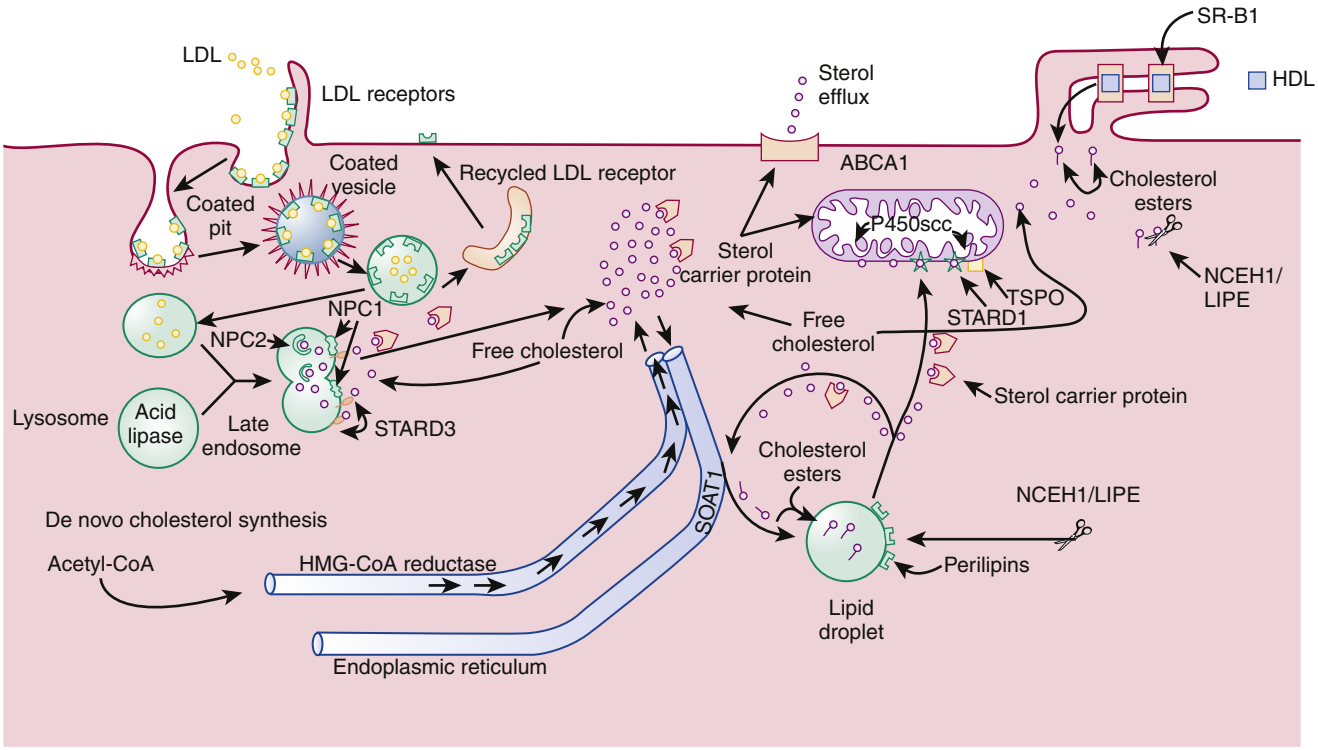


FIGURE 4.2 The acquisition, storage, and trafficking of cholesterol in steroidogenic cells. ABCA1, ATP-binding cassette transporter A1; FFA, free fatty acid; HDL, high-density lipoprotein; HMG-COA, 3-hydroxy-3-methyl-glutaryl-coenzyme A; LDL, low-density lipoprotein; LIPE, hormone-sensitive lipase; NCEH1, neutral pH cholesterol ester hydrolase; SR-B1 scavenger receptor type B; STARD1, steroidogenic acute regulatory protein; STARD3; (steroidogenic acute regulatory protein)-related lipid transfer domain 3; sterol carrier proteins include sterol carrier protein$_2$, STARD4, and STARD5; SOAT1, sterol-O-acyltransferase-1; TSPO, translocator protein.

Severe acid lipase deficiency (Wolman's disease) is associated with lysosomal accumulation of cholesterol esters and triglycerides, which can lead to damage of steroidogenic cells and compromised hormone production.[5] Free cholesterol is released from lysosomes through a system of sterol binding proteins encoded by genes *(NPC1, NPC2)* that when mutated cause the cholesterol storage disorder, Neiman-Pick Type C disease. NPC2, a soluble protein in the lysosome, delivers free cholesterol to NPC1, a membrane-associated cholesterol binding protein that controls sterol efflux. Other sterol binding proteins, including steroidogenic acute regulatory protein (STAR)-related lipid transfer (START) domain protein 3 (STARD3), also known as metastatic lymph node 64 protein (MLN64), may participate in this process.[5] Stimulation of steroidogenic cells by trophic hormones increases the number of LDL receptors on the cell surface, and also accelerates the rate of LDL internalization and degradation.

High-density lipoproteins (HDL) can also provide cholesterol for hormone synthesis.[3,9] Receptors for HDL (scavenger receptor type B, class 1 [abbreviated SR-B1]) are located in closely apposed microvilli that form "microvillar channels" in which HDL particles are lodged.[3] Endothelial lipases (hepatic lipase or endothelial cell–derived lipases) may facilitate uptake of the HDL-carried sterols by steroidogenic cells, including the selective uptake the HDL sterol esters. Like LDL receptor expression, SR-B1 expression is up-regulated in response to trophic stimulation, facilitating the usage of HDL-delivered substrate. Individuals with a SR-B1 missense mutation (Pro297Ser) that reduces uptake function, have increased HDL levels and attenuated adrenal steroidogenesis in response to ACTH stimulation.

The process by which cholesterol is accumulated by the "HDL pathway" differs from that of the "LDL pathway." HDL cholesterol esters are selectively internalized by SR-B1, leaving the apolipoproteins on the cell surface. The internalized HDL cholesterol esters are then cleaved, by a cytosolic, neutral pH optimum sterol esterase (either *hormone-stimulated lipase* [LIPE] or *neutral cholesterol ester hydrolase*, NCEH1), thereby releasing free cholesterol.[3,10,11]

De novo synthesis of cholesterol, a process that involves at least 17 enzymes, takes place primarily in the abundant smooth endoplasmic reticulum (SER).[5] Steroidogenic cells have up to tenfold more SER by volume than rough endoplasmic reticulum. In certain cells, the SER takes on unique forms, exemplified by the whorls found in testicular Leydig cells. Enzymes involved in steroid formation and metabolism are also embedded in the SER. Trophic hormones that stimulate steroidogenesis generally increase both cellular cholesterol synthesis and lipoprotein uptake. Of note, biosynthetic intermediates between lanosterol and cholesterol stimulate oocyte maturation in *in vitro* assays. These 4,4-dimethyl sterols, referred to as *meiosis-activating sterols*, contain 29 carbons and are found in the testis and follicular fluid in low micromolar concentrations. However, their physiological role in gamete maturation *in vivo* and their potential use in clinical settings remain subjects of debate.

The quantitative importance of circulating cholesterol carried by LDL, HDL, and other lipoproteins as steroid hormone precursor in adults, as opposed to de novo cholesterol

synthesis, is demonstrated by the fact that radiolabeled plasma cholesterol in humans is almost fully equilibrated with the steroidogenic pool of cholesterol. Additional evidence for an important role of circulating lipoprotein cholesterol in steroidogenesis comes from the study of hypobetalipoproteinemia, a disorder in which there is virtually no circulating LDL.[12,13] This rare metabolic disease is associated with reduced adrenocortical steroid production and diminished progesterone levels in the luteal phase and in pregnancy, although the lower levels of progesterone elaborated are still sufficient to achieve a term pregnancy.

Individuals with familial hypercholesterolemia due to inactivating mutations in the LDL receptor have only modest impairment of steroidogenic gland function, reflecting the capacity of alternative sterol acquisition mechanisms to compensate for LDL receptor deficiency. The commonly used cholesterol-lowering statins [which inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme in de novo cholesterol synthesis] do not impair adrenal, testicular, or luteal steroidogenesis in adult humans despite the lowering of plasma LDL levels.[14,15]

Smith-Lemli-Opitz syndrome, an autosomal recessive disease, offers interesting insight into the relationship of plasma cholesterol and de novo sterol synthesis for the supply of precursors for fetal steroidogenesis.[16] The disease is caused by inactivating mutations in an enzyme involved in the terminal steps of cholesterol synthesis, 3β-hydroxysteroid Δ^7-reductase (DHCR7). As a result, cholesterol levels are quite low and 7-dehydrocholesterol levels are elevated. Hypospadias or ambiguous genitalia is a frequent finding in affected male neonates, reflecting diminished fetal testicular testosterone synthesis. Estrogen production during pregnancy is also reduced, resulting from impaired fetal adrenal hormone production. Adrenal insufficiency has been reported in some affected individuals, although others compensate with modest elevations of ACTH secretion.[16,17] Interestingly, B-ring unsaturated equine-like steroids (1,3,5[10], 7-estratetrenes) are produced from the 7-dehydrocholesterol that accumulates, showing that the steroidogenic enzymes do not have an absolute requirement for cholesterol as a starting material.[18] Desmosterolosis, a rare autosomal recessive disease caused by mutations in the 3β-hydroxysterol Δ^{24} reductase, is also associated with ambiguous genitalia in affected males, presumably as a result of impaired fetal testicular testosterone synthesis.[16]

Cytoplasmic lipid droplets represent another major depot of substrate in steroidogenic cells: as much as 80% of the total cholesterol content of steroidogenic cells can be found esterified in these droplets. The sterol esters are synthesized in the endoplasmic reticulum from cholesterol acquired from lipoproteins or de novo synthesis by sterol O-acyltransferase 1 (SOAT1, previously named acyl-coenzyme A, cholesterol acyltransferase-1 or ACAT1), encoded by one of two related genes.[19] The esters generated by SOAT1 accumulate within the SER, and subsequently bud off as lipid droplets (Fig. 4.2). A targeted mutation in the *Soat1* gene in mice results in markedly reduced sterol ester storage in the adrenal cortex without impairment of basal or ACTH-stimulated corticosterone production by adrenal cells. These findings suggest that transit through the sterol ester pool is not part of an obligatory itinerary for steroidogenic cholesterol.

The limiting membranes of the lipid droplets contain a family of proteins, called perilipins.[20,21] These proteins protect the droplet contents from hydrolysis in the basal state. They also serve as scaffolds, anchoring lipases to the lipid droplet surface, as well as mediating physical and functional interactions between lipid droplets and other organelles, including mitochondria (Perilipin 5). Mobilization of cholesterol ester stores occurs when cells are stimulated by trophic hormones. An adenosine 3′,5′cyclic monophosphate (cAMP) mediated process leads to phosphorylation of perilipins by protein kinase A, and subsequent detachment of perilipins from the droplet surface, giving lipases access to the sterol esters.

The lipases that free cholesterol from lipid droplet sterol esters are hormone sensitive lipase (LIPE) and neutral cholesterol esterases (NCEH1) (Fig. 4.2).[10,11,22,23] Protein kinase A activates LIPE by phosphorylation of serine residues, promoting binding of the sterol esterase to lipid droplets. This enzyme's role in steroidogenesis was suggested by reduced production of corticosterone under ACTH stimulation associated with an accumulation of lipid droplets in the adrenal cortex of mice deficient in *Lipe*. Others described targeted mutation of both the *Lipe* and *Nceh1* genes resulting in adrenal enlargement and lipid accumulation, but no impairment in ACTH-stimulated corticosterone production. The different mouse models demonstrate a role for both hormone sensitive lipase and neutral cholesterol ester hydrolase activities in mobilization of sterol esters. The variable impact of enzyme deficiency on steroidogenesis in these murine models may be related to the time of study since lipid accumulation may have secondary deleterious effects on cell function beyond the disruption of sterol ester hydrolysis.

The size and number of lipid droplets change as the ester pool expands or contracts.[24] The quantity of sterol ester stored is determined by the availability of cholesterol to the cell through de novo synthesis, through accumulation of lipoprotein-carried cholesterol, and by the steroidogenic activity of the cell. Trophic stimulation promotes cholesterol ester hydrolysis and diverts cholesterol into the steroidogenic pool away from SOAT1, preventing re-esterification and resulting in a net depletion of cholesterol from the lipid droplets. Conversely, pharmacological blockade of steroid hormone synthesis (e.g., with the cholesterol side-chain cleavage inhibitor, aminoglutethimide) or defects in cholesterol use for steroidogenesis (e.g., congenital lipoid adrenal hyperplasia) increase sterol ester storage by increasing the amount of cholesterol available to SOAT1.

The exact intracellular itinerary of lipoprotein-derived cholesterol, free cholesterol from the plasma membrane, or free cholesterol released from lipid droplets, remains to be elucidated. In particular, much is still unknown about the ways in which sterol is presented to the mitochondria, where the first step in steroidogenesis takes place. It is likely that sterol distribution to and from organelles occurs through a dynamic vesicular–tubular late endosomal compartment, as well as through the assistance of lipid transfer proteins.[5] The lipid transfer proteins involved in this process may include ATP binding cassette transporter G1 (ABCG1), proteins with a structure resembling the *steroidogenic acute*

regulatory protein (STARD1), including STARD4, STARD5 (Fig. 4.2) and *sterol carrier protein-2* (SCP2).

The mitochondria of steroidogenic cells are frequently found in close association with cytoplasmic lipid droplets, which may facilitate movement of substrate from these depots to the mitochondria.[21,24] They have tubulovesicular cristae, in contrast to the lamellar cristae that are characteristic of mitochondria in other cells. The remodeling of cristae in the human syncytiotrophoblast is believed to facilitate steroidogenesis. The inner mitochondrial membranes contain the cholesterol side-chain cleavage enzyme, which catalyzes the first step in cholesterol metabolism into steroid hormones leading to the formation of pregnenolone. The hydrophobic cholesterol substrate must move from the mitochondrial outer membrane across the aqueous intermembranous space to reach the inner membrane. This translocation process is the major rate-limiting step in steroidogenesis. The capacity to produce large amounts of steroid hormone in rapid response to trophic stimulation requires the action of STARD1, the prototypic member of the START domain family, which greatly enhances the flux of substrate to the side-chain cleavage system (Fig. 4.2).[5,6] The mitochondrial cholesterol side-chain cleavage system is also juxtaposed to downstream enzymes in the steroidogenic pathway on the endoplasmic reticulum, allowing for efficient metabolism of pregnenolone.

Regulation of Cellular Cholesterol Balance

Cellular free (non-esterified) cholesterol balance is highly regulated by transcriptional and post-translational mechanisms. The expression of genes involved in cholesterol biosynthesis (e.g., the gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase, *HMGCR*) and the uptake of plasma cholesterol *(LDLR)* are controlled by master transcription factors, the sterol regulatory element-binding proteins (SREBF1 and SREBF2).[25] The SREBPs are synthesized as inactive precursors that are bound to the endoplasmic reticulum. When cells are depleted of sterols, SREBP cleavage-activating protein (SCAP) transports the SREBPs from the endoplasmic reticulum to the Golgi apparatus where they are cleaved through the action of two different proteases, releasing an NH_2-terminal domain transcription factor which enters the nucleus, where it activates genes controlling lipid synthesis and uptake. Cholesterol loading inhibits the movement of SREBPs to the Golgi and consequently the proteolytic processing, resulting in reduced transcription of the cholesterol synthesis and uptake genes. A micro ribonucleic acid RNA, miR-33, derived from an intron located within the gene encoding SREBF2, post-transcriptionally modulates expression of genes involved in cellular cholesterol balance.

Metabolites of cholesterol, the side-chain oxygenated sterols including 22-, 24-, 25-, and 27-hydroxycholesterol, and 7-hydroxylated sterols, are endogenous regulators of cellular cholesterol metabolism through the liver X receptors (LXRalpha and LXRbeta, also known as NR1H3 and NR1H2, respectively).[26,27] In addition to LXRs, *steroidogenic factor 1* (SF-1, also known as NR5A1) and a related protein liver receptor homolog-1 (LRH-1, also known as NR5A2), play roles in regulating genes encoding cholesterol metabolizing enzymes including *SOAT1*, *LIPE*, and *STARD1*.

Post-translational mechanisms influencing sterol synthesis and uptake include cholesterol-induced ubiquitination of HMG-CoA reducatase, which tags the protein for degradation by proteasomes. Another post-translational mechanism by which cells control sterol balance is the degradation of LDL receptors by proprotein convertase subtilisin/kexin 9 (PCSK9), a secreted serine protease that binds to LDL receptors on the cell surface and interferes with their recycling so that they are directed to lysosomes for degradation.

Cells also control their sterol economy by reverse cholesterol transport (sterol efflux), mediated by members of the ATP-binding cassette subfamily A1 (ABCA1), which transfers cholesterol to plasma lipoproteins.

In the primate corpus luteum undergoing functional luteolysis, a fall in STARD1 expression results in diminished progesterone production.[28,29] To maintain free cholesterol balance, expression of lipoprotein receptors is reduced (reduced uptake), and expression of ABCA1 is increased (increased efflux), perhaps by hydroxysterol activation of LXR transcription factors. Excess cholesterol not available for steroidogenesis is esterified and deposited in cytoplasmic lipid droplets. These "homeostatic" adjustments reflect, in part, changes in gene transcription, post-transcriptional and post-translational processes.

Overview of Steroidogenesis

The cellular manufacture of steroid hormones involves the action of several classes of enzymes: the cytochromes P450, hemeprotein mixed-function oxidases (named because of their distinct absorption peak at 450 nm when reduced in the presence of carbon monoxide), the hydroxysteroid dehydrogenases and reductases.[6,30]

Cytochrome P450s catalyze the major alterations in the sterol framework, cleavage of the side chain, hydroxylations, and aromatization. These hemeproteins require molecular oxygen and a source of reducing equivalents (i.e., electrons) to complete a catalytic cycle. Each member of the steroidogenic cytochrome P450 family of genes is designated "CYP," followed by a unique identifying number that usually refers to the carbon atom at which the enzyme acts.

The hydroxysteroid dehydrogenases reduce ketone groups or oxidize hydroxyl functions, employing pyridine nucleotide cofactors, usually with a stereospecific substrate preference and reaction direction. In addition to being involved in hormone biosynthesis in steroidogenic cells, this family of enzymes works with the reductases, steroid sulfotransferases, and steroid sulfatase to regulate the level of bioactive hormone in target tissues. The hydroxysteroid dehydrogenases are key determinants of the cellular response to endogenous steroid hormones as well as steroidal drugs.

The reductases, using the pyridine nucleotide nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor, produce saturated ring A steroids from Δ^4-steroids (again, with stereospecificity). Table 4.1 lists the key steroidogenic enzymes by class and the respective gene designation. Figure 4.3 outlines the pathways of steroid hormone synthesis, indicating where specific enzymes act.

Table 4.1 Key Human Steroidogenic Proteins and Their Genes

Protein	Chromosomal Gene	Chromosomal Locus	Substrates*	Major Activities	Known Deficiency States
StAR STARD1	*STARD1*	8p11.2	Cholesterol flux within mitochondria	Sterol delivery to P450scc	Congenital lipoid adrenal hyperplasia
P450scc	*CYP11A1*	15q23-q24	Cholesterol Hydroxysterols	Cholesterol side chain cleavage	Side chain cleavage enzyme deficiency
P450c17	*CYP17A1*	10q24.3	Preg, 17OH-Preg Prog, [17OH-Prog] DHEA	17α-Hydroxylase 1 6α-Hydroxylase 17,20-Lyase	17α-Hydroxylase deficiency Isolated 17,20-lyase deficiency
P450c21	*CYP21A2*	6p21.1	Prog, 17OH-Prog	21-Hydroxylase	21-Hydroxylase deficiency
P450c11β	*CYP11B1*	8q21-q22	11-Deoxycortisol	11-Hydroxylase	11-Hydroxylase deficiency
P450c11AS	*CYP11B2*	8q21-q22	Corticosterone 11-DOC 18OH-Corticosterone	11-Hydroxylase 18-Hydroxylase 18-Oxidase	CMO I deficiency CMO II deficiency
P450arom	*CYP19A1*	15q21.1	Androstenedione	19-Hydroxylase	Aromatase deficiency
P450 oxido reductase	*POR*	7q11.2	Androgens/corticoids Testosterone	17α-hydroxylase/17-20 lyase and 21-hydroxylase Aromatization	Combined partial 17α-hydroxylase/17-20 desmolase and 21-hydroxylase deficiencies
3β-HSDI	*HSD3B1*	1p13	Preg, 17OH-Preg DHEA, Adiol	3β-Dehydrogenase $\Delta^{5,4}$-Isomerase	
3β-HSD2	*HSD3B2*	1p13	Preg, 17OH-Preg DHEA, Adiol	3β-Dehydrogenase Δ^{5-4}-Isomerase	3β-HSD deficiency
17β-HSD1	*HSD17B1*	17q21	Estrone, [DHEA]	17β-Ketosteroid reductase	
17β-HSD2	*HSD17B2*	16q24	Estradiol, testosterone DHT, 20α-OH-prog	17β-Hydroxysteroid dehydrogenase 20α-Hydroxysteroid dehydrogenase	
17β-HSD3	*HSD17B3*	9q22	Androstenedione	17β-Ketosteroid reductase	Male 17-ketosteroid reductase deficiency
17β-HSD5	*HSD17B5* *AKR1C3*	10p14-15	Androstenedione, DHT, 3α-Asdiol 3α-Androstanediol Asone, Asdione	17β-Ketosteroid reductase 3α-Hydroxysteroid dehydrogenase	
5α-Reductase type 1	*SRD5A1*	5p15	Testosterone, C21 steroids	5α-Reductase	
5α-Reductase type 2	*SRD5A2*	2p23	Testosterone, C21 steroids	5α-Reductase	5α-Reductase deficiency
11β-HSDI	*HSD11B1*	1q32.2	Cortisol, cortisone, corticosterone, 11-dehydrocorticosterone	11β-Ketosteroid reductase	Cortisone reductase deficiency
11β-HSDII	*HSD11B2*	16p22	Cortisol, cortisterone	11β-Hydroxysteroid dehydrogenase	Syndrome of apparent mineralocorticoid excess
Estrogen sulfotransferase	*SULT1E1*	4q13.1	Estradiol, estrone	Sulfonation	
DHEA sulfotransferase	*SULT2A1* *SULT2B1*	19q13.4	DHEA, Preg	Sulfonation	
Steroid sulfatase	*STS*	Xp23.3	DHEA sulfate Cholesterol sulfate	Sulfatase	Sulfatase deficiency

*Bracketed compounds are minor substrates.

Adiol, Δ^5,3β-androstanediol; 3α-Asdiol, 3α-androstanedione; 11-DOC, 11-deoxycorticosterone; Asdione, androstanedione; Asone, androsterone; COA, coenzyme A; CMO, corticosterone methyl oxidase; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; HSD, hydroxysteroid dehydrogenase; Preg, pregnenolone; Prog, progesterone.

Key Proteins in the Biosynthesis and Catabolism of Steroid Hormones

Steroidogenic Acute Regulatory Protein (STARD1): the Principal Regulator of Gonadal and Adrenal Steroidogenesis

Translocation of cholesterol from the outer mitochondrial membranes to the relatively sterol-poor inner membranes is the critical step in steroidogenesis.[5,6] This translocation process occurs at modest rates in the absence of specific effectors. It is markedly enhanced by STARD1, a protein with a short biological half-life. The following evidence established STARD1 as the key mediator of substrate flux to the side-chain cleavage system:

1. Expression of STARD1 is directly correlated with steroidogenesis.
2. Co-expression of STARD1 and the cholesterol side-chain cleavage enzyme system in cells that are not normally steroidogenic results in substantial pregnenolone synthesis above that produced by cells with

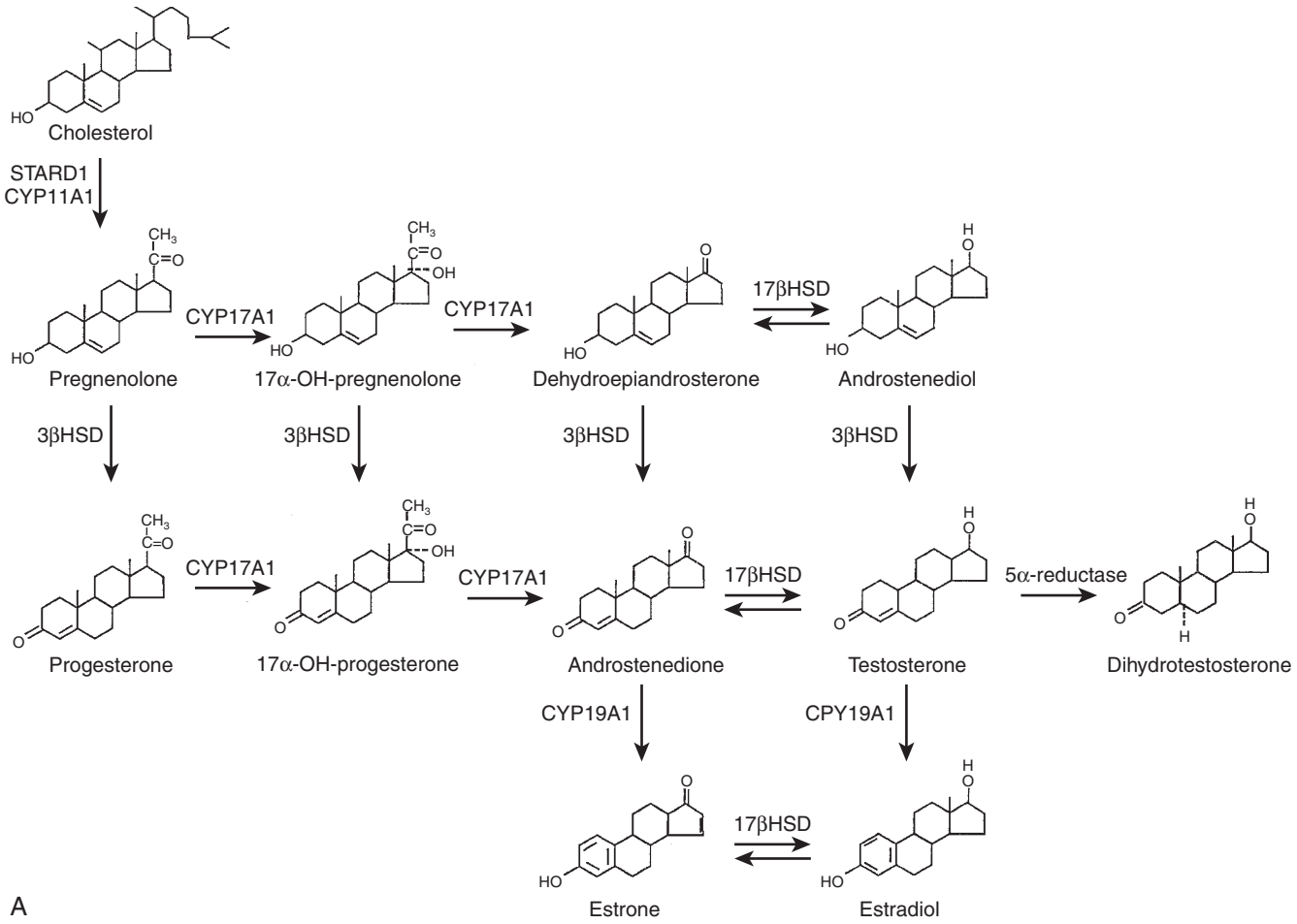


FIGURE 4.3 **A,** *Biosynthetic pathway for sex steroid hormones.*

the cholesterol side-chain cleavage enzyme system alone.

3. Mutations that inactivate STARD1 cause congenital lipoid adrenal hyperplasia, a rare autosomal recessive disorder in which the synthesis of all adrenal and gonadal steroid hormones is severely impaired before the cholesterol side-chain cleavage step.
4. Targeted deletion of the murine *StarD1* gene results in a phenotype in nullizygous mice that mimics human congenital lipoid adrenal hyperplasia.

Human STARD1 is synthesized as a 285–amino acid protein. The N-terminus of STARD1 is characteristic of proteins synthesized in the cytoplasm and then imported into mitochondria, with the first 26 amino acid residues predicted to form an amphipathic helix. Newly synthesized STARD1 preprotein (37 kDa) is rapidly imported into mitochondria and processed to the "mature" 30-kDa form. The preprotein has a very short half-life (minutes), but the mature form is longer-lived (hours).

STARD1 contains two consensus sequences for cyclic AMP (cAMP)-dependent protein kinase phosphorylation at Ser57 and Ser195. Ser195 of human STARD1 must be phosphorylated for maximal steroidogenic activity in model systems.

Tissues that express STARD1 at high levels carry out trophic hormone–regulated mitochondrial sterol hydroxylations through the intermediacy of cAMP. STARD1 messenger RNA (mRNA) and protein are not present in the human placenta, an observation that is consistent with the fact that pregnancies hosting a fetus affected with congenital lipoid adrenal hyperplasia go to term. Although estrogen production is impaired in these pregnancies as a result of diminished fetal adrenal androgen production, placental progesterone synthesis is not significantly affected, indicating that the trophoblast cholesterol side-chain cleavage reaction is independent of STARD1.

The abundance of STARD1 protein in steroidogenic cells is determined primarily by the rate of *STARD1* gene transcription, which is influenced by transcription factors involved in the control of other genes involved in cholesterol metabolism (e.g., SREBFs, NR5A1, NR5A2, LXRs), although STARD1 mRNA stability and translational mechanisms may also contribute. In differentiated cells, the *STARD1* gene is activated by the cAMP signal transduction cascade within 15 to 30 minutes. In differentiating cells (e.g., luteinizing granulosa cells), the induction of *STARD1* transcription takes hours and requires ongoing protein synthesis.

Originally, STARD1 was believed to stimulate cholesterol movement from the outer to the inner mitochondrial membrane as it was imported into the mitochondria. The importation process was proposed to create contact sites between the two membranes, allowing cholesterol to flow down a chemical gradient. However, a STARD1 protein lacking the

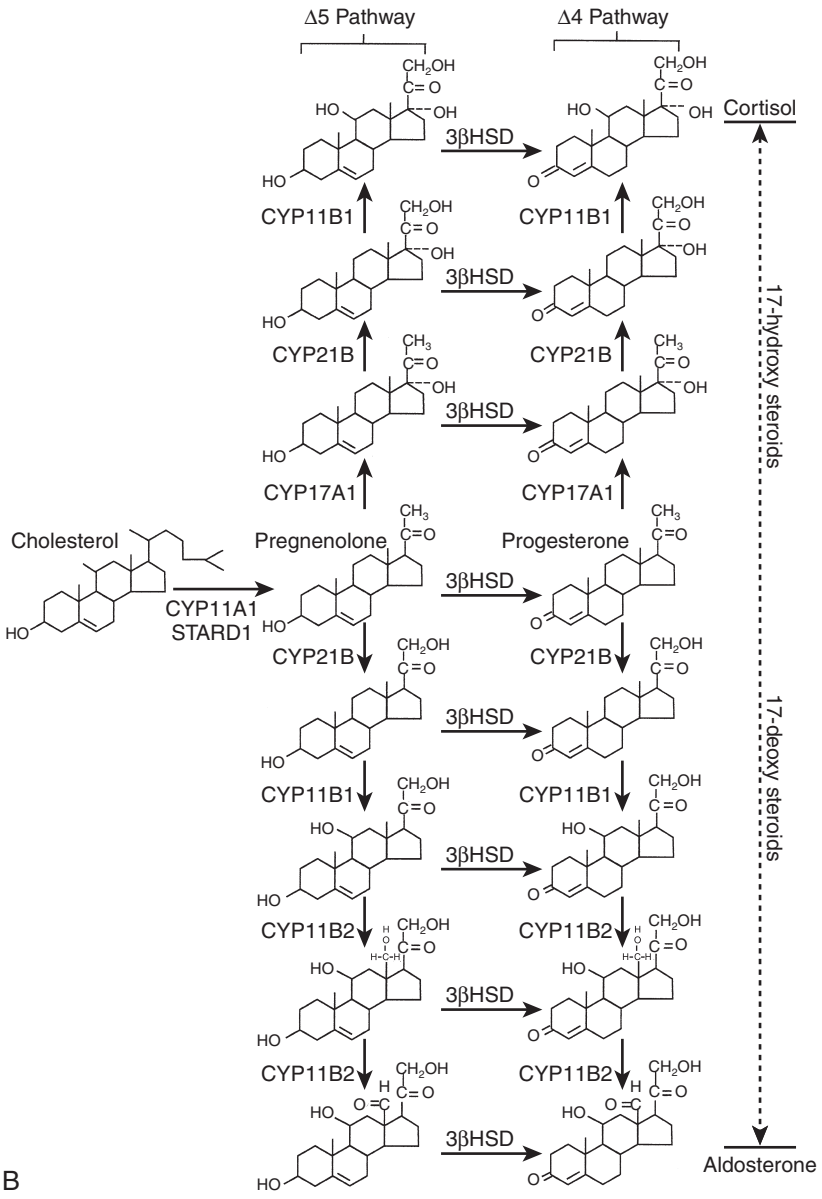


FIGURE 4.3, CONT'D B, Biosynthetic pathway for adrenal steroid hormones. Note that the 3β-hydroxysteroid dehydrogenase in the gonads and adrenal cortex is the type 2 enzyme (HSD3B2), whereas the type 1 enzyme is responsible for this activity in the placenta. Note also that the 17β-hydroxysteroid dehydrogenases involved in the reduction and oxidation of steroids are different enzymes, as described in the text. CYP, cytochrome P; HSD, hydroxysteroid dehydrogenase; STARD1, steroidogenic acute regulatory protein.

N-terminal 62 amino acids (N-62 STARD1), which contain the mitochondrial targeting sequence, was found to be as effective as STARD1 in stimulating steroidogenesis. Other STARD1 constructs engineered for prolonged tethering to the surface of the mitochondria were very active in stimulating pregnenolone production, suggesting that the residency time of the protein on the mitochondrial surface determines the duration of the steroidogenic stimulus. Recombinant human N-62 STARD1 in nanomolar concentrations enhanced pregnenolone production by isolated ovarian mitochondria in a dose- and time-dependent fashion, with significant increases in steroid production observed within minutes. Collectively, these findings strongly suggest that STARD1 acts on the outer mitochondrial membrane to promote cholesterol translocation. This perspective implies that import of the protein into the mitochondrial matrix, rather than being the *trigger* to steroid production, is actually the "off" mechanism because it removes STARD1 from its site of action (Fig. 4.3). Consequently, on-going production of STARD1 preprotein is required to sustain steroidogenesis.

The findings of the previously described experiments are most consistent with the idea that STARD1 enhances desorption of cholesterol from the sterol-rich outer mitochondrial membrane to the relatively sterol-poor inner membranes. The desorption process may involve a pH-dependent conformational change (molten globule transition). Even though STARD1 contains a hydrophobic pocket that binds cholesterol, sterol binding is not required for steroidogenic activity.

The molecule or structure on the mitochondrial outer membrane that STARD1 acts on has not yet been identified. It could be a lipid configuration or a protein. One protein candidate is the translocator protein (TSPO), also known as the peripheral-type benzodiazepine receptor (PBR), an outer mitochondrial membrane protein that also binds cholesterol.[5,6] Knock-down of TSPO expression

blocks steroidogenesis in cultured cells, even in the presence of STARD1, suggesting that TSPO is required for STARD1 action and that it may serve to activate a pore, comprised of other mitochondrial membrane proteins including the voltage-dependent anion channel (VDAC), through which cholesterol could flow to the inner mitochondrial membrane in the presence of STARD1. However, the nature of STARD1–TSPO-VDAC interactions remain to be elucidated.

As noted above, mutations in the *STARD1* gene cause congenital lipoid adrenal hyperplasia, a rare autosomal recessive disease. Exceptions occur in Japan and Korea, however, where the mutation accounts for at least 5% of all cases of congenital adrenal hyperplasia.[5,6,31] The pathophysiology of the disease entails a two-step process in which impaired use of cholesterol for steroidogenesis leads to accumulation of sterol esters in lipid droplets. These droplets ultimately compress cellular organelles, causing damage through the formation of lipid peroxides. This damage occurs prominently in the adrenal cortex and Leydig cells.

Mutations found in the *STARD1* gene, which is composed of seven exons, include frameshifts caused by deletions or insertions, splicing errors, and nonsense and missense mutations. All of these mutations lead to the absence of STARD1 protein or the production of functionally inactive protein. Several nonsense mutations were shown to result in C-terminus truncations of STARD1. One of these mutations, Gln258Stop, results in the deletion of the final 28 amino acids of the STARD1 protein and accounts for 80% of the known mutant alleles in the affected Japanese population. Known point mutations that produce amino acid substitutions occur in exons 5 to 7 of the gene, the exons that encode the C-terminus. Mutations that cause partial loss of STARD1 activity are associated with a milder disease phenotype.

Although affected XY subjects are pseudohermaphrodites (46,XY disorder of sexual development [DSD]) because of an inability to generate sufficient fetal testicular testosterone to masculinize the external genitalia, XX subjects have normal external genitalia, develop female secondary sexual characteristics, and experience menarche. They are, however, anovulatory and unable to produce large amounts of estradiol and progesterone in a cyclic fashion. The fact that some ovarian estradiol synthesis occurs reflects the existence of STARD1-independent substrate movement to the cholesterol side-chain cleavage system.

Other START Domain Proteins

A family of proteins exists sharing a domain that is similar to the C-terminus of STARD1, the *StAR-related lipid transfer (START) domain proteins*.[32] The absence of STARD1 from the human placenta, an organ that produces a significant amount of pregnenolone, documented the existence of STARD1-independent steroidogenesis, but raised the possibility that another protein, possibly STARD3, might subserve the function of STARD1 in the placenta. Other cytosolic START domain proteins (STARD4, STARD5) could be involved in the movement of cholesterol to the mitochondria as sterol carrier proteins, although their specific roles in sterol trafficking remain to be elucidated (Fig. 4.2).

The Cholesterol Side-Chain Cleavage Enzyme (P450scc Encoded by *CYP11A1*)

Cholesterol side-chain cleavage is catalyzed by cytochrome P450scc and its associated electron transport system, consisting of a flavoprotein reductase (ferredoxin or adrenodoxin reductase) and an iron sulfoprotein (ferredoxin or adrenodoxin), encoded by the *FDX1* gene, which shuttles electrons to cytochrome P450scc.[6,33,34] The side-chain cleavage reaction involves three catalytic cycles: the first two lead to the introduction of hydroxyl groups at positions C-22 and C-20, and the third results in scission of the side chain between these carbons (Fig. 4.4). Each catalytic cycle requires one molecule of NADPH and one molecule of oxygen so that the formation of one mole of the cleavage products, pregnenolone and isocroapraldehyde, uses three moles of NADPH and three moles of oxygen.

The slowest step of the reaction is the binding of cholesterol to the hydrophobic pocket of P450scc, where the heme resides. The sterol substrate remains bound to a single active site on cytochrome P450scc for all three cycles because of the tight binding of the reaction intermediates. The dissociation constant (K_d) for binding of cholesterol, a measure of the enzyme's affinity for its substrate, is approximately 5000 nM, whereas the K_d for the binding of the intermediate product 22-hydroxycholesterol is 4.9 nM; the K_d for 20,22-dihydroxycholesterol is 81 nM. However, the estimated K_d for pregnenolone, the end product, is 2900 nM, which permits its dissociation from the enzyme at the end of the reaction.

Reducing equivalents are shuttled to cytochrome P450scc by ferredoxin in cycles of reduction and oxidation, facilitated by differential affinities of the proteins, depending on their state of oxidation or reduction.[34] Ferredoxin forms a 1:1 complex with ferredoxin reductase, which catalyzes reduction of the iron-sulfur protein. The reduced ferredoxin then dissociates and forms a 1:1 complex with cytochrome P450scc and is subsequently oxidized when it donates its electrons to P450scc. Oxidized ferredoxin returns to ferredoxin reductase for electron recharging. This recharging is facilitated by the fact that ferredoxin reductase has a greater affinity for oxidized over reduced ferredoxin. The binding of cholesterol to cytochrome P450scc increases its affinity for reduced ferredoxin, which enhances the shuttle of electrons to substrate-loaded enzyme.

The rate of formation of pregnenolone is determined by:

1. Access of cholesterol to the inner mitochondrial membranes
2. The quantity of cholesterol side-chain cleavage enzyme, and secondarily, its flavoprotein and iron-sulfur protein electron transport chain
3. Catalytic activity of P450scc, which can be influenced by post-translational modification

Acute alterations in steroidogenesis generally result from changes in the delivery of cholesterol to P450scc, whereas long-term alterations involve changes in the quantity of enzyme proteins as well as cholesterol delivery.

The *CYP11A1* gene, located on chromosome 15q23-q24, consists of nine exons, an organization shared by the other mitochondrial steroidogenic P450 enzymes, 11β-hydroxylase and aldosterone synthase.

Mutations in the *CYP11A1* gene that result in significantly diminished cholesterol side-chain cleavage activity have been reported in association with adrenal insufficiency and XY sex reversal, phenotypes that are similar to those

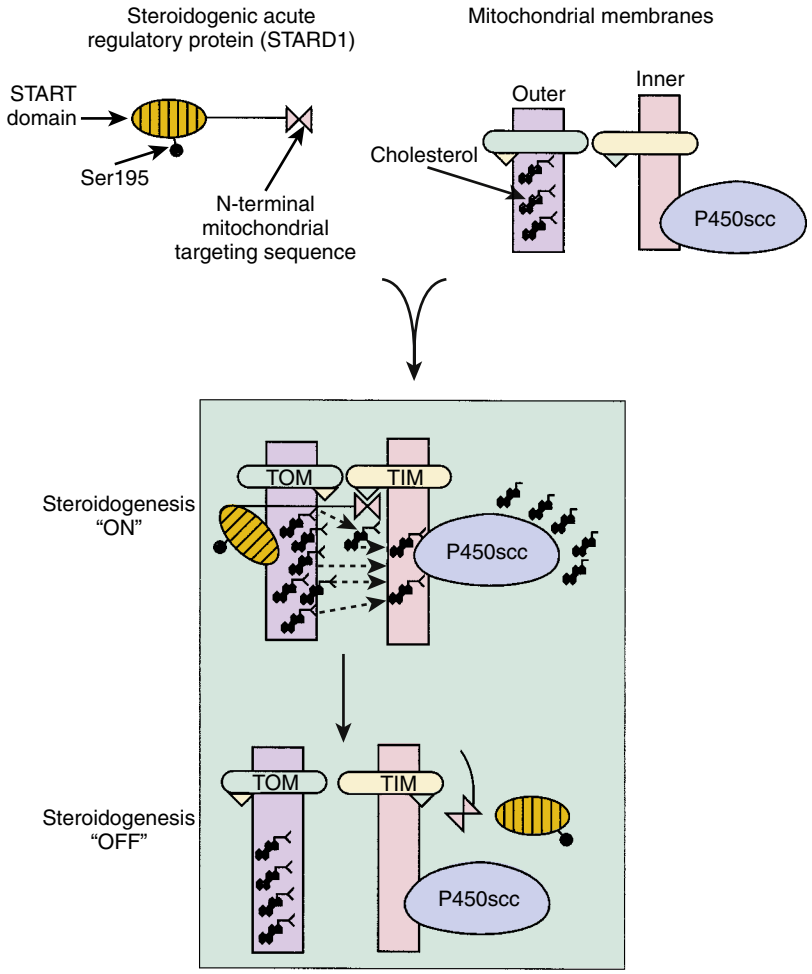


FIGURE 4.4 Structure of steroidogenic acute regulatory protein (STARD1) and model for its mechanism of action on intramitochondrial cholesterol translocation. TIM, inner mitochondrial membrane translocator; TOM, outer mitochondrial membrane protein translocator.

associated with inactivating mutations in the *STARD1* gene.[6,35-37] An interesting *CYP11A1* mutation was discovered in an XY subject, born prematurely with sex reversal and adrenal failure. The subject was homozygous for a single-nucleotide deletion leading to a premature termination codon at codon 288, predicted to delete the C-terminal 242 amino acids, including the heme-binding site, which should result in a nonfunctional protein.

The discovery of mutations causing severe P450scc deficiency in humans challenges the notion that absence of P450scc activity in the fetus and placenta is incompatible with pregnancy progressing beyond the usually limited period of luteal progesterone support. Therefore, compensatory mechanisms exist to maintain sufficient progestational activity to sustain pregnancy and fetal viability in women hosting a fetus with *CYP11A1* mutations. Another surprising and distinguishing feature of subjects with *CYP11A1* mutations is the absence of adrenal hypertorophy, as seen in *STARD1* mutations.

17α-Hydroxylase/17,20-Lyase (P450c17; CYP17A1)

P450c17 is an endoplasmic reticulum enzyme that catalyzes two reactions: hydroxylation of pregnenolone and progesterone at carbon 17, and conversion of pregnenolone into C19 steroids (in the case of the human enzyme, progesterone is also converted, but to a much lesser extent).[6] The 17α-hydroxylation reaction requires one pair of electrons and molecular oxygen. The lyase reaction requires a second electron pair and molecular oxygen. The reducing equivalents are transferred to the P450c17 heme iron from NADPH by NADPH cytochrome P450 reductase (POR). The hydroxylase and lyase reactions are both believed to proceed through a ferryl oxene mechanism, with the substrate bound to the enzyme in the catalytic pocket in the same orientation. P450c17 also catalyzes 16α-hydroxylation of progesterone and dehydroepiandrosterone.

The importance of POR in steroid metabolism catalyzed by endoplasmic reticulum cytochrome P450 enzymes has been illuminated by the phenotype of individuals with POR deficiency, which causes a form of congenital adrenal hyperplasia.[38,39] The autosomal recessive disorder results in a steroid profile suggestive of combined 21-hydroxylase and 17-hydroxylase/17-20 lyase deficiency, which presents as a range of phenotypes, including adrenal insufficiency, ambiguous genitalia, and Antley-Bixler skeletal malformation syndrome.

Several factors determine whether substrates only undergo 17α-hydroxylation or subsequent scission of the 17,20 bond, including:

1. The nature of the substrate
2. The amount of POR and flux of reducing equivalents
3. Allosteric effectors
4. Post-translational modification of P450c17 (Fig. 4.5)

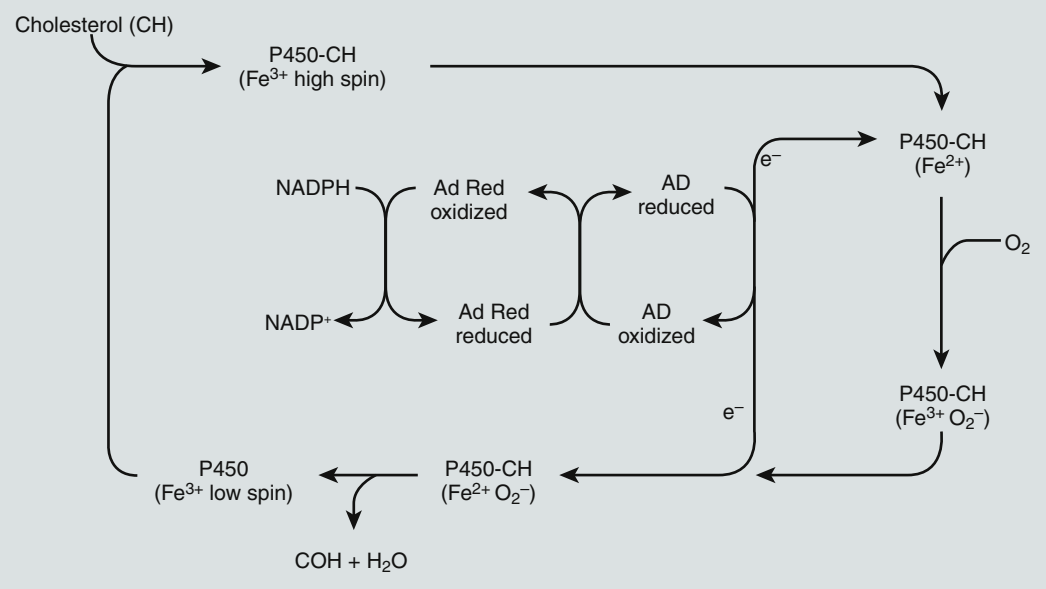


FIGURE 4.5 Catalytic cycle for P450scc. Cholesterol (CH) binds to P450scc. Reducing equivalents are shuttled to P450scc by adrenodoxin (AD), which receives electrons from adrenodoxin reductase (Ad Red), which oxidizes nicotinamide adenine dinucleotide phosphate (NADPH). P450scc goes through three sequential catalytic cycles with substrate and reaction intermediates remaining bound to the enzyme (not reflected in the scheme shown above) to convert 1 mole of cholesterol to 1 mole of pregnenolone and isocaproic acid, using 3 moles of NADPH and molecular oxygen. The P450scc heme iron undergoes oxidation and reduction, changing its electron spin resonance state. NADP, nicotinamide adenine dinucleotide.

Collectively, these factors determine the nature of the products produced by the enzyme, which, in the gonads and zona reticularis, favor androgens through augmentation of lyase activity. In contrast,17α-hydroxylation required for glucocorticoid and mineralocorticoid synthesis is favored in the zona fasciculata and glomerulosa.

Human P450c17 preferentially uses Δ^5 substrates for 17,20 bond cleavage. Cytochrome b_5 (CYB5A) promotes electron transfer for the lyase reaction acting as an allosteric effector, and not as an electron donor per se, because the apo b_5 protein is also effective.[40] Cytochrome b_5 also increases the use of 17α-hydroxyprogesterone as a substrate for androstenedione synthesis. The distribution and regulation of cytochrome b_5 expression in the adrenal cortex, being greatest in the adult zona reticularis, supports a role for this protein in the regulation of lyase activity. The product of a second cytochrome b_5 gene (type 2 cytochrome b_5, CYB5B), found in human testis and expressed in the adrenals, also increases lyase activity.

Phosphorylation of P450c17 at serine and threonine residues by a yet-to-be-identified protein kinase appears to be necessary for maximal 17,20-lyase activity.[41] The phosphorylated P450c17 protein is evidently a substrate for protein phosphatase 2A (PP2A), since inhibitors of PP2A enhance lyase activity in cultured adrenal tumor cells.

Adrenarche, the increased production of adrenal androgens in the absence of increased production of cortisol or levels of ACTH, may result from enhanced P450c17 lyase activity due to increased expression of cytochrome b_5 or the state of P450c17 phosphorylation. The availability of electrons to P450c17 has been proposed to influence the relative ratio of 17,20-lyase activity to 17α-hydroxylase activity. However, increasing the ratio of P450-oxidoreductase to P450c17 augments both the 17α hydroxylation and the lyase reactions, making this an unlikely mechanism by which lyase-to-hydroxylase activity is differentially regulated.

A P450c17-independent mechanism for conversion of pregnenolone into dehydroepiandrosterone in glial tumor cell homogenates promoted by $FeSO_4$ has been described. This conversion presumably results from the fragmentation of tertiary hydroperoxides, which are probably derived from pregnenolone molecules oxygenated at carbons 17 and 20.[42] The physiological significance of this pathway, if any, to the formation of C19 steroids in the brain or other tissues is unknown.

The *CYP17A1* gene, located on band 10q24.3, consists of eight exons. Mutations in this gene cause combined or isolated deficiency states for each activity of P450c17.[6,39] Individuals with combined deficiency have a marked diminution in the production of C19 and C18 steroids, low levels of cortisol that result in elevated ACTH secretion, and excess production of steroids proximal to the P40c17 reaction. Hypertension from sodium retention and hypokalemia are a consequence of the increased production of 11-deoxycorticosterone. The inability to produce sex steroid hormones prevents adrenarche, and puberty in females, and results in incomplete or absent development of male genitalia (46, XY DSD).

Four recurrent mutations have been described that cause combined 17α-hydroxylase and lyase deficiency in different populations: a 4-bp insertion resulting in a frame shift and altered C-terminal sequence (Holland); in-frame deletions of amino acid residues 487 through 489 (Southeast Asia); a deletion of phenylalanine in the N-terminus of the protein (Japan); and non-synonymous mutations (Tryp406Arg and Arg362Cys) (Brazil). Isolated 17,20-lyase deficiency is very rare.[6,39] The documented cases result from point mutations that permit pregnenolone or progesterone to be bound and undergo 17α-hydroxylation, but prohibit the efficient

receipt or usage of a second pair of electrons provided by POR to support the 17,20-lyase reaction.

Aromatase (P450aro, CYP19A1)

Aromatase, an endoplasmic reticulum enzyme, catalyzes three sequential hydroxylations of a C19 substrate by using three molecules of NADPH and three molecules of molecular oxygen to produce one molecule of C18 steroid with a phenolic A ring.[6,43] The first hydroxylation yields a C19 hydroxyl derivative, which is converted in a second hydroxylation to a gem diol that collapses to yield a C19 aldehyde. The final hydroxylation involves the formation of a 19-hydroxy-19-hydroperoxide intermediate that results in the elimination of the C19 methyl group as formic acid and concurrent aromatization. This sequence of reactions takes place at a single active site on the enzyme, with reducing equivalents transferred to P450aro by POR. The crystal structure of aromatase in complex with androstenedione revealed hydrogen bonding and tight packing of hydrophobic side chains that explain the enzyme's specificity for androgen substrates.

The aromatase protein is encoded by a single large gene, *CYP19A1*, on band 15q21.1. This gene gives rise to cell-specific transcripts from different promoters (Fig. 4.6).[6,44] The promoter driving ovarian aromatase expression lies adjacent to the exon encoding the translation start site (promoter IIa). In granulosa cells, FSH stimulates transcription of the genes encoding both aromatase and POR, which provides its reducing equivalents. A separate promoter lying approximately 100 kb upstream from the start of translation controls placental *CYP19A1* transcription. Expression of aromatase in adipose tissue, skin, and brain is driven from other promoters. Cytokines (including interleukin-11, interleukin-6, oncostatin-M, and leukemia-inhibiting factor) increase P450arom expression in adipose tissue via the I.4 promoter.

Several cases of aromatase deficiency have been described.[6,45-47] Pregnancies in which the fetus is affected with aromatase deficiency are characterized by low maternal urinary estrogen excretion, maternal virilization, and ambiguous genitalia or female pseudohermaphroditism (46, XX DSD) in affected genetic females. The maternal and fetal virilization in the absence of placental aromatase activity highlights the importance and efficiency of the placenta in converting maternal and fetal androgens into estrogens.

Among the mutations identified in the *CYP19A1* gene are an 87-bp insertion at the splice junction between exon 6 and intron 6, causing the addition of 29 in-frame amino acid residues, with the other mutations being mainly missense or nonsense mutations in exons 4, 9, and 10. The mutant protein with the 29 in-frame amino acid residues displayed less than 3% of normal aromatase activity. Expression of the mutant complementary DNA confirmed that the protein had only a trace of aromatase activity. Compound heterozygous mutations in coding sequences found in patients with aromatase deficiency have also been shown to have minimal activity. When aromatase activity has been measured in placenta from offspring with *CYP19A1* mutations, activities have been markedly reduced to 21% of normal values.

Mice deficient in aromatase have been created by targeting the *Cyp19a1* gene.[48] The aromatase-deficient (ArKO) mice show many of the features of human aromatase deficiency and the consequential lack of estrogens, including a profound bone phenotype with reductions in all indices of bone mineralization.

Families with autosomal dominant estrogen excess resulting from over-expression of aromatase have been reported.[49-52] The phenotypes include severe prepubertal gynecomastia in males and macromastia and premature puberty in females. The aromatase over-expression is caused in some families by heterozygous genomic rearrangements. In some cases an inversion results in a constituitively active cryptic promoter placed in control of the *CYP19A1* gene, driving excess aromatase production.

Inappropriate expression of aromatase in neoplastic and non-neoplastic tissue has also been found. In these pathological conditions, exemplified by breast cancer, there appears to be a shift in promoter use to favor the stronger gonadal (promoter IIa or I.3) over the weaker adipose tissue promoter (promoter I.4). This shift allows for activation of a cAMP-dependent signaling pathway, accounting for excessive aromatase expression and the resulting increase in estrogen synthesis.

11β-Hydroxylases (P450c11β and P450c11AS)

The human genome contains two genes located on band 8q24.3 that encode related mitochondrial enzymes involved in 11β-hydroxylation and aldosterone synthesis, respectively, P45011β, encoded by *CYP11B1*, and P450c11AS (also referred to as "P450aldo," "P450c18," or "P450cmo"), encoded by *CYP11B2*.[6] These two genes are located 40 kb apart; each containing nine exons. The encoded proteins differ in only 33 amino acid residues. Both enzymes display 11β-hydroxylase activity, but P450c11AS can also carry out the two oxygenation steps at carbon 18 required for the production of aldosterone. They require molecular oxygen as well as reducing equivalents for catalysis, the latter shuttled to the enzymes by the adrenodoxin reductase–adrenodoxin system.

CYP11B1, a gene whose transcription is stimulated by ACTH-triggered cAMP signaling pathways, is expressed in the zonae fasciculata and reticularis of the adrenal cortex. In contrast, *CYP11B2* expression is restricted to the zona glomerulosa. Transcription of this gene is stimulated by protein kinase C signaling pathways, which are turned on by angiotensin II.

Mutations in the *CYP11B1* gene cause 11β-hydroxylase deficiency, whereas mutations in *CYP11B2* cause 18-hydroxylase or corticosterone methyl oxidase I deficiency and 18-oxidase or corticosterone methyl oxidase II deficiency.[6,53,54] Unequal crossover of the adjacent *CYP11B1* and *CYP11B2* genes creates a third hybrid gene, in which the cAMP-regulated promoter of the *CYP11B1* gene drives expression of a chimeric protein with aldosterone synthase activity. This leads to a state of glucocorticoid-suppressible aldosteronism.

Accounting for 5% to 8% of cases of congenital adrenal hyperplasia in European ancestry populations and 15% in Middle Eastern populations, 11β-hydroxylase deficiency is characterized by high levels of 11-deoxycortisol and deoxycorticosterone, resulting in salt retention and hypertension. Affected females are virilized by the excess production of adrenal androgens driven by elevated ACTH levels. *CYP11B1* mutations causing 11β-hydroxylase deficiency

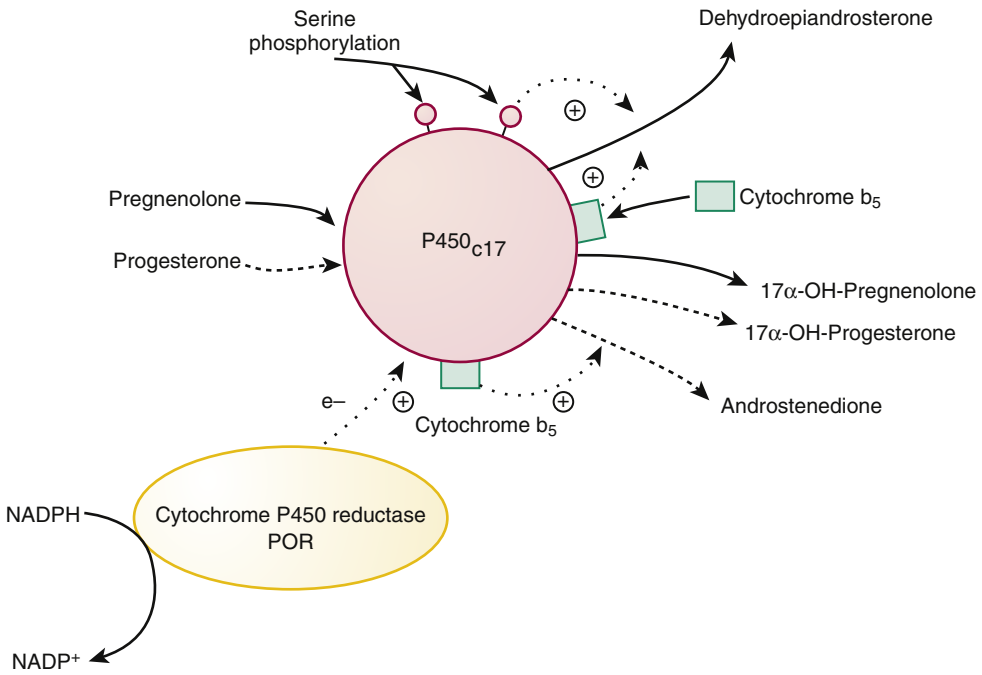


FIGURE 4.6 Factors modulating 17α-hydroxylase and 17,20-lyase activities of P450c17. Phosphorylation of serine/threonine residues and cytochrome b5 stimulate *(designated by plus symbol)* lyase activity, with pregnenolone being the preferred substrate for the lyase reaction. Dephosphorylation of the serine/threonine residues by protein phosphatase 2A reduces lyase activity. Flow of reducing equivalents (e^-, electrons) from cytochrome P450 reductase (POR) stimulates both 17α-hydroxylase and lyase activities. H6PDH, hexose-6-phosphate dehydrogenase; NADP, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate.

include those resulting in non-synonymous amino acid substitutions and a premature stop codon.

Corticosterone methyl oxidase I deficiency results from the complete absence of P450c11AS activity. Aldosterone synthesis is absent, but the production of corticosterone and cortisol is retained. An increased ratio of corticosterone to 18-hydroxycorticosterone in the presence of low aldosterone and elevated plasma renin activity is diagnostic. Corticosterone methyl oxidase II deficiency is caused by mutations that inactivate 18-methyl oxidase while retaining 18-hydroxylase activity. This causes elevated 18-hydroxycorticosterone and low aldosterone levels.

21-Hydroxylase (P450c21,CYP21A2)

P450c21 is an adrenal endoplasmic reticulum enzyme that catalyzes the 21-hydroxylation of progesterone and 17α-hydroxyprogesterone in the pathway of mineralocorticoid and glucocorticoid biosynthesis.[6,53,55,56] The Michaelis constant (K_m) for 17α-hydroxyprogesterone (1.2 μM) is lower than that for progesterone (2.8 μM), and the apparent maximum velocity (V_{max}) for the former substrate is twice that for progesterone. The enzyme requires 1 mole of molecular oxygen and reducing equivalents (generated from NADPH through POR) to accomplish the hydroxylation of carbon 21. Mutations inactivating POR cause a partial deficiency in 21-hydroxylase activity, as well as a partial deficiency in 17α-hydroxylase/17-20 lyase activity.[6,38] The primary regulator of *CYP21A2* expression in the zona fasciculata is ACTH by way of a cAMP-mediated signal transduction cascade.

The *CYP21A2* gene is adjacent to a pseudogene *(CYP21A1P)*, separated by the complement *C4B* gene. These genes are embedded in the human leukocyte antigen region on band 6p21.1. Frequent unequal crossovers and gene conversions make 21-hydroxylase deficiency one of the most common autosomal recessive metabolic diseases, occurring in 1:10,000 to 1:15,000 births.[55] Unequal crossover, with the complete loss of the *C4B* gene and a net deletion of *CYP21A2*, along with gene conversion events in which mutations in the pseudogene are introduced into the expressed gene, result in reduced 21-hydroxylase enzyme levels or impaired catalytic activity (Fig. 4.7). Large-scale deletions/gene conversions may extend into the adjacent gene encoding tenascin-X, which when mutated in both alleles, causes a form of Ehlers-Danlos syndrome.[56]

The signs and symptoms of congenital adrenal hyperplasia caused by 21-hydroxylase deficiency reflect deficits in cortisol (because of inability to convert 17α-hydroxyprogesterone into 11-deoxycortisol) and aldosterone (because of inability to convert progesterone into deoxycorticosterone). Another contributing factor is the accumulation of adrenal androgens that results from elevated ACTH levels, due to the absence of cortisol-negative feedback on the hypothalamic–corticotrophin axis.

The clinical phenotypes are, however, variable and dependent on the severity of the 21-hydroxylase deficiency.[6,55] The non–salt-wasting, salt-wasting, and non-classic forms are associated with certain mutations that affect the amount of residual 21-hydroxylase activity, with the salt-wasting form being characteristic of severe enzyme deficiency (deletions and large gene conversions). The simple virilizing (non–salt-wasting) form is associated with mutations that substantially reduce activity (e.g., the missense mutation resulting in a non-synonymous amino acid substitution Ile172Asp), and the non-classic (or *late-onset*) form is caused by mutations that do not severely impair the level of expression or activity of P450c21 (e.g., Val28Leu, Pro30Leu).

Hydroxysteroid Dehydrogenases and Reductases

Hydroxysteroid dehydrogenases (HSDs) or oxidoreductases catalyze the interconversion of alcohol and carbonyl functions in a position- and stereospecific manner on the steroid nucleus and side chain, using oxidized (+) or reduced (H) nicotinamide adenine dinucleotide, NAD(H), or nicotinamide adenine dinucleotide phosphate NADP(H)

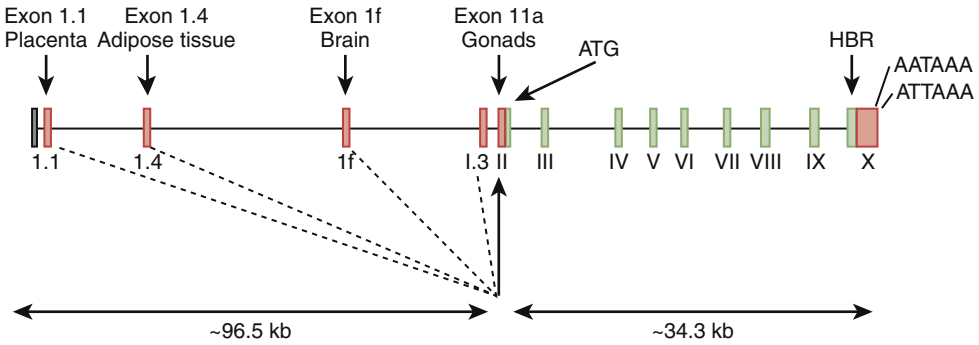


FIGURE 4.7 Structure of the human *CYP19A1* gene showing the protein coding exons indicated by Roman numerals and the location of the different 5′-UTR-coding exons and tissue-specific promoters. The P450arom heme-binding region (HBR) and polyadenylation signals in exon 10 are indicated. ATG, methionine codon. *(Modified from Kamat A, Hinshelwood MM, Murry BA, et al. Mechanisms in tissue-specific regulation of estrogen biosynthesis in humans.* Trends Endocrinol Metab *13:122, 2002.)*

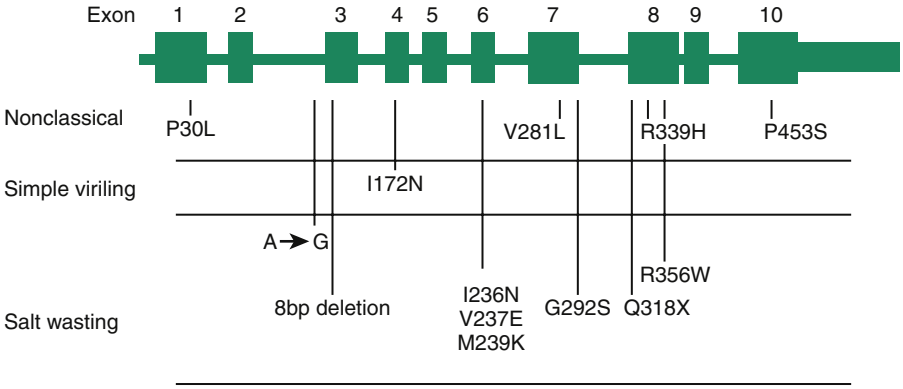


FIGURE 4.8 Structure of the *CYP21A2* gene and selected mutations causing phenotypes of 21-hydroxylase deficiency. *(Modified from White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency.* Endocr Rev *21:245, 2000.)*

as cofactors.[6,30,57] In some instances, HSDs display bifunctionality (e.g., they oxidize or reduce 17β and 20α oxy functions), such as HSD17B2. Although the HSDs can catalyze both the oxidation and reduction reactions *in vitro* under different conditions (e.g., substrate, pH and cofactor), *in vivo* they catalyze reactions in one direction and can be classified as dehydrogenases or reductases. The enzymes are members of the short chain dehydrogenase reductase or aldo-keto superfamilies (www.med.upenn.edu/akr/).

The importance of cofactor availability to HSDs is exemplified by inactivating mutations in the hexose-6-phosphate dehydrogenase *(H6PDH)* gene, which regenerates NADPH in the endoplasmic reticulum required for the HSD11B1 reaction. Individuals with these mutations have apparent cortisone reductase deficiency due to impaired HSD11B1 activity.[6,58]

Multiple isoforms of HSDs exist, and coupled with their tissue-specific expression, account for the ability of specific enzymes to act predominantly as reductases (ketone reduction) or dehydrogenases (alcohol oxidation). In steroidogenic tissues, HSDs catalyze the final steps in progestin, androgen, and estrogen biosynthesis. In steroid target tissues, HSDs can regulate the occupancy of steroid hormone receptors by converting active steroid hormones into inactive metabolites or relatively inactive steroids to molecules with greater binding activity.

This is best exemplified by the human type 2 11β-HSD (HSD11B2), which controls mineralocorticoid activity in the kidney by converting cortisol (which has high affinity for both the glucocorticoid and mineralocorticoid receptors) to cortisone (which does not bind to the mineralocorticoid receptor). Thus, the specificity of mineralocorticoid receptor activation is not determined by the receptor, but by activity of the HSD that removes the more abundant potential mineralocorticoid receptor ligand, leaving aldosterone as the controlling activator (Fig. 4.8).[59] Because of their tissue-specific roles in controlling the bioavailability of steroids, HSDs are interesting targets for pharmacological manipulation.[30]

3β-Hydroxysteroid Dehydrogenase/$\Delta^{5\text{-}4}$ Isomerases

The 3β-HSD/$\Delta^{5\text{-}4}$ isomerases are membrane-bound enzymes localized to the endoplasmic reticulum and mitochondria that use nicotinamide adenine dinucleotide (NAD+) as a cofactor. These enzymes catalyze dehydrogenation of the 3β-hydroxyl group and the subsequent isomerization of the Δ^5 olefinic bond to yield a Δ^4 ketone structure. They convert pregnenolone into progesterone, 3-17α-hydroxypregnenolone into 17α-hydroxyprogesterone, and dehydroepiandrosterone into androstenedione.[6,60]

The dehydrogenase and isomerase reactions are performed at a single bifunctional catalytic site that adopts different conformations for each activity. The 3β-hydroxysteroid dehydrogenase step is rate-limiting in the overall reaction sequence, and the NADH formed in this reaction is believed to alter the enzyme conformation to promote the isomerase reaction.

The human genome has two active 3β-HSD/$\Delta^{5\text{-}4}$ isomerase genes: one encodes a protein predominantly expressed in the placenta, liver, breast, and brain (HSD3B1), the other a protein expressed in the adrenal cortex and gonads (HSD3B2). These two genes, each consisting of four exons each, lie 100 kb

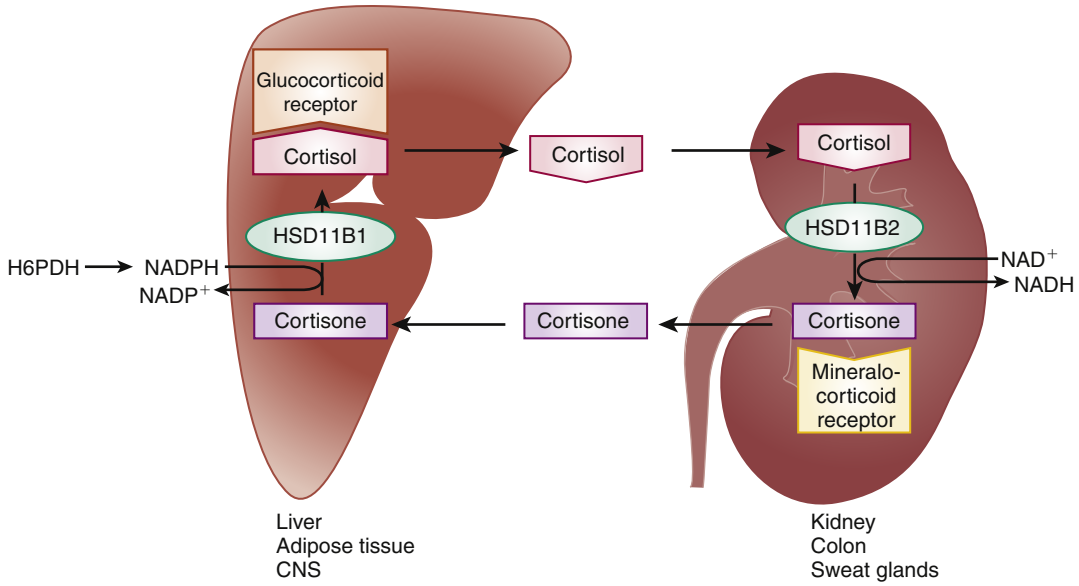


FIGURE 4.9 The roles of 11β-HSD types 1 (HSD11B1) and 2 (HSD11B2) in controlling levels of bioactive glucocorticoids. HSD11B1 reduces inactive cortisone into cortisol in liver and other tissues, whereas HSD11B2 oxidizes cortisol to cortisone. Cortisone cannot activate the mineralocorticoid receptor, thus allowing aldosterone (a steroid that is less abundant than cortisol) to specifically regulate the mineralocorticoid receptor. The supply of NADPH to HSD11B1 is a major determinant of the reaction and inactivating mutations in the H6PDH gene, which generates NADPH, cause cortisone reductase deficiency *(Modified from Seckl J, Walker B. 11β-Hydroxysteroid dehydrogenase type I-A tissue-specific amplifier of glucocorticoid action.* Endocrinology *142:1371, 2001.)*

apart on band 1p13.1. The human genome also contains five unprocessed pseudogenes closely related to *HSD3B1* and *HSD3B2* on band 1p13.1, with two of them lying between the expressed genes. The DNA sequences of the exons of the two active genes are very similar, and the encoded proteins differ in only 23 amino acid residues. HSD3B1, however, has a lower K_m for substrate than HSD3B2 (<1 μM vs 1–4 μM), which facilitates metabolism of lower concentrations of Δ^5 substrate. Electron microscope cytochemistry has localized HSD3B2 activity to the perimitochondrial endoplasmic reticulum and in subcellular fractions containing STARD1 and P450scc. In some cell types, HSD3Bs appear to be localized in the inner mitochondrial membrane. Thus, these enzymes are positioned to act on pregnenolone produced by the cholesterol side-chain cleavage system.

Because most steroidogenic cells have a large capacity to generate progesterone when presented with exogenous pregnenolone, the 3β-HSD/$\Delta^{5\text{-}4}$ isomerases are not believed to be rate-determining enzymes. However, mutations causing deficiency of HSD3B2 cause a form of congenital adrenal hyperplasia characterized by impaired adrenal and gonadal steroidogenesis with accumulation of Δ^5 steroids in the circulation. The presence of active HSD3B1 in extra-adrenal and extra-gonadal tissues results in the formation of some Δ^4 steroids (e.g., 17α-hydroxyprogesterone).[6]

In its severest form, HSD3B2 deficiency is associated with salt wasting because of insufficient mineralocorticoid production.[6,53,60] Kinetic analysis of mutant proteins associated with the salt-wasting and non–salt-wasting forms of the disease showed a four to fortyfold reduction in catalytic efficiency for the conversion of pregnenolone into progesterone. The salt-wasting form of the disease is associated with frameshift mutations resulting in protein truncation and a variety of missense mutations that affect affinity for the cofactor and protein stability. The greater instability of the mutant proteins found in subjects with salt-wasting disease compared with those proteins found in the non–salt-wasting form appears to account, in part, for the different clinical phenotypes.

A so-called attenuated, or late-onset, form of 3β-HSD deficiency, diagnosed by steroid measurements, has been described in the literature. However, no mutations have yet been discovered in the genes encoding *HSD3B1* and *HSD3B2* in subjects with this clinical diagnosis. Mutations in the distal promoter or epigenetic factors that might alter enzyme expression cannot be ruled out. The apparent reduced 3β-HSD activity could also be the result of alterations in the membrane environment that affect catalytic activity or post-translational modifications to the enzyme that diminish its function.

Mutations in *HSD3B1* have not been detected, although several sequence variants that are evidently without functional significance have been described. Because the HSD3B1 is the primary 3β-HSD/$\Delta^{5\text{-}4}$ isomerase in the placenta, it is possible that mutations that inactivate HSD3B1 would lead to miscarriage or preterm birth as a result of insufficient placental progesterone production.

11β-Hydroxysteroid Dehydrogenases: Key Regulators of the Activity of Glucocorticoids

The biological activity of cortisol in target tissues is controlled by the action of two different 11β-hydroxysteroid dehydrogenases that are members of the short-chain alcohol dehydrogenase family (Fig. 4.9). These enzymes catalyze the interconversion of active glucocorticoids and their inert 11-keto metabolites.[6,59] The type 2 enzyme (HSD11B2) is an endoplasmic reticulum protein with reversible oxidoreductase activity *in vitro*, but preferentially catalyzes the

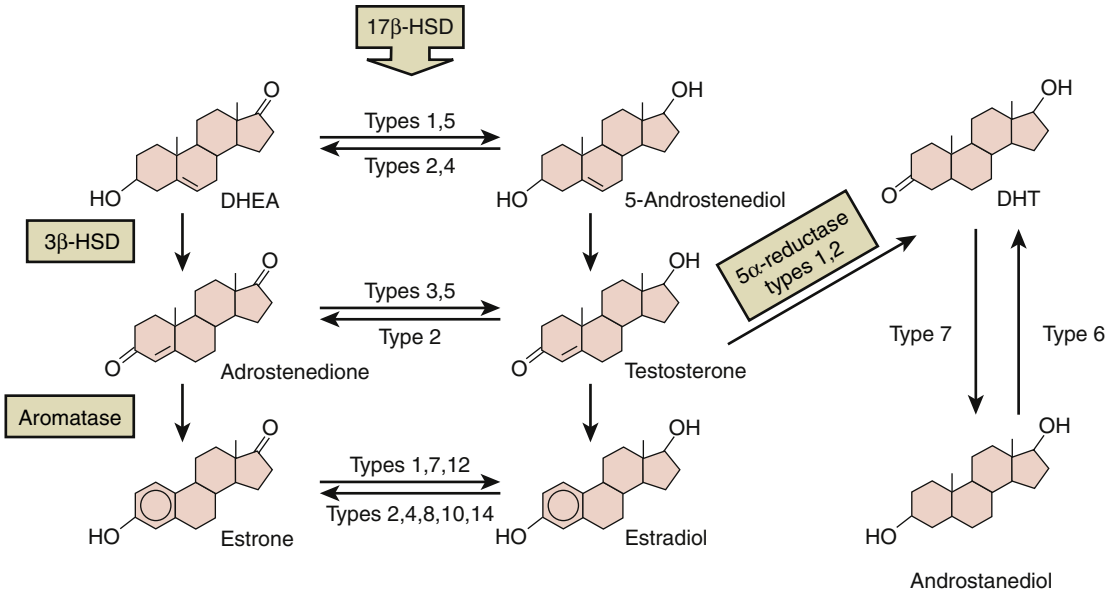


FIGURE 4.10 The family of 17*β*-HSDs and some of their roles in androgen and estrogen synthesis and metabolism. DHT, dihydrotestosterone. *(Modified from Luu-The V. Analysis and characteristics of multiple types of human 17β-hydroxysteroid dehydrogenase.* Steroid Biochem Mol Biol *76:143, 2001.)*

reduction of the 11-keto group, using NADPH as a cofactor *in vivo*. This enzyme is expressed in the liver, lung, adipose tissue, brain, vascular tissue, and gonads, where it regenerates cortisol from 11-ketosteroids. In the case of the placenta, the activity ensures transport of biologically active cortisol to the fetus in the first half of pregnancy.

Targeted deletion of the type 1 enzyme gene *(Hsd11b1)* in mice results in animals with lower blood glucose levels in response to overfeeding and stress, impaired activation of gluconeogenesis, and blunted sensitivity to natural glucocorticoids. This finding substantiates a role for HSD11B1 in the amplification of cortisol and corticosterone action. HSD11B1 also plays a key role in the pharmacology of glucocorticoids. Hepatic HSD11B1 converts cortisone and prednisone, inactive prohormones, into active cortisol and prednisolone.

As noted above, mutations in the *H6PDH* gene cause a syndrome characterized by high ratios of cortisone to cortisol, impaired cortisol negative feedback resulting in elevated ACTH secretion, and increased production of adrenal androgens leading to hyperandrogenism, sexual precocity and a polycystic ovary syndrome-like phenotype. Mice with targeted mutations in *H6pdh* share many of the phenotypes of mice with *Hsd11b1* mutations. Heterozygous mutations in the *HSD11B1* gene causing a mild form of cortisone reductase deficiency have recently been described.[61]

HSD11B2, also an endoplasmic reticulum enzyme, has a higher affinity for its substrate than HSD11B1 and catalyzes the oxidation of cortisol with NAD^+ as a cofactor. It shares only modest amino acid sequence identity (21%) with HSD11B1. HSD11B2 is highly expressed in the kidney, colon, salivary glands, and placenta, all tissues that respond to aldosterone, or in the case of the placenta, tissues that act to separate the maternal and fetal endocrine systems (which is important in the third trimester). By converting cortisol and corticosterone to 11-keto compounds, HSD11B2 protects the renal mineralocorticoid receptors,which cannot distinguish cortisol or corticosterone from aldosterone, from inappropriate activation by the glucocorticoids.

Mutations that inactivate HSD11B2 produce a syndrome of apparent mineralocorticoid excess in humans, which is mimicked in the mice deficient in the enzyme that display hypertension, hypokalemia, and renal structural abnormalities. Glycyrrhizic acid, a component of licorice, and its metabolite carbenoxolone, are competitive inhibitors of HSD11B2, but also cause reduced expression of the HSD11B2 mRNA when administered *in vivo*. As a result, a drug-induced syndrome of apparent mineralocorticoid excess is produced.[62,63]

17β-Hydroxysteroid Dehydrogenases: Multiple Enzymes with Specific Biosynthetic and Catabolic Roles

The adrenals, gonads, and placenta reduce 17-ketosteroids into 17β-hydroxysteroids (which have greater biological potency), whereas target tissues usually oxidize 17β-hydroxysteroids, inactivating them.[6,57,64-66] In humans, these metabolic processes are mediated by at least 7 of the 14 known mammalian 17β-HSDs, designated types 1 through 14, according to the chronological order in which they were identified (Fig. 4.10). They are all members of the short-chain dehydrogenase–reductase family, except the type 5 enzyme, which is an aldo-keto reductase. They have different cofactor and substrate specificities, including molecules that are not steroids, subcellular locations, and tissue-specific patterns of expression.

The structures of the genes encoding the 17β-HSDs differ, and their nucleotide sequence homology is low. They can be grouped into enzymes that catalyze NAD^+-dependent oxidation (types 2, 4, 6, 8, 9, 10, 11, and 14) and those that catalyze NADPH-dependent reduction (types 1, 3, 5, and 7). Because of the broad substrate specificities, the

primary roles of several of these enzymes are in basic metabolic pathways unrelated to steroid metabolism, and deficiencies of these enzymes cause metabolic disease.

The type 1 enzyme (HSD17B1) is referred to as the "estrogenic" 17β-HSD because it catalyzes the final step in estrogen biosynthesis by preferentially reducing the weak estrogen estrone to yield the potent estrogen 17β-estradiol. The enzyme is a cytosolic protein that uses either NADH or NADPH as a cofactor. It has 100-fold higher affinity for C18 steroids than for C19 steroids. It also converts 16α-hydroxyesterone into estriol and shows modest 20α-HSD activity.

The structural gene encoding HSD17B1 consists of six exons and is located on bands 17q11-12 in tandem with a highly homologous pseudogene. It is expressed in granulosa cells of the ovary and the placental syncytiotrophoblast. HSD17B1 is also expressed at higher levels in breast cancer cells relative to the HSD17B2, which converts estradiol into estrone. Amplification of *HSD17B1* in estrogen receptor-positive breast cancers is associated with lower survival compared to subjects without amplification. The crystal structure of HSD17B1 has been determined to a resolution of 2.2 Å with and without bound substrates, providing a molecular framework for the design of specific inhibitors which might be employed in chemotherapy of estrogen-responsive cancers.[66,67]

HSD17B2 is an endoplasmic reticulum enzyme that inactivates hormones; it preferentially oxidizes testosterone into androstenedione, and it oxidizes estradiol into estrone using NAD+ as its cofactor. HSD17B2 also has the ability to convert 20α-hydroxyprogesterone into progesterone.

The gene encoding HSD17B2, located on band 16q24, contains five exons. It is expressed in liver, secretory endometrium, and the fetal capillary endothelial cells of the placenta, as well as the endothelial cells of the larger vessels. This expression pattern is consistent with its role of inactivating testosterone and estradiol. HSD17B2 in the fetal capillary endothelium protects the fetal compartment from estradiol formed in the syncytiotrophoblast, and from any testosterone escaping aromatization. Its expression in the secretory endometrium permits the conversion of estradiol to estrone, whereas 20α-hydroxyprogesterone is converted back into progesterone, resulting in progestational dominance. In normal breast tissue, HSD17B2 expression predominates over HSD17B1 expression.

HSD17B3 is referred to as the "androgenic" 17β-HSD because it catalyzes the final step in androgen biosynthesis in the Leydig cells, reducing androstenedione to testosterone using NADPH as cofactor. It also can reduce estrone to estradiol. HSD17B3, an endoplasmic reticulum enzyme, is not expressed in the ovary, requiring androgen-producing cells of the ovary to employ another enzyme, probably HSD17B5, to synthesize testosterone. The *HSD17B3* gene, comprised of 11 exons, is located on band 9q22.

In the absence of HSD17B3 activity, the testes are unable to convert androstenedione into testosterone, resulting in male pseudohermaphroditism (46, XY DSD) associated with ten to fifteenfold elevations in the ratio of blood androstenedione to testosterone.[6,68] Females with mutations in the *HSD17B3* gene are asymptomatic, and produce normal amounts of estrogens and androgens. Molecular analysis of the *HSD17B3* gene in affected individuals revealed mutations that affect splicing, cause amino acid replacements in exons 9 and 10, and a small deletion leading to a frameshift. Many of the missense mutations result in proteins that are devoid of catalytic activity when expressed in eukaryotic cells.

HSD17B5, located on chromosome 10p15-14, is a member of the aldo-keto reductase family (AKR1C3). It has 3α-HSD and 20α-HSD activity in addition to 17β-HSD activity that produces testosterone from androstenedione. It is expressed in the adrenal cortex and may serve as the "androgenic" 17β-HSD of ovarian theca cells.[69,70] HSD17B5 is also expressed in prostate, mammary gland, and Leydig cells.

HSD17B6 (also known as 11-cis retinol dehydrogenase, RODH), encoded by a gene on chromosome 12q13.3 has 3α-HSD activity and catalyzes the conversion of androstanediol to dihydrotestosterone in the prostate. It is also a 3α-3β epimerase. Polymorphisms in the *HSD17B6* gene have been associated with polycystic ovary syndrome phenotypes in some, but not all, studies.

Human HSD17B7 is involved in cholesterol synthesis but also thought to produce active estrogens and inactivates androgens. It transforms estrone into estradiol, and has 3-keto reductase activity for dihydrotestosterone. The gene located on band 10p11.2 is expressed in the ovary, breast, placenta, testes, prostate, and liver cells.

3α- and 20α-Hydroxysteroid Dehydrogenase Activities

A number of HSDs catalyze reactions at both the 3 and 20 positions of steroid hormones (e.g., AKR1C1).[6,30] The enzymes that are 3α-reductases are encoded by tandemly duplicated genes on chromosome 10p14-p15, and are all members of the aldo-ketoreductase family. They are expressed in liver, prostate, breast, and uterus, but some are also expressed in the testis and adrenals. Recessive mutations in one of these genes, AKR1C2, was discovered in four 46,XY individuals with disordered sexual development, implicating this enzyme in a pathway of bioactive androgen biosynthesis through conversion of androsterone to androstanediol, followed by HSD17B6-mediated conversion of androstanediol to dihydrotestosterone.[71]

The enzymes with 20α-HSD activity that are members of the aldo-keto reductase family reduce progesterone to yield the inactive steroid, 20α-hydroxyprogesterone. They are cytosolic proteins with a molecular weight of approximately 34 kDa, exemplified by genes expressed in human keratinocytes and cells of the liver, prostate, testis, adrenal gland, brain, uterus, and mammary gland. These enzymes prefer NADPH to NADH as a cofactor.[6,30]

Enzymes with 3α-hydroxysteroid oxidative activity are members of the short-chain dehydrogenase/reductase family. As noted previously, enzymes of the short-chain dehydrogenase–reductase family have 20α-HSD activity, including HSD17B1 and HSD17B2. HSD17B2 preferentially oxidizes 20α-hydroxyprogesterone into progesterone.

$\Delta^{4\text{-}5}$ Reductases

The $\Delta^{4\text{-}5}$ reductases are membrane-associated enzymes that reduce the Δ5-4 double bond in steroid hormones by catalyzing direct hydride transfer from NADPH to the carbon 5 position of the steroid substrate.[6,72,73] They produce either 5α or 5β-dihydrosteroids.

5α-Reductases

Two different human 5α-reductases sharing 50% similarity in amino acid sequence and a molecular weight of approximately 29 kDa have been identified.[67,68] The genes for type 1 *(SRD5A1)* and 2 *(SRD5A2)* 5α-reductases each have five exons. The substrate-binding domain of the 5α-reductases is encoded by exon 1, and the cofactor-binding domain is encoded by exons 4 and 5. The type 2 5α-reductase gene, *SRD5A2*, is located on chromosome 2p23, whereas the type 1 5α-reductase gene, *SRA5A1*, is located on chromosome 5p15, with a pseudogene on Xq24-qter. A third enzyme containg a 5α-reductase domain, SRD5A3, has been identified but its role in steroid metabolism has not been established.

SRD5A2 is predominantly expressed in male genital structures, including genital skin and prostate, where it reduces testosterone to yield the more potent androgen, 5α-dihydrotestosterone. SRD5A1, which catalyzes a similar reaction on both C21 and C19 steroid hormones, is expressed in the liver, kidneys, skin, and brain. Although this enzyme can also make 5α-dihydrotestosterone, its tissue distribution suggests that its predominant function is to inactivate steroid hormones.

SRD5A2 has an acidic pH optimum and a K_m for testosterone in the nanomolar range, whereas SRD5A1 has a broad alkaline pH optimum and a lower affinity for its substrates in the micromolar range. SRD5A2 can also be distinguished from the type 1 enzyme by its selective inhibition by finasteride, with an inhibition constant (K_i) of 3 nM.

Inactivating mutations in *SRD5A2* cause male pseudohermaphrodism (46, XY DSD). The enzyme defect is characterized by abnormal testosterone-to-5α-dihydrotestosterone ratios. Affected males have varying degrees of abnormal development of the external genitalia, ranging from mild hypospadias to severe defects in which the external genitalia are essentially female. Wolffian ducts develop normally in response to adequate levels of testosterone.

Females carrying mutations in the *SRD5A2* gene have a normal phenotype and normal menstrual cycles. They have a low incidence of hirsutism and acne, and like males with the disease, have low ratios of 5α- to 5β-dihydrosteroid metabolites in the urine. The infrequency of acne in both affected males and females, the rarity of hirsutism in affected females, the absence of male pattern baldness, and the finding of an atrophic prostate in affected males indicates that SRD5A2 plays an important role in androgen metabolism in skin and in the androgen-dependent growth of the prostate.

Among the mutations reported are deletions that inactivate SRD5A2, and missense mutations that impair enzyme activity by affecting substrate or cofactor binding. A *SRD5A2* variant (Ala49Thr) that has increased catalytic activity has been associated with increased risk of prostate cancer.

Mutations have not been described in the human *SRD5A1* gene. However, targeted deletion of the murine counterpart results in a female phenotype of reduced fecundity and a parturition defect caused by failed cervical ripening. This defect can be reversed by administration of 5α-androstanediol.[74,75]

The only known human 5β-reductase (SRD5B1 or AKR1D1) is a member of the aldo-keto reductase superfamily.[76] This enzyme is involved in steroid hormone inactivation in the liver. Its reaction mechanism is similar to that described for 5α-reductase, except that an A/B *cis*-fused ring product is formed. The enzyme efficiently catalyzes the NADPH-dependent reduction of the Δ5-4 double bond in C27, C21, and C19 steroids to yield the 5β-dihydrosteroids, with a distinct preference for C27 steroids. In keeping with this observation, mutations in the *SRD5B1 (AKR1D1)* gene on chromosome7q32-q33 that encodes this enzyme result in abnormal bile acid synthesis, along with a marked reduction in the primary bile acids and 5β-reduced steroid metabolites.

Sulfotransferases

A family of enzymes introduce the sulfonate (SO_3^-) anion from an activated donor, *3′-phosphoadenosine-5′-phosphosulfate* (PAPS), to a steroid hydroxyl acceptor, inactivating the hormone.[6,77,78] The major enzymes carrying out this reaction include estrogen sulfotransferase (SULT1E1), encoded by a gene on chromosome 4q13.1, an enzyme that sulfonates the 3-hydroxyl function of phenolic steroids, and the hydroxysteroid sulfotransferases, encoded by the closely linked *SULT2A1* and *SULT2B1* genes on chromosome 19q13.4.

The SULT2A1 enzyme, also known as *dehydroepiandrosterone sulfotransferase*, has a broad substrate range including the 3α-, 3β-, and 17β-hydroxy functions of steroid hormones. SULT2A1 enzyme is expressed at high levels in the fetal zone of the adrenal cortex, as well as in the zona reticularis after adrenarche, and in the liver, gut, and testes. This enzyme is developmentally regulated in the adrenal cortex, with expression increasing between ages 5 and 13 years in association with adrenarche. The GATA-binding factor 6 (GATA6) transcription factor, which is known to increase transcription of other genes involved in androgen biosynthesis, also activates expression of the *SULT2A1* gene.

The *SULT2B1* gene gives rise to two protein isoforms through alternative splicing. The SULT2B1a isoform sulfonates pregnenolone, whereas the SULT2B1b isoform preferentially sulfonates cholesterol. They both sulfonate dihydrotestosterone. Unlike the *SULT2A1* gene that is expressed in a limited number of tissues, SULTB1 isoforms are present in a variety of hormone-producing and hormone-responsive tissues, including the placenta, ovary, uterus, and prostate.[79]

SULT1E1 is expressed in many tissues, including the adrenal gland, liver, kidneys, muscle, fat, and uterus. In the endometrium, progesterone increases its activity, contributing to the inactivation of estradiol in the secretory phase. The importance of estrogen sulfotransferase in modulating local levels of bioactive estrogens has been shown in mice with targeted deletions of the gene.[80] Male mice with estrogen sulfotransferase deficiency have Leydig cell hyperplasia and become infertile with age, due to elevated testicular levels of bioactive estrogen. Adipose tissue mass also increases. Hydroxylated metabolites of polychlorinated biphenyls are potent inhibitors of estrogen sulfotransferase, with IC50 values in the picomolar range.[81] The blockade of estradiol inactivation by these compounds may account for the reported "estrogenic" activity of polychlorinated biphenyls.

Mutations in the gene that encodes one of the enzymes that synthesizes the sulfate donor, PAPS, (PAPS synthase

type 2 [PAPSS2]), results in increased free DHEA levels, with the subsequent conversion of DHEA into biologically active androgens.[6]

Steroid Sulfatase

The sulfonate function on steroids is cleaved by steroid sulfatase, an enzyme encoded by the *STS* gene on chromosome Xp22.3.[6,79,82-84] This enzyme plays a key role in controlling the generation of biologically active steroids from the inactive sulfated molecules (e.g., estrone sulfate and dehydroepiandrosterone sulfate). The sulfated steroid substrates, which are hydrophilic and not membrane-permeable, are thought to be taken up by cells via specific transport proteins, including organic anion transport polypeptide B (OATP-B).

The syncytiotrophoblast is enriched in steroid sulfatase, which plays a key role in placental estrogen synthesis by liberating sulfonated androgen precursors produced in the fetal compartment prior to aromatization. The enzyme is also expressed in skin, where it metabolizes cholesterol sulfate and sulfated estrogens.

Sulfatase deficiency is associated with marked impairment of placental estrogen synthesis during pregnancy and ichthyosis developing after birth.[83,84] It occurs mostly in males because of the X chromosome location of the sulfatase gene, at a frequency of 1:2000 to 1:6000 live born males. The majority of subjects with steroid sulfatase deficiency have a deletion of the entire gene that results from recombination of repetitive elements that flank the locus. The large deletions of the *STS* gene occur in association with mutations in the adjacent Kallmann's syndrome gene *(KAL1)*. Partial deletions in the *STS* gene causing enzyme deficiency have also been described.

Characteristically, in pregnancies hosting an affected fetus, maternal plasma estriol and urinary estriol excretion are quite low, approximately 5% of the levels found in normal pregnancies. Excretion of estrone and estradiol are also reduced, at approximately 15% of normal. Maternal serum 16α-hydr oxydehydroepiandrosterone levels are elevated, and intravenous administration of dehydroepiandrosterone sulfate to the mother does not lead to an increase in estrogen excretion, whereas administration of dehydroepiandrosterone does.

Steroid sulfatase is expressed in estrogen target tissues, including endometrium, bone, and breast. Increased sulfatase expression may contribute to greater bioavailability of estradiol in certain tumors, including breast and endometrial cancers. Inhibitors of sulfatase have been developed and tested in clinical trials (Irosustat).[85]

UDP-Glucuronosyl Transferases

Glucuronidation, catalyzed by a family of uridine 5'-diphospho UDP-glucuronosyl transferases, is part of the metabolic clearance mechanism for steroid hormones by the liver and extrahepatic tissues.[86-89] There are 18 known UDP-glucuronosyl transferases that have been divided into three subfamilies: UGT1A , UGT2A, and UGT2B. UGT1A enzymes are encoded by a single gene that gives rise to alternatively spliced products capable of acting on estrogens. The UGT2 enzymes are products of separate genes *UGT2A* and *UGT2B*. *UGT2A* is expressed in olfactory epithelium, and *UGT2B* is expressed in the liver, kidney, breast, lung, and prostate. At least seven members of the UGT2B family have been identified with different steroid substrate specificities. Three UGT2B enzymes, UGT2B7, UGT2B15, and UGT2B17, appear to be responsible for glucuronidation of 5α-dihydrotestosterone, androsterone, and androstane-3α-17β-diol, Of these, UGT2B15 and UGT2B17 are expressed in the prostate where they are proposed to modulate steroid action.[90] Polymorphisms in genes encoding UDP-glucuronosyl transferases have been associated with estrogen levels, suggesting a role for these phase II biotransformation enzymes in regulating the bioactive steroid economy.

Other Steroid Hormone Metabolical Pathways

There are several metabolical fates of steroid hormones that have a significant role in the synthesis of bioactive hormones. A pathway for androgen production, referred to as a "backdoor pathway" is one example.[71] Among the other fates of steroid hormones are esterification to long-chain fatty acids, formation of catechol estrogens, and the 7α-hydroxylation of androgens. Equine steroidogenic tissues also have a novel pathway for biosynthesis of estrogens, resulting in a phenolic A ring and an unsaturated B ring.

An Alternative Pathway for Androgen Synthesis

Fetal androgen synthesis in marsupials involves a novel pathway that leads from 17α-hydroxyprogesterone to 5α-dihydrotestosterone without going through androstenedione or testosterone as intermediates (Fig. 4.11). This pathway, recently identified in humans, is initiated when either progesterone or 17α-hydroxyprogesterone is 5α-reduced. The resulting 5α-C_{21} steroids are reduced by 3α-HSDs (AKR1C2/4) yielding substrates for P450c17. Androsterone produced by P450c17 can ultimately be converted to 5α-dihydrotestosterone by HSD17B6.[71] This pathway also contributes to androgen excess in the context of 21-hydroxylase deficiency.

Catechol Estrogens

Catechol estrogens are generated by the actions of genes encoded by *CYP1A1* and *CYP1A2* (which catalyze 2-hydroxylation of estrogens), and *CYP1B1*, which is an estrogen 4-hydroxylase.[91-93] Although the catechol estrogens are short-lived *in vivo* and are postulated to have physiological functions as locally generated signaling molecules, they also yield potent genotoxic molecules implicated in carcinogenesis. The 4-hydroxyestrogens can be oxidized to quinone intermediates that react with purine bases of DNA, resulting in depurinating adducts that generate highly mutagenic apurinic sites (Fig. 4.12). Quinones derived from the 2-hydroxyestrogens produce stable DNA adducts and are presumed to be less genotoxic. Metabolism of catechol estrogens may also generate oxygen-free radicals.

Catechol estrogens are methylated by catechol-O-methyltransferase, resulting in a catecholamine-like substance. The methylated catechol estrogens have anti-angiogenic and antitumor activity through inhibition of hypoxia-inducible factor-1α (HIF-1α), a pro-angiogenic transcription factor. There is evidence that altered production of 2-methoxyestradiol may contribute to the pathogenesis of preeclampsia through its modulation of anti-angiogenic factor production by the placenta.[94,95]

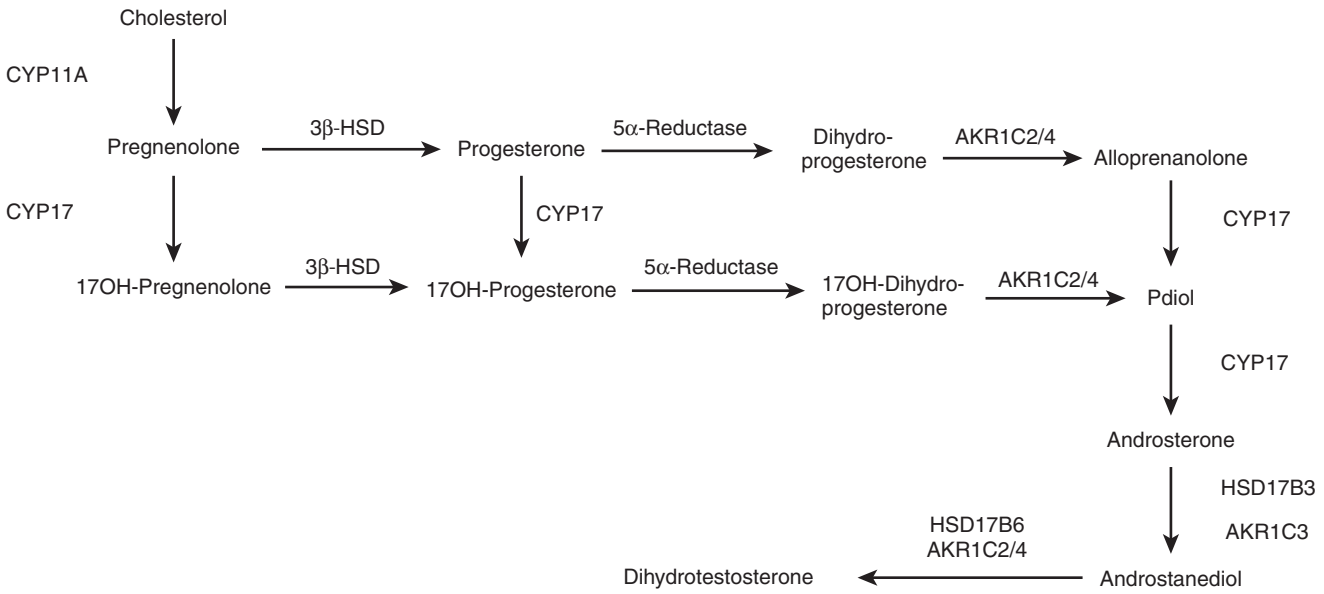


FIGURE 4.11 Alternative pathway or "backdoor pathway" for fetal testicular androgen synthesis. *(Modified from R.J. Auchus and W.L. Miller (2012) Defects in androgen biosynthesis causing 46,XY disorders of sexual development.* Seminars in Reproductive Medicine *30: 417–426.)*

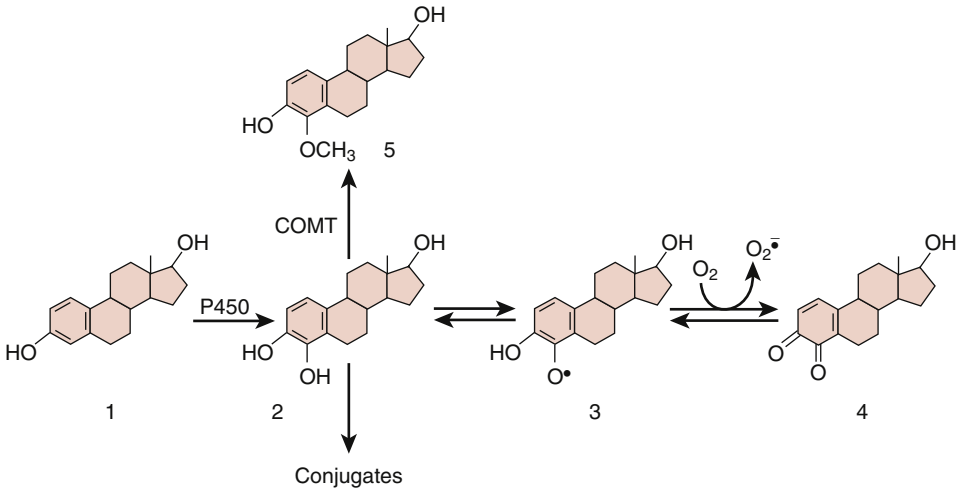


FIGURE 4.12 Metabolism of estradiol (1) by P450 enzymes, including P4501B1 to 4-hydroxyestradiol (2). Metabolic cycling between 4-hydroxyestradiol and estradiol 3,4-quinone (4) can be catalyzed by P4501A1 for the oxidation step and cytochrome P450 reductase for the reduction step. The semiquinone intermediate (3) is a free radical that can react with molecular oxygen to form superoxide radical and quinone. 4-Hydroxyestradiol can be converted to 4-methoxyestradiol (5) by catechol-O-methyltransferase (COMT). *(From Liehr JG. Catecholestrogens in the induction of tumors in the kidney of the Syrian hamster. In Goldstein DS, Eisenhofer G, McCarty R [eds].* Advances in pharmacology: catecholamines. bridging basic science with clinical medicine, *vol 42. San Diego, Academic Press, 1998, pp 824–828.)*

Steroid Fatty Acid Esters

Steroids esterified to long-chain fatty acids are present in blood bound to lipoproteins, or are found in tissue, particularly steroidogenic glands and fat.[96,97] These hydrophobic molecules may serve as a depot form of steroid, but they also have unique biochemical attributes. Estradiol 17-esters are produced in blood by the action of lecithin-cholesterol acyl transferase and in tissues by SOATs. The fatty acid esters of estradiol have pronounced antioxidant activity. Fatty acid esters of other steroids, including pregnenolone, testosterone, dehydroepiandrosterone, and glucocorticoids, have also been described.

Steroid 7α-Hydroxylation

Substituents on carbon 7 of the steroid nucleus can have a significant effect on activity. A cytochrome P450 encoded by the *CYP7B* gene catalyzes 7α-hydroxylation of steroid hormones and oxysterols. The 7α-hydroxylation of dehydroepiandrosterone produces a molecule with enhanced immunostimulatory activity, a property demonstrable in animal bioassays.[98]

B-Ring Unsaturated Steroids

The pregnant mare produces estradiol and estrone, but also B-ring unsaturated estrogens (equilin [with an 8,9 olefinic

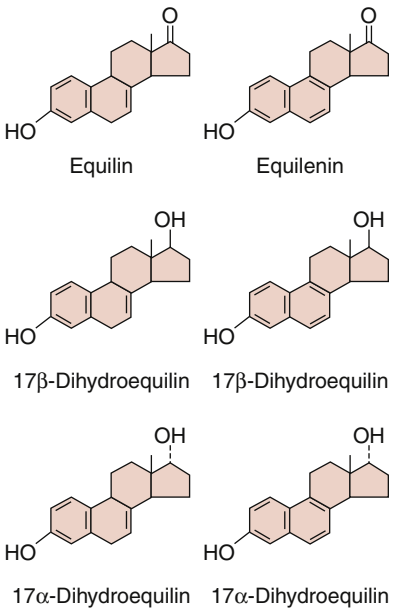


FIGURE 4.13 Structures of equine estrogens.

bond], equilenin [with a phenolic B ring], 17α-dihydroequilin, 17α-dihydroequilenin, 17β-dihydroequilin, and 17β-dihydroiequilenin) (Fig. 4.13).[99]

These B-ring unsaturated compounds are potent estrogens *in vivo*. Although 7-dehydrocholesterol can be converted into B-ring unsaturated estrogens (as occurs in Smith-Lemli-Opitz syndrome), the biosynthesis of these compounds in the pregnant mare occurs by a pathway not requiring the synthesis of squalene or cholesterol, and thus does not involve 7-dehydrocholesterol. Evidently, they are derived from a C25 sesterterpene pathway that coexists with the normal biosynthetic route of "standard" estrogens from a cholesterol precursor.

Vitamin D Synthesis and Metabolism

Mounting epidemiological evidence indicates that in addition to calcium and bone metabolism the secosteroid, vitamin D, plays important roles in immune function and reproduction, and that vitamin D deficiency is associated with reproductive disorders. The impact of vitamin D on reproduction is demonstrable in genetically engineered mice. Vitamin D receptor (Vdr) and 1α-hydroxylase *(Cyp27b1)* knockout female mice display reduced fecundity, impaired folliculogenesis, and anovulation. These reproductive abnormalities are evidently due to dysfunctional calcium homeostasis since high calcium diets partly restore fertility and increase the rate of conception in *Vdr* null mice.

Vitamin D_3 is produced in the innermost layers of the skin (stratum basale and stratum spinosum) through the action of ultraviolet light on 7-dehydrocholesterol (Fig. 4.14).[100,101] Vitamin D_3 travels to the liver where it is converted into 25-hydroxyvitamin D_3 by an endoplasmic reticulum P450, CYP2R1, a highly conserved and substrate-specific 25-hydroxylase. Mutations in the *CYP2R1* gene lead to deficient 25-hydroxylation of D_3. 25-Hydroxyvitamin D_3 in plasma is mainly bound to vitamin D binding protein (also called group specific protein). Megalin and cubilin, endocytic receptors, participate in the uptake of vitamin D binding protein and its 25-hydroxyvitamin D_3 cargo for further metabolism. The 25-hydroxyvitamin D_3 is converted into the biologically active 1,25-dihydroxyvitamin D_3 by the action of a mitochondrial enzyme, CYP27B1, in the kidney, but also in monocytes, maternal decidua and placental trophoblast cells. Inactivating mutations of the *CYP27B1* gene are associated with clinical and radiographic features of rickets, hypocalcemia, and low serum concentrations of 1,25-dihydroxyvitamin D_3. The *CYP27B1* gene and the formation 1,25-dihydroxyvitamin D_3 by the kidney are regulated by negative feedback by 1,25-dihydroxyvitamin D_3, stimulated by parathyroid hormone (PTH), whose levels reflect calcium status, and inhibited by fibroblast growth factor 23 (FGF23), which reflects phosphate homeostasis.

The 24R-hydroxylase, CYP24A1, acts on 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3. This enzyme is thought to play the key role in inactivation of 1,25-dihydroxyvitamin D_3, although some have argued that the 24-hydroxylated vitamin D molecules may have biological roles.

Regulation of Expression of the Steroidogenic Machinery

The regulation of expression of genes encoding proteins involved in steroidogenesis in the ovary, testes, and adrenal cortex shares a number of similarities with respect to the involvement of *cis* elements and transcription factors. SF-1, a nuclear receptor, also known as Ad4BP and by the nuclear receptor family designation NR5A1, is essential for development of steroidogenic glands. Most of the genes encoding key proteins involved in steroidogenesis (e.g., *SRB1*, *STARD1*, *CYP11A1*, *CYP11B2*, *CYP17A1*, *CYP19A1*, *CYP21A2*) contain one or more NR5A1 response elements in their proximal promoters. These elements are important for basal as well as stimulated expression of these genes, generally by a cAMP-mediated signal transduction pathway.

The importance of cAMP signaling in steroidogenic tissues is reflected in increased steroid production when specific phosphodiesterases are blocked, which elevates intracellular cAMP levels, or when mutations occur in the gene encoding the regulatory subunit of protein kinase A *(PRKAR1A)*, resulting in unrestrained protein kinase A activity.[6,102] There appear to be several mechanisms by which NR5A1 action is modified by cAMP action, including phosphorylation of the protein by kinases, providing a link between this transcription factor and molecules that transduce signals from plasma membrane receptors.[103]

The importance of NR5A1 to the regulation of steroidogenic tissues was documented by gene targeting. Mice deficient in NR5A1 lacked adrenal glands and gonads, and males were consequently sex-reversed. Haploinsufficiency of *Nr5α1* in the mouse resulted in an impaired adrenal steroidogenic response to stress, although basal steroidogenesis was not affected due to compensatory hypertrophy.[103] *NR5A1* haploinsufficiency has been reported in humans with primary adrenal failure and XY sex reversal.[104] It is now

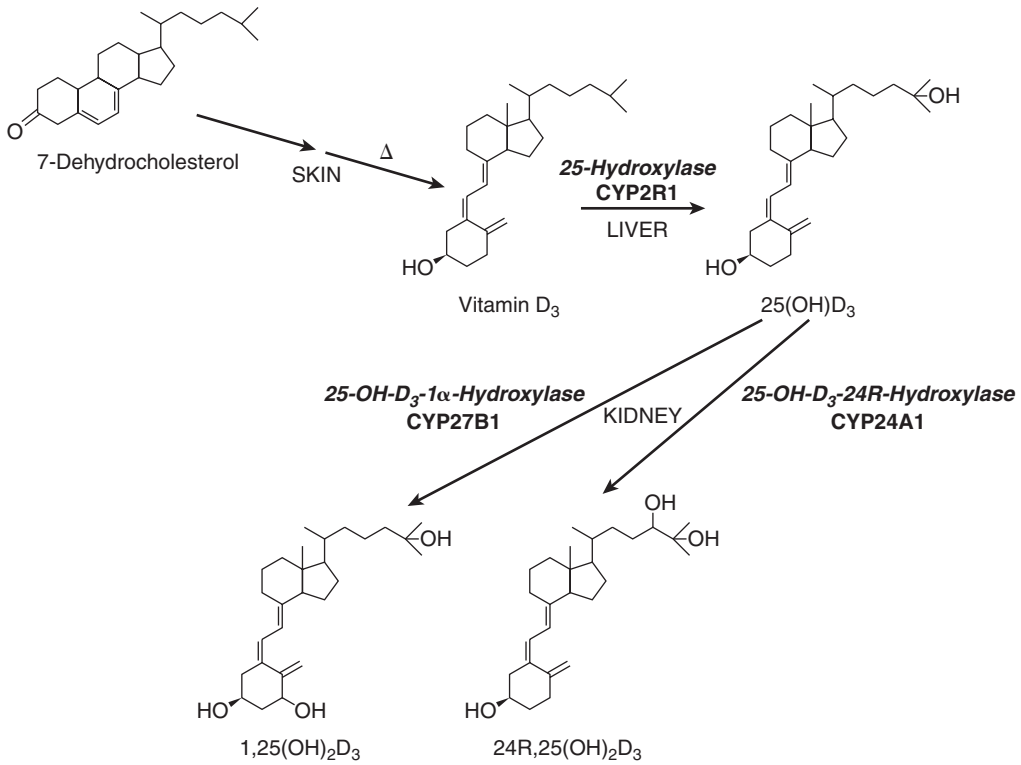


FIGURE 4.14 Metabolism of vitamin D3. 7-Dehydroxholesterol is converted to vitamin D3 in the skin triggered by ultraviolet light (Δ). Vitamin D3 is transported to the liver, mostly bound to protein, where it undergoes hydroxylation at the C25-position catalyzed by CYP2R1. 25-Hycroxyvitamin D3, the major form circulating vitamin D, undergoes hydroxylation in the kidney at either the C1 position, catalyzed by CYP27B1, or the C24 position, catalyzed by CYP24A1, resulting in 1a,25-dihydroxyvitamin D3, or 24R,25-dihydroxyvitamin D3. *(Modified from Henry, HL. Regulation of vitamin D metabolism.* Best Pract Res Clin Endocrinol Metab. *2011;25(4):531–541.)*

evident that variation in the NR5A1 gene is associated with a range of phenotypes such as 46,XY disorders of sex development, hypospadias, anorchia, male factor infertility, and primary ovarian insufficiency. Among the *NR5A1* mutations reported are missense mutations within the DNA-binding region, a nonsense mutation, a and a frameshift mutation predicted to disrupt RNA stability or protein function. Functional studies of the missense mutants (Cys33Ser, Arg84His) and of one nonsense mutant (Tyr138Stop) showed impaired activation of NR5A1–responsive target genes.[104]

Although NR5A1 is clearly an important regulator of embryological development of steroidogenic glands and control of transcription of proteins comprising the steroidogenic machinery, other transcription factors participate in the latter process. A related transcription factor, liver receptor homologue-1 (SF-2 or NR5A2) recognizes the same canonical DNA motif to which NR5A1 binds and may share functions with NR5A1 in certain tissues, including the adrenal cortex, testis, and ovary.[105] Both NR5A1 and NR5A2 have been crystallized and found to contain phospholipid-binding pockets, with phosphatidyl inositols being the presumed ligands. These observations suggest that phospholipids may be regulatory molecules controlling expression of genes involved in steroidogenesis.[106]

The tissue-specific regulation of genes expressed in multiple steroidogenic glands (e.g., *CYP17A1*) requires the action of other transcription factors working either independently or in concert with NR5A1 in a combinatorial fashion. In addition, the activity of NR5A1 is regulated by transcription factors that either bind to NR5A1 response elements and prevent activation of transcription (chicken ovalbumin upstream promoter-transcription factor [COUP-TF]) or bind to NR5A1 and block its ability to transactivate promoters (DAX-1, also known as NR0B1). Interestingly, expression of the latter gene is up-regulated by NR5A1, so there is a complex control mechanism in place for modulating these antagonistic molecules.

Other transcription factors that are known to be important for the expression of genes involved in steroidogenesis include GATA4 and GATA6, members of the GATA family of transcription factors originally identified as being central to hematopoiesis and endoderm development, and LXRalpha.[27,107,108]

It is notable that the human placenta is an outlier in many respects in terms of the regulation of steroidogenesis.[109] First, the placenta does not express certain genes that are pivotal to gonadal and adrenal steroid hormone synthesis, including *NR5A1*, *STARD1*, *HSD3B2*, and *CYP17A1*. Moreover, HSD3B1 replaces HSD3B2 in the placenta, and a START domain protein, STARD3 (which is not subject to acute regulation), may subserve STARD1's role in cholesterol movement to the placental side-chain cleavage system.

Unlike the gonads and adrenal cortex, the placenta's capacity to produce progestins is believed to be largely determined by levels of adrenodoxin reductase (which governs the availability of reducing equivalents) and P450scc. In addition, *CYP19A1* transcription in the placenta is driven by a different promoter than that used by the gonads. Thus, placental

steroidogenesis is controlled in a distinctly different way than gonadal and adrenal steroid production. The mechanism is more tonic, with steroidogenic capacity determined primarily by differentiation of trophoblast cells and growth of the placenta as opposed to tight regulation by trophic hormones.

Examples of Extraglandular Steroidogenesis

Although steroid hormone synthesis traditionally has been studied in the classic steroidogenic glands (ovaries, testes, adrenal cortex, and placenta), it is now evident that production of bioactive steroids, albeit at much lower levels, occurs at extraglandular sites, such as the brain, vascular tree, and adipose tissue.[110,111] Synthesis also occurs in pathological conditions affecting the endometrium (endometriosis and endometrial cancers), breast (breast cancer) and prostate (prostate cancer).[82,90,110,112]

Synthesis of Neurosteroids

The notion that steroid hormones could be synthesized in the central nervous system evolved from the discovery of appreciable levels of pregnenolone and dehydroepiandrosterone and their fatty acid esters in the brains of animals, even after gonadectomy or adrenalectomy.[113,114] It was subsequently shown that enzymes required for steroid hormone synthesis were expressed in the brain, spinal cord, and peripheral nervous system at the mRNA and protein levels. Included in this category are STARD1, P450scc, P450c17, P450arom, P450c11β, and P450c11AS, HSD17B1, and HSD17B2, as well as 3β-HSD, 5α-reductase, 3α-HSD, 11β-HSD activities.

The enzymes and their associated activities are distributed in different brain regions and cell types, including glia (astrocytes and oligodendrocytes) and neurons. They probably can act on circulating "prohormones" as well as participate in the de novo synthesis of steroids. The expression of the steroidogenic enzymes in the brain is developmentally regulated, although little is known about the mechanisms that control expression.

The known neurosteroids may act via the classic nuclear hormone receptors, but there is also good evidence that nonclassic signaling pathways are involved, including actions on gamma-aminobutyric acid $(GABA)_A$, N-methyl-D-aspartic acid (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), glycine, serotonin, Sigma type 1, nicotinic acetylcholine, and oxytocin receptors. The actions of neurosteroids on these receptors have been implicated in stress responses, anxiolysis, seizure disorders, memory, unipolar and postpartum depression, and protection against neuronal injury. The evidence supporting these notions has mostly been derived from *in vitro* studies and animal studies. However, pharmacological evidence from human studies based on synthetic neurosteroids (e.g., the short-acting anesthetic alphaxolone) substantiates the concepts derived from animal experimentation.

Steroid Hormone Metabolism in Skin

Skin is an important site of production of vitamin D precursors.[100,101] It also makes a contribution to testosterone production in women by metabolizing prohormones, such as dehydroepiandrosterone sulfate and androstenedione into testosterone.[115] The *HSD3B1*, *17BHSD3*, and *SRD5A1* and *SRD5A2* genes are expressed in skin, allowing for local formation of androgens that can activate the androgen receptors present in the stroma, sebocytes, and dermal papillae. Recent studies suggest that expression of STARD1 and aromatase in skin is correlated with skin androgen and estrogen levels.

Secretion, Production, and Metabolic Clearance Rates of Steroid Hormones

The concentration of a steroid in the circulation is determined by the rate at which it is secreted from glands, the rate of metabolism of precursor or prehormones into the steroid, and the rate at which it is extracted by tissues and metabolized. Steroid metabolism is influenced by sex, age, weight, and thyroid function. The *secretion rate* of a steroid refers to the total secretion of the compound from a gland per unit time.

Secretion rates have been assessed by sampling the venous effluent from a gland over time and subtracting out the arterial or peripheral venous hormone concentration. Although seemingly simple in concept, this procedure is quite challenging in practice. Much of the difficulty originates from the potential of the catheterization process to disturb gland function (e.g., endocrine changes resulting from the stress of the procedure) and the possibility of dilution or contamination from blood draining other glands. For example, the role of the postmenopausal ovary in androgen production has been challenged because of potential contamination of adrenal venous blood in ovarian venous samples.[116]

The *metabolic clearance rate* of a steroid is defined as the volume of blood that has been completely cleared of the hormone per unit time. The *whole-body metabolic clearance rate* is usually measured, reflecting the sum of clearance rates for each tissue or organ. Experimentally, this measurement is accomplished by infusion of an isotopically labeled steroid at a constant rate.[117] At equilibrium, the concentration of the infused steroid in peripheral venous blood is constant, and the rate of clearance from the blood equals the rate of entry. The metabolical clearance rate is calculated by dividing the infusion rate by the concentration of the steroid isotope in peripheral blood, giving the metabolical clearance rate in milliliters per day or liters per day.

Most of the circulating steroids are removed from blood by the liver. Hepatic blood flow in humans is approximately 1500 L/day, so metabolical clearance rates exceeding this level generally reflect extraction of steroids by other organs in addition to the liver. The lung, with its high rate of blood flow, is another potentially important site of C21 and C19 steroid metabolism.[118,119] In addition to the amount of sex hormone binding globulin (SHBG), and therefore the amount of bound steroid, obesity, irrespective of SHBG levels, increases the metabolical clearance rate. The uptake of steroids into the liver as well as other organs is highly influenced by their affinity for plasma steroid–binding proteins and albumin. Binding of steroid hormones SHBG and corticosteroid-binding globulin (CBG) reduces peripheral metabolism.[120,121] The binding of free steroids to albumin

Table 4.2 Blood Production Rates, Secretion Rates, Metabolical Clearance Rates, and Normal Serum Concentration of Sex Steroid Hormones

Steroid Hormone	Reproductive Phase	MCR (L/Day)	PR (mg/Day)	SR (mg/Day)	Reference Values
Men				*Testes*	
Androstenedione		2200	2.8	1.6	2.8-7.3 nmol/L
Testosterone		950	6.5	6.2	6.9-34.7 nmol/L
Estrone		2050	0.15	0.11	37-250 nmol/L
Estradiol		1600	0.06	0.05	<37-210 pmol/L
Estrone sulfate		167	0.08	Insignificant	600-2500 pmo/L
Women				*Ovary*	
Androstenedione		2000	3.2	2.8	3.1-12.2 nmol/L
Testosterone		500	0.19	0.06	0.7-2.8 nmol/L
Estrone	Follicular	2200	0.11	0.08	110-400 pmol/L
	Luteal	2200	0.26	0.15	310-660 pmol/L
	Postmenopausal	1610	0.04	Insignificant	22-230 pmol/L
Estradiol	Follicular	1200	0.09	0.08	<37-360 pmol/L
	Luteal	1200	0.25	0.24	699-1250 pmol/L
	Postmenopausal	910	0.006	Insignificant	<37-140 pmol/L
Estrone sulfate	Follicular	146	0.1	Insignificant	700-3600 pmol/L
	Luteal	146	0.18	Insignificant	1100-7300 pmol/L
Progesterone	Follicular	2100	2	1.7	0.3-3 nmol/L
	Luteal	2100	25	24	19-45 nmol/L

MCR, metabolic clearance rate; PR, production rate; SR, secretion rate.

is of relatively low affinity; consequently, metabolical clearance rates of albumin-bound unconjugated steroids are relatively high compared with those of hormones, such as testosterone and cortisol, which bind, respectively, to SHBG and CBG with high affinity.[122] Sulfoconjugated steroids are an exception because they bind tightly to albumin, and as a result, are cleared very slowly from the blood. Consequently, concentrations of sulfated steroids in blood are usually several-fold higher than their respective unconjugated forms. In contrast, steroid glucuronates are weakly bound to albumin and are rapidly cleared.

The *production rate* of a steroid hormone refers to entry into the blood of the compound from all possible sources, including secretion from glands and conversion of prohormones into the steroid of interest.[113] At steady state, the amount of hormone entering into the blood from all sources will be equal to the rate at which it is being cleared (metabolical clearance rate) multiplied by blood concentration (production rate = metabolic clearance rate × concentration). If there is little contribution of prohormone metabolism to the circulating pool of steroid, then the production rate will approximate the secretion rate.

The fraction of prohormone that is metabolized into the steroid of interest, known as the *rho (ϱ) value*, can be estimated by infusing an isotope of the prohormone at a constant rate until equilibrium is reached, then determining the blood concentrations of the unconjugated prohormone isotope and the isotopic product.[123] The amount of prohormone entering into the circulation can be calculated using the ϱ value and by the production rate.

Table 4.2 shows the secretion, production, and metabolical clearance rates of the major steroids, and Table 4.3 provides the ϱ values of selected sex steroid hormones. Note that there are some differences in these values between sexes and in the favored direction of interconversion, which reflects the integrated activities of the different 17β-hydroxysteroid dehydrogenases that selectively oxidize or reduce androgens and estrogens.

Table 4.3 Mean ϱ Values for Interconversion of Key Sex Steroid Hormones

Interconversion			ϱ Values: Female	ϱ Values: Male
Androstenedione	→	Testosterone	0.03	0.052
Testosterone	→	Androstenedione	0.122	0.076
Androstenedione	→	Estrone	0.007	0.0114
Testosterone	→	Estradiol	0.0014	0.0033
Estrone	→	Estradiol	0.041	0.05
Estradiol	→	Estrone	0.176	0.156

Plasma Steroid Hormone–Binding Proteins

Greater than 97% of the circulating fractions of testosterone, estradiol, cortisol, and progesterone are bound by plasma proteins of hepatic origin. SHBG and albumin bind testosterone and estradiol, whereas CBG and albumin bind cortisol and progesterone, and to a far lesser extent testosterone. High affinity binding of natural steroid hormones to SHBG requires a 17β-hydoxy group, and a 5α-hydrogen function. An olefinic bond in the A ring or an aromatic A ring reduces binding affinity. Consequently, testosterone has a greater affinity for SHBG than does estradiol; 65% and 78% of circulating testosterone is bound to SHBG in men and women, respectively, whereas only 30% and 58% of estradiol in men and women, respectively, is associated with SHBG. The remainder is mostly bound to albumin.

The protein-bound steroid hormone is generally considered a reservoir, restrained from free diffusion into cells, where it can act and can be metabolized. This notion is substantiated by the discovery of variants in the SHBG gene that include a missense mutation (Pro156Leu) that causes abnormal glycosylation and impaired secretion. Women with this

variant display symptoms of hyperandrogenemia, reflecting a greater proportion of bioavailable testosterone.[124]

Association studies and genome-wide linkage scans suggest that variation in plasma SHBG levels is influenced by polymorphisms in the *SHBG* gene, as well as several other genes at different loci in different ethnic groups.[125,126] Interestingly, the level of SHBG in plasma, as influenced by these polymorphisms, is associated with risk for developing type 2 diabetes mellitus with higher SHBG levels being protective. The molecular mechanisms underlying this association remain to be elucidated. [127]

Although SHBG is generally believed to reduce the entry of sex steroids into target tissues, it has been argued that the steroid bound to binding globulins may be selectively accumulated by some cell types by "receptors," and that target tissues also synthesize SHBG that acts locally to facilitate signal transduction by way of a cAMP mechanism.[128] However, there is no evidence for a specific SHBG receptor, suggesting that uptake of SHBG may be related to its binding to extracellular matrix elements of receptors for other proteins.

Because of the importance of plasma steroid–binding proteins in influencing the amount of bioavailable hormone, any genetic or physiological variation or pharmacologically induced change in the production of these proteins by the liver can have a significant effect on steroid hormone action and metabolism. The clinical evaluation of subjects with suspected disorders of hormone production or action may require an assessment of the level of binding protein or a measurement of the bioavailable or free fraction of hormone to clarify the basis for the clinical presentation. Conditions with increased SHBG levels are associated with increased free estradiol/testosterone ratios and those with reduced SHBG with decreased free estradiol to testosterone ratios. For example, suppression of SHBG levels associated with obesity, hyperinsulinemia and hyperglycemia contributes to the symptoms of hyperandrogenemia associated with polycystic ovary syndrome, which is frequently accompanied by these metabolical phenotypes. Table 4.4 summarizes changes in SHBG- and CBG-binding capacity under different physiological and pathophysiological conditions, as well as the influence of certain pharmacological agents.

The gene encoding vitamin D binding protein (GC) is polymorphic with alleles showing different population distributions that are associated with skin pigmentation. Binding protein levels are increased by estrogen and are unaffected by age or body mass index (BMI).

Table 4.4 Factors Influencing the Binding Capacity of Sex Hormone-Binding Globulin and Cortisol-Binding Globulin

	Binding Capacity	
Factors and Endocrine Status	*SHBG*	*CBG*
Exogenous estrogen	↑	↑
Pregnancy	↑	↑
Exogenous androgens	↓	↓
Anabolic steroids	↓	NC
Synthetic progestins (androgenic properties)	↓	NC
Thyroid hormone (hyperthyroidism)	↑	↓
Prolactin (hyperprolactinemia)	↓	NC
Growth hormone (acromegaly)	↓	NC
Old age (men)	↑	NC
Postmenopausal	↓	↓
Obesity	↓	NC
Hyperinsulinemia	↓	↓

CBG, Cortisol-binding globulin; NC, no change; SHBG, Sex hormone-binding globulin.

Inhibitors of Steroidogenic Enzymes

The inhibition of steroidogenic enzymes has been shown to be an effective strategy for terminating pregnancy and treating disorders of excessive hormone production (including Cushing's syndrome, steroid hormone–secreting malignancies, and hormone-dependent cancers of the prostate, endometrium, and breast). Inhibitors of aromatase are also used in the induction of ovulation. The inhibitors include steroid-based and non–steroid-based molecules that act as competitive inhibitors or mechanism-based enzyme poisons (Fig. 4-15).

Endocrine-disrupting chemicals, often believed to act through direct effects on steroid hormone receptors, are now known to interfere with steroidogenic enzymes and enzymes involved in steroid metabolism. These actions may alter endogenous steroid hormone production and catabolism and lead indirectly to altered responses of hormone-responsive target tissues.[129]

Inhibitors of P450scc

Aminoglutethimide, a drug originally introduced as an antiepileptic, is a non-steroidal competitive inhibitor of P450scc and P450arom.[130] The free amine of the drug (essential to the drug's inhibitory activity) interacts with the P450 heme to prevent reduction of Fe^{3+}, which is an obligatory step in the P450 catalytic mechanism. Ketoconazole and related compounds also inhibit P450scc as well as other P450bsteroidogenic enzymes.

P450c17 Inhibitors

Ketoconazole, an antifungal agent that blocks P450s involved in ergosterol biosynthesis, is an effective inhibitor of the 17,20-lyase activity and blocks androgen biosynthesis.[123] Other compounds that are used in experimental systems to block 17α-hydroxylase/17,20-lyase activity are too toxic for clinical application. Newer compounds, including the 17-heteroarylsteroids, abiraterone acetate, and VN/124-1 (TOK-001) ([3β]-17-[1H-benzimidazol-1-yl]androsta-5,16-dien-3-ol) are less toxic and are used in the treatment of "castration-resistant" prostate cancer.[131]

P450c11 Inhibitors

Metyrapone, an 11β-hydroxylase inhibitor, reduces cortisol production, with a concomitant increase in 11-deoxycortisol. It is used in diagnostic testing of the hypothalamic–pituitary–adrenal axis and has also been used to treat Cushing's syndrome, but its utility in this regard is limited by side effects.[132]

Aromatase Inhibitors

Two major classes of aromatase inhibitors have been developed, the nonsteroidal imidazole and triazole analogs

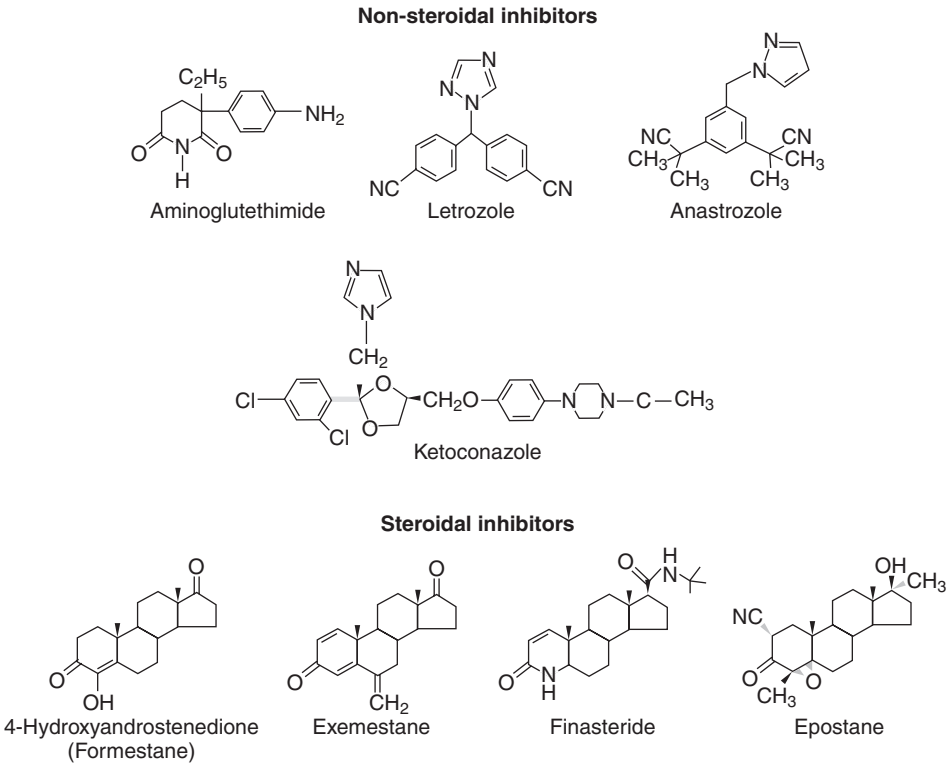


FIGURE 4.15 Structures of nonsteroidal and steroidal enzyme inhibitors.

based on ketoconazole, and the steroidal mechanism-based inactivators.[133] The first class of compounds is relatively nonspecific because they can inhibit other steroidogenic P450s. Two triazole compounds that are reversible competitive inhibitors of P450arom are in clinical use (letrozole [Femara] and anastrozole [Arimidex]). These drugs bind to the iron atom of the heme protein and exclude the substrate from the catalytic pocket.

The steroidal mechanism–based inhibitors include 4-hydroxyandrostenedione (formestane) and 6-methylandrosta-1,4-diene-3,17-dione (exemestane) (Fig. 4.15). They are fundamentally innocuous by themselves, but are activated by the catalytic mechanism of P450arom to produce electrophilic species, which then covalently modify the active site. Inactivation requires both NADPH and oxygen. Inhibition by these drugs is long-lasting because new aromatase must be synthesized to overcome the inactivation event. The mechanism-based inhibitors are selective because they only inactivate the target enzyme. In the case of 4-hydroxyandrostenedione, the ultimate electrophilic species responsible for enzyme inactivation is unknown.

5α-reductase Inhibitors

The 4-azasteroids represented by finasteride were developed as selective inhibitors of type 2 5α-reductase to prevent the formation of the potent androgen 5α-dihydrotestosterone.[134] These inhibitors contain a heterocyclic A ring with a nitrogen substitution at C4. The potent competitive inhibition of SRD5A2 (K_i = 3 nM) seen with finasteride was originally attributed to the ability of this compound to produce a mimetic of the enolate transition state. It is now evident that finasteride acts as a mechanism-based inactivator of 5α-reductase to form an isocitrate dehydrogenase (NADP+)–dihydrofinasteride bisubstrate analog with a K_i of 10^{-13} M (Fig. 4.16).

Other steroidal-based inhibitors include steroid acrylates, which contain a carboxylic acid substituent at C3. The carboxylate moiety again mimics the enolate transition state. Interestingly, these compounds are potent noncompetitive inhibitors because they form an abortive enzyme—NADP+–acrylate complex. Dual-enzyme 5α-reductase inhibitors (e.g., GI198745) that are under development can suppress dihydrotestosterone production by 99% 24 hours after oral administration.

3β–Hydroxysteroid Dehydrogenase/$\Delta^{5\text{-}4}$ Isomerase Inhibitors

Compounds originally developed to target 3β-HSD were derivatives of 2α-cyanoketone (2α-cyano-4,4,17α-trimethylandrost-5-en-17β-ol-3-one).[135] The subsequently developed compounds trilostane and epostane are relatively specific competitive inhibitors for blocking steroidogenesis in the adrenal cortex and placenta, respectively.[136]

HSD11B1 inhibitors

Inhibitors of HSD11B1, which prevent conversion of cortisone into cortisol, are being developed to treat type 2 diabetes, insulin resistance, dyslipidemia, and obesity.[137,138]

Other Enzyme Targets for Reducing Sex Steroid Production

Inhibition of HSD17B1, and steroid sulfatase is an approach to the reduction of bioavailable estradiol levels.[30,66,67,85] Tibolone, a drug used in Europe for hormone replacement

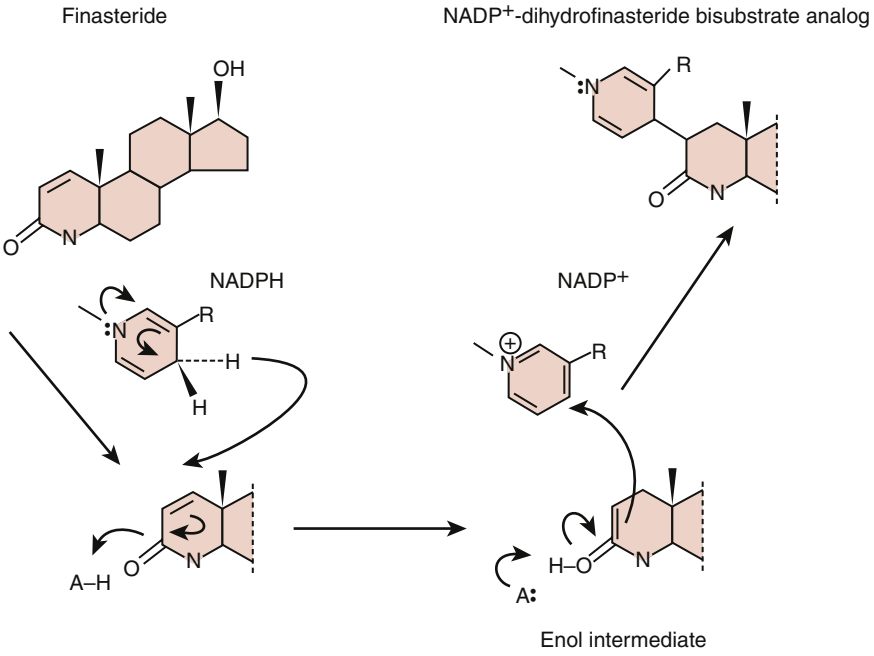


FIGURE 4.16 Inhibition of 5α-Reductase type 2 by finasteride. Finasteride is reduced by 5α-reductase via an enol intermediate, which then reacts with NADP+ to produce a NADP+–dihydrofinasteride bisubstrate analog, which is a potent enzyme inhibitor. NADP, nicotinamide adenine dinucleotide phosphate. *(Modified from Strauss JF III, Penning TM. Synthesis of the sex steroid hormones: molecular and structural biology with application to clinical practice. In Fauser BCJM, Rutherford AJ, Strauss JF III, et al [eds]. Molecular biology in reproductive medicine. New York, Parthenon, 1999, pp 201–232.)*

therapy, has the interesting property of inhibiting sulfatase activity in breast cancer cells, but not in bone cells, a tissue-selective pattern of action that could reduce estradiol bioavailability in breast, but not in bone.[139] As noted previously, hydroxylated metabolites of polychlorinated biphenyls are potent inhibitors of estrogen sulfotransferase and thus increase the bioavailability of estradiol.[81]

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 5

Steroid Hormone Action

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Steroid Hormone Receptors Act as Ligand-Dependent Transcription Factors or Repressors

Steroids are small, lipophilic hormones synthesized from a common precursor molecule, cholesterol, within the adrenal glands or the gonads (see Chapter 4). The adrenal cortex is the primary source of circulating mineralocorticoids and glucocorticoids, while the gonads are the main source of circulating active sex steroids called *estrogens*, *progestins*, and *androgens*. Despite their shared molecular origin and basic structural similarities, mineralocorticoids, glucocorticoids, estrogens, progestins, and androgens are distinct classes of steroid hormones that interact with specific, high affinity receptors to exert their biological effects. These hormones control diverse physiological and cellular processes and affect virtually every aspect of vertebrate biology, from sexual differentiation, growth, and reproduction to immunity, brain function, and behavior. Consequently, a clear and complete understanding of the basic mechanisms of steroid hormone action is of critical importance for reproductive health and general well-being. Also of significance are the unique mechanisms that lead to hormone-specific effects and function and to differences in hormone responsiveness between species.

This chapter reviews what is known about the mechanisms of steroid action. Briefly, the classic mode of action of steroid hormones is to enter cells, interact with cognate receptors, and stimulate or inhibit transcription of target genes (Fig. 5.1).[1] Hormone-dependent changes in receptor conformation ultimately influence transactivation and transrepression of gene expression by (1) disrupting interactions with molecular chaperones that keep the receptor in a ligand-independent state; (2) promoting the formation of receptor dimers; (3) promoting interactions with specific DNA sequences in the promoter of target genes; and (4) facilitating recruitment of coactivator or corepressor proteins that alter chromatin structure and contact the basal transcription machinery.

To fully understand the commonalities of steroid hormone action and appreciate the specificity of signaling by different classes of steroids, it is important to understand the evolution of receptors (mineralocorticoid [MR], glucocorticoid [GR], estrogen [ER], progestin [PR], and androgen [AR]) from a common ancestral protein. Next is a discussion of hormone-dependent activation and repression of gene expression and the physiological roles played by each of these receptors. This is followed by a discussion of general factors that influence steroid hormone action. Finally, the chapter examines more recent work, which has begun to elucidate alternative modes of action for steroid hormones and their receptors. Such mechanisms include interactions with other transcription factors and nongenomic effects mediated by second messenger signaling pathways.

Evolution of Steroid Hormone Receptor Structure and Function

Steroid receptors belong to a larger family of structurally and evolutionarily related proteins called *nuclear receptors*.[2-4] A bioinformatic analysis of the human genome identified 49 genes encoding for nuclear receptors, which appears to represent the total number of paralogs found in humans.[5] Paralogs are related genes found within a single genome that evolved by gene duplication. Through alternative splicing and alternative translation initiation, it is predicted that these 49 genes could result in 75 receptors in the human proteome.[5] Although a complete review of the nuclear receptor family of transcription factors is beyond the scope of this chapter, a brief discussion is in order.

All nuclear receptors, including steroid receptors, exhibit a modular structure composed of distinct domains (Fig. 5.2). In general, these receptors contain a variable amino-terminal region (A/B), a highly conserved DNA-binding region (C), a highly variable hinge region (D), and a moderately conserved hormone or ligand-binding domain (E). Some receptors also contain a carboxy-terminal F domain. The primary structure of each human steroid receptor is shown, along with its physiological ligand (Fig. 5.2). Specific residues within the DNA- (C) and ligand-binding (E) domains play an important part in receptor dimerization, which is critical because most nuclear receptors are only transcriptionally active as homo- or heterodimers. Finally, nuclear receptors have one or two regions called *activation function 1 and 2* (AF1 and AF2) that are required to transactivate gene expression. Whereas the activity of AF1 is usually ligand-independent and located in the A/B domain, AF2 is found in the ligand-binding domain and is predominantly regulated by hormone binding.

Some nuclear receptors have defined natural ligands, such as steroid hormones, thyroid hormones, retinoids, or vitamin D, but others have no identified ligand and are called *orphan receptors*. The finding that diverse compounds

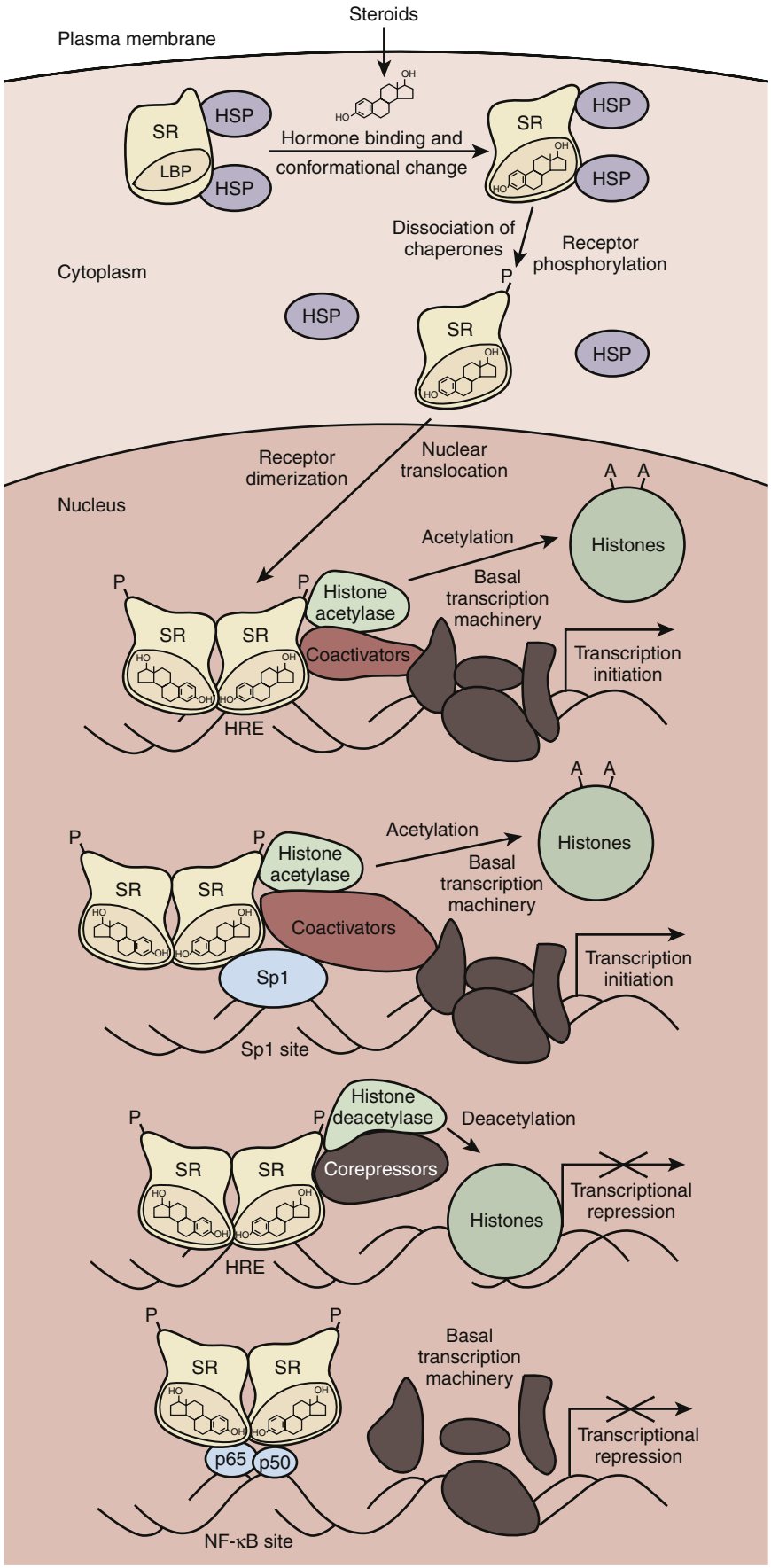


FIGURE 5.1 General mechanism of action for cytoplasmic steroid receptors as described in the text. The two subunits of nuclear factor kappa B are p50 and p65. A, acetyl group; HSP, heat shock protein; HRE, hormone response element; LBP, ligand-binding pocket; NF, nuclear factor; P, phosphate group; SR, steroid receptor.

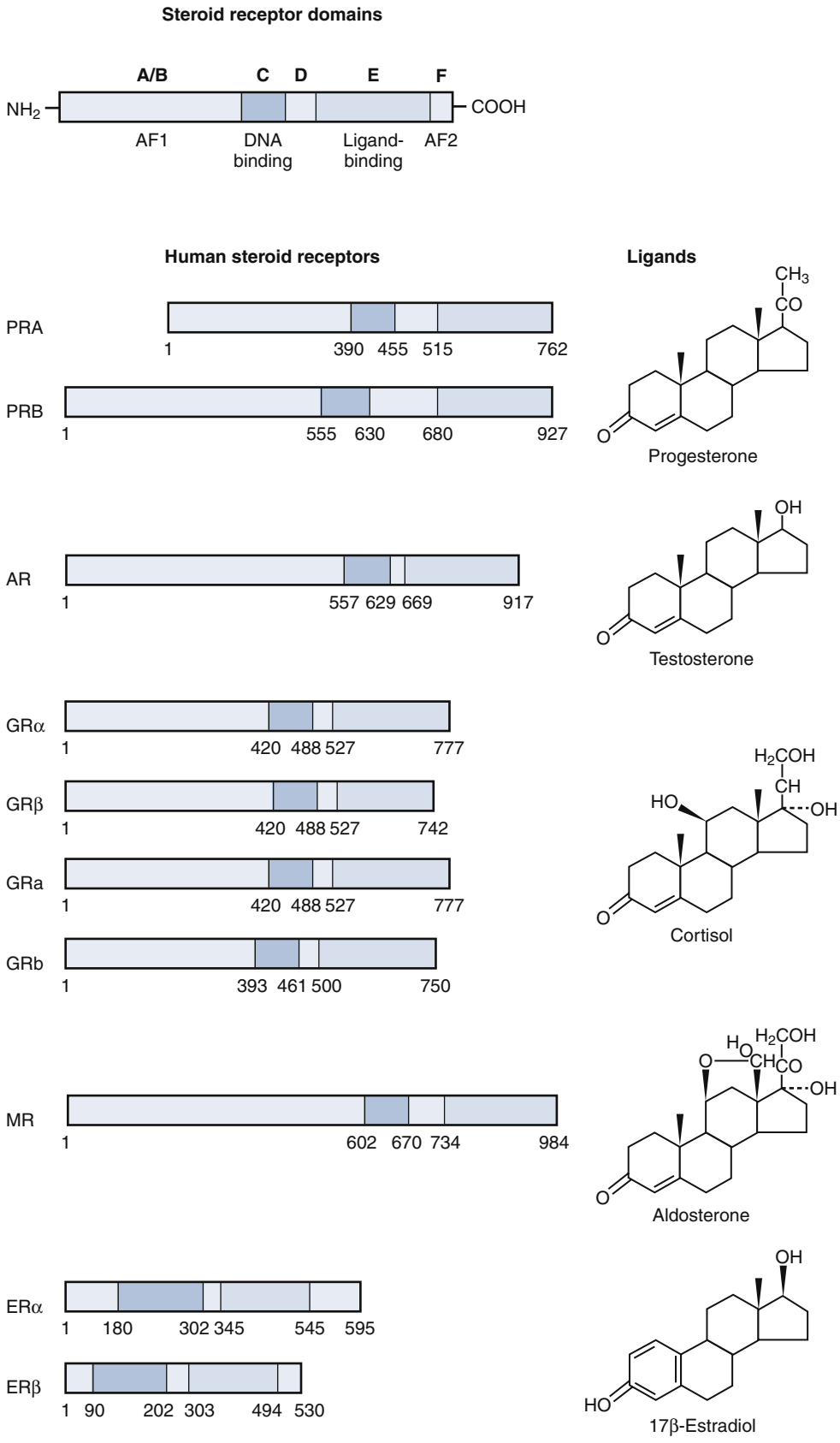


FIGURE 5.2 Schematic diagram of the primary structure of a generic steroid receptor and its functional domains. Region A/B contains transactivation function 1 domain (AF1). Region C contains the DNA-binding domain. Region D is the hinge region. Region E contains the ligand-binding domain. Region F contains the transactivation function 2 (AF2) domain. The primary structure of human steroid receptors, their isoforms, and their physiological ligands: AR, androgen receptor; ER, estrogen receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PRA, progestin receptor isoform A; PRB progestin receptor receptor isoform B.

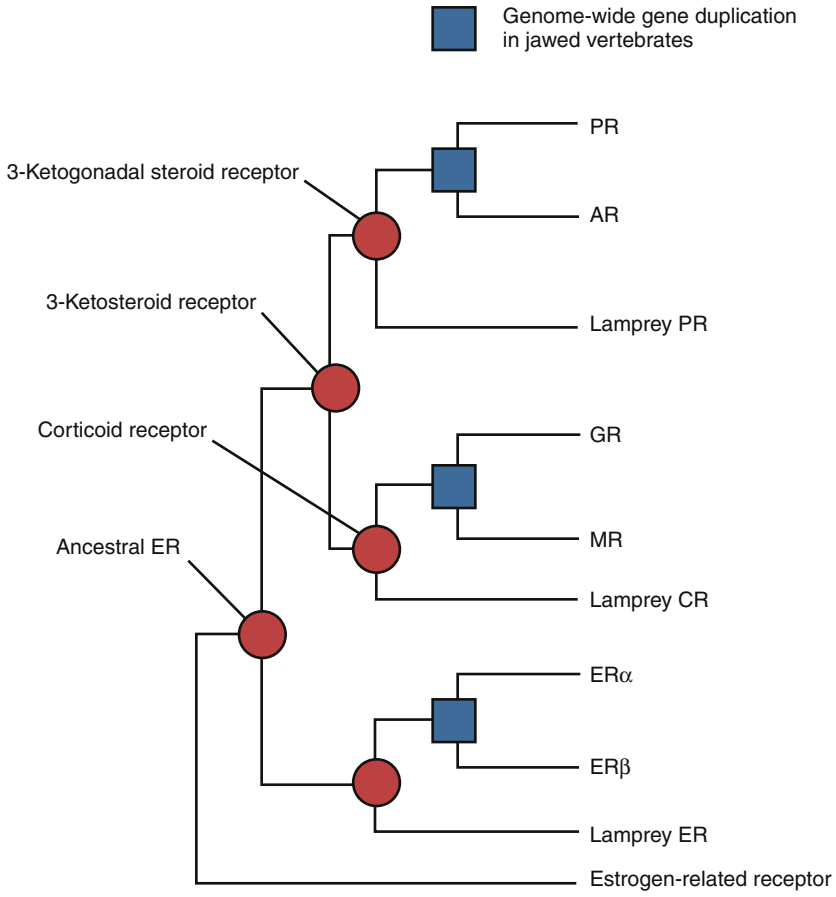


FIGURE 5.3 Phylogeny of the steroid receptor gene family. AR, androgen receptor; CR, corticoid receptor; ER, estrogen receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR progestin receptor.

act as ligands for nuclear receptors and that some receptors have no apparent ligand led to the hypothesis that ancestral nuclear receptors were constitutive transcription factors that independently evolved the ability to bind ligand.[6,7] However, a second hypothesis posits that ancestral receptors were ligand-dependent transcription factors that evolved specificity for different ligands by gene duplication, mutation, and functional divergence. There are several lines of evidence that favors the latter hypothesis for the evolution of ligand binding in the steroid receptor family. First, the primary, secondary, and tertiary structures of the ligand-binding domain of different steroid receptors are highly similar.[8-11] Second, detailed sequence, structural, and functional analyses strongly support the hypothesis that the ancestral steroid receptor bound estrogens and specificity for other steroids evolved by serial and parallel duplications of the ancestral gene, mutation of nucleotides coding for specific amino acids, and structural and functional divergence of the paralogs.[12-14] Finally, steroid hormone receptors are nuclear receptors unique to the chordate lineage, indicating that they originated when the first chordates evolved.

Gene Duplication of Ancestral Steroid Hormone Receptors

Phylogenetic analysis of the primary amino acid sequences of 73 steroid receptors from jawed vertebrates (e.g., fish, amphibians, reptiles, birds, and mammals) and a jawless fish (the sea lamprey) indicate that there were two serial duplications of an ancestral steroid receptor before the divergence of these lineages approximately 450 million years ago.[13] Maximum likelihood reconstructions of the ancestral amino acid sequence indicate that the first steroid receptor was probably an ER-like molecule (Fig. 5.3). After this gene was duplicated, one copy was constrained by natural selection and retained its function as an "estrogen receptor," whereas the other copy evolved specificity for 3-ketosteroid–like ligands (Fig. 5.3). Duplication of the latter gene then produced a corticoid receptor–like protein and a receptor for 3-ketogonadal steroid–like molecules (e.g., androgens, progestins, or both). Whereas these three steroid receptors (i.e., the ER, corticoid, and 3-ketogonadal steroid receptors) are present in the sea lamprey, six steroid receptors are present in fish and tetrapods, suggesting a genome-wide duplication in the last common ancestor of jawed vertebrates (indicated by blue boxes in Fig. 5.3).

This final duplication then led to the evolution of the true androgen and progesterone receptors from the ancestral 3-ketogonadal steroid receptor, glucocorticoid and mineralocorticoid receptors from the ancestral corticoid receptor, and ERα and ERβ from the ancestral estrogen receptor. The number of paralogs for other gene families, such as the homeobox *(Hox)* genes, supports the hypothesis that many genes were duplicated in parallel in the ancestor of jawed vertebrates.

Structural and Functional Divergence of Steroid Hormone Receptors

Experiments deleting large regions of different steroid receptors have clearly shown that domain C is responsible for

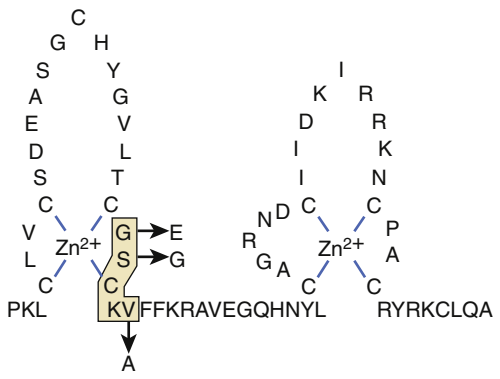


FIGURE 5.4 Amino acid sequence of the DNA-binding domain of human glucocorticoid receptor showing two zinc fingers and the P box, which is outlined. The three mutant residues that change glucocorticoid receptor binding specificity from glucocorticoid-responsive element and estrogen-responsive element are indicated by the *arrows*.

DNA binding and that domain E is responsible for ligand binding (Fig. 5.2). Furthermore, swapping the entire ligand-binding domain among different receptors show that this region determines specificity for particular classes of steroid hormone. Nevertheless, crystal structures of various steroid hormone receptors show that all ligand-binding domains fold into a highly homologous three-layered structure with a small ligand-binding pocket in the center. This pocket is composed of roughly 30 amino acids that are in close proximity or make direct contact with hormones when bound to their cognate receptors. In agreement with structural studies, experiments using site-directed mutagenesis indicate that specific, but minor, changes of amino acids within the ligand-binding pocket can lead to dramatic changes in the hormone-binding specificity of steroid receptors. For example, the human PR, GR, and MR contain a conserved cysteine residue that appears to be critical for contacting the C20 keto group found in progestins, glucocorticoids, and mineralocorticoids.[13,15] Mutation of the corresponding threonine to cysteine in the AR reduces its affinity for androgens and allows the receptor to transactivate gene expression in the presence of progesterone and corticoids.[16] Based on structure-function studies of this sort and phylogenetic analyses, Thornton[13] proposed a series of relatively minor amino acid changes that may account for broad changes in hormone specificity during the evolution of steroid receptors.

Similar studies of the DNA-binding domain have defined the molecular basis for interactions between steroid receptors and particular DNA sequences.[17,18] The DNA-binding domain of nuclear receptors contains two zinc fingers. The first finger interacts with the major groove of DNA, whereas the second is involved in receptor dimerization. Mutation of three residues within a five residue motif known as the Proximal box *(P box)* of the first zinc finger of the GR to the corresponding residues in ER changes the binding specificity from DNA sequences called *glucocorticoid-responsive* elements to *estrogen-responsive* elements and vice versa (Fig. 5.4). Although the DNA-binding domain is very highly conserved among nuclear receptors, these residues are variable among different receptors, which may in part account for receptor-specific regulation of distinct sets of genes. These examples illustrate that site-directed mutagenesis and fine-scale comparison of amino acid sequences among receptors, in the context of the tertiary structure of the DNA- and ligand-binding domains, can lead to testable hypotheses about the evolution of signaling and regulation of gene expression by different classes of steroids.[19]

Activation and Repression of Gene Expression

Given this background on the evolution of different steroid receptors, we now focus on the general function of these receptors and the activation and repression of gene expression by each class of steroid hormone. Steroid receptors have taken on more or less distinct physiological roles during evolution. ERα, ERβ, PR, and AR are primarily involved in sexual differentiation and reproduction. Estrogens and progestins are essential for normal female development and reproduction. Androgens are involved in various aspects of male reproductive physiology and development. Although androgens, estrogens, and progestins are sex-typical hormones, they are not sex-limited, and they play a physiological role in both sexes. In contrast, GR and MR principally regulate nonreproductive traits, although glucocorticoids have recently been implicated in general reproductive function.[20] Glucocorticoids are considered stress hormones that control the function of many tissues, whereas mineralocorticoids play a more restricted role in regulating electrolyte balance and a few other traits. Despite the general separation of these functions, each class of steroid can also modulate the action of other steroids. Cross-talk among steroid hormones is therefore an important subject that will be addressed subsequently.

Estrogen Receptor

The biological effects of estrogens were believed to be mediated by a single receptor (i.e., ERα) until the cloning of a second receptor (i.e., ERβ); both receptors, ERα and ERβ, are products of different genes.[21] Subsequently, it has been shown that these two receptors play distinctive roles in estrogen signaling and that the genes encoding ERα and ERβ are differentially expressed in different tissues (Table 5.1).[22,23] Although both ERα and ERβ are required for normal ovarian function, the phenotypes of ERα and ERβ knockout mice are dissimilar. The ERα knockout mouse is anovulatory and accumulates cystic follicles, whereas the ERβ knockout mouse contains histologically normal ovaries, but still display impaired ovulation. Based on the available evidence, it appears that ERα bears much of the load for mediating the effects of estrogen in other tissues of both sexes. For instance, only ERα is required for the effects of estrogen on growth and differentiation of the uterus and mammary gland development. In males, knocking out ERα results in infertility, whereas males lacking ERβ reproduce normally. Despite the fact that both ERα and ERβ bind natural estrogens with similar affinity (Table 5.2),[22] in vitro studies have identified that their unique amino terminal (A/B) domain is responsible for the observed functional differences between the receptors.

A number of estrogen-regulated genes have been identified in the uterus. Lactoferrin is strongly induced by

Table 5.1 Tissue-Specific Patterns of ERα and ERβ mRNA Expression in the Rat

	Receptor*	
Tissue	*ERα*	*ERβ*
Epididymis	+++	+
Prostate	+	+++
Testis	+++	+
Pituitary	++	+
Ovary	+++	+++
Uterus	+++	++
Bladder	+	++
Lung	0	+
Liver	+	0
Kidney	++	0
Thymus	+	+
Adrenal	++	0
Olfactory lobe	0	+
Cerebellum	0	+
Brain stem	0	+
Spinal cord	0	+
Heart	+	0

*Relative levels of expression are indicated by the number of plus signs: 0, not detected; +, low; ++, medium; +++, high.
From Kuiper GGJM, Carlsson B, Grandien K, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology* 138:863–870,1997.

Table 5.2 Binding Affinity of Various Ligands for ERα and ERβ Relative to Binding Affinity of E2

	Relative Binding Affinity*	
Ligand	*ERα*	*ERβ*
E2	100	100
Diethylstilbestrol	468	295
Hexestrol	302	234
Dienestrol	223	404
Estrone	60	37
17α-Estradiol	58	11
Moxestrol	43	5
Estriol	14	21
4-OH-Estradiol	13	7
2-OH-Estradiol	7	11
Estrone-3-sulfate	<1	<1
4-OH-Tamoxifen	178	339
ICI-164384	85	166
Nafoxidine	44	16
Clomifene	25	12
Tamoxifen	7	6
Coumestrol	94	185
Genestein	5	36
Bisphenol A	0.05	0.33
Methoxychlor	0.01	0.13

*Relative binding affinity is the ratio of concentrations of E2 and competitor required to displace 50% of specific radioligand binding. Relative binding affinity was set to 100% for E2.
From Kuiper GGJM, Carlsson B, Grandien K, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology* 138:863–870,1997.

estrogen and is commonly used as a marker for the effects of estrogen in the uterus. However, the functional role that lactoferrin plays in the uterus is currently unknown.[24] Estrogens enhance insulin-like growth factor 1 (IGF-1) signaling by inducing the expression of IGF-1 and down-regulating IGF binding proteins 3 and 5, resulting in the proliferation of uterine epithelial cells. Estrogen also induces the expression of the glycoprotein mucin 1 (Muc-1), which plays a role in blastocyst attachment and provides a barrier against uterine infections.[25,26]

In addition to their normal role in regulating uterine function, estrogens are a risk factor for the initiation and progression of breast cancer. Moreover, ER antagonists slow or stop the growth of breast cancer by blocking estrogen-regulated gene expression. The carcinogenic effects of estrogen in breast tissue may, in part, be mediated by the induction of vascular endothelial growth factor (VEGF),[27] as tumor growth is generally dependent on angiogenesis and a steady blood supply. Interestingly, the breast cancer susceptibility gene *(BRCA1)*, can directly interact with ER and block the induction of VEGF. Mutations that inactivate *BRCA1* and confer increased risk for breast cancer, may be due to a loss of the ability to antagonize the actions of estrogen.

Somatic mutations and genetic polymorphisms in the human ER itself are also associated with various disease states. An adult male without functional ERα (i.e., the mutation produces a premature stop codon) had decreased bone mineral density, signs of increased bone turnover, and incomplete closure of bone epiphyses, indicating that estrogens (and ERα) play an important role in bone growth and homeostasis in humans.[28] This finding is in general accord with studies of male ERα knockout mice, which have decreased bone mineral density. While genetic polymorphisms within ER have been associated with increased risk of osteoporosis in humans, the underlying mechanisms responsible for these associations are not clear (reviewed in Gennari et al.[29]). A number of ER mutations have been detected in breast cancer.[30] For example, deletion of exon 5, which falls within the ligand-binding domain, generated a receptor that was constitutively active in the absence of estrogens. Deletion of exon 7, which also falls within the ligand-binding domain, produced a receptor that displayed dominant negative activity when co-expressed with wild-type ER. Additional mutations, including base pair insertions and deletions or that result from alternative splicing result in deletion of other exons and alter signaling through the ER. However, it is not currently known whether the mutations occurred before or after the development of malignancy.

Progesterone Receptor

Like estrogens, progestins have broad effects, including the establishment and maintenance of pregnancy, regulation of mammary gland development, control of ovulation, and regulation of female reproductive behavior. Interestingly, the PR has two isoforms, "PRA" and "PRB," which are derived from the same gene. PRA and PRB are identical except for an additional 164 amino acids at the amino-terminal end of PRB due to an alternative translation initiation site. Female mice with a targeted disruption of the PR gene (i.e., both A and B isoforms are inactive) are unresponsive to progesterone, strongly indicating that the pleiotropic effects of progestins are mediated by the PR.[31] In contrast to females, male mice that lack a functional PR appear normal and are able to reproduce as well as wild-type males. Analyses of PRA and PRB activity using transient transfection assays have shown that the two isoforms have distinct transcriptional activities within the same promoter and are each able

to recognize unique promoters. Whereas PRB is a transcriptional activator in most promoter contexts and cell types, PRA appears to have promoter and cell-specific effects on progestin target genes, as well as inhibiting PRB action in contexts where PRA alone is inactive.

Mice with targeted deletion of either PRA or PRB has led to an initial characterization of the specific roles of each PR isoform in different tissues *in vivo*.[32] PRA is required for uterine development and reproduction, whereas PRB is required for normal development of the mammary gland. However, transgenic mice carrying an extra copy of PRA have abnormal morphological development of mammary glands.[33] These results suggest that overexpression of PRA, or an increased ratio of PRA:PRB, can have important physiological consequences. Although the ratio of PRA:PRB changes during development and as a function of reproductive stage in different tissues,[34,35] it remains to be determined whether these changes influence progesterone signaling. A third potential PR isoform, "PRC," has also been cloned and appears to result from translation initiation at a downstream methionine.[36] Although PRC lacks the first zinc finger in the DNA-binding domain, it modulates the transcriptional activity of PRA and PRB on a reporter gene.

In accord with the finding that PRB is necessary for full differentiation and branching of the mammary ducts, progesterone has also been shown to regulate a number of genes that may be involved in breast cancer.[37] The PROGINS allele of PR, characterized by an Alu insertion in intron G, is linked to mutations in exons 4 and 5 that increase the stability and transcriptional activity of the receptor *in vitro*. Interestingly, there is epidemiological evidence that individuals carrying the PROGINS allele have reduced risk of breast cancer,[38] while it was found at a higher frequency in patients with ovarian cancer.[39] Subsequent work confirmed an association of this polymorphism with increased risk of ovarian cancer, but only in women who had not used oral contraceptives.[40]

In the uterus, progesterone up-regulates expression of regulators of cell cycle progression, growth factors and their receptors, while repressing cell cycle arrest proteins.[41] Localized suppression of the immune system in both nonpregnant females and during gestation of the semi-allogenic embryo is another essential role played by progesterone and PR. While it is not known which genes are involved in nonpregnant females, the down-regulation of regulated on activation, normal T cell expressed and secreted (RANTES) in the endometrial stromal cells may be involved during pregnancy.[42] The induction of osteopontin and the repression of the oxytocin receptor are two other key genes regulated by progesterone because of their role in uterine physiology and partuition.[43,44]

Androgen Receptor

Androgens produced by the testes during and after the sex-determining period coordinate the development and differentiation of many sexually dimorphic tissues by specifically activating the AR.[45] Only one isoform of the AR has been recognized to date. Testosterone produced by Leydig cells in embryonic testes directs the development of the male external genitalia, vas deferens, and related structures from the wolffian ducts. Evidence indicates that the early surge in testosterone also directs male-typical patterns of neural development and behavior that are expressed later in life. In the absence of androgens and in AR knockout mice, the wolffian ducts regress and female external genitalia develop.[46] Likewise, mutations in the human AR can lead to complete androgen insensitivity and development of female genitalia or partial androgen insensitivity and ambiguous genitalia. Given the readily observed phenotype, a large number of AR mutations have been described.[47,48] For example, mutations that substitutes a proline at residues 689 and 865 greatly reduces or eliminates dihydrotestosterone binding and abolishes androgen-dependent transactivation.[49,50] It is interesting to note that different mutations at a given residue can generate different phenotypes. Substitution of threonine for methionine at residue 807 produces partial androgen insensitivity and ambiguous genitalia by reducing but not completely blocking androgen binding to AR. In contrast, arginine or valine substitutions at the same site totally abrogate androgen binding and produce complete androgen insensitivity syndrome.[51]

Androgens are secreted at low levels in immature males, but are again produced at high levels later in development (i.e., at puberty and in sexually mature males). At this point, androgens govern the development of secondary sexual characteristics, activate male reproductive and aggressive behavior, and allow spermatogenesis. Meiosis in germ cells begins at puberty in males and is continuous thereafter. The rate of programmed cell death (or apoptosis) in germ cells during spermatogenesis has a large influence on sperm output and male fertility. Androgens play an important role as a survival factor because testosterone withdrawal from mature males increases germ cell apoptosis.[52] *Bax*, a well-known proapoptotic gene, could be involved in germ cell death as it is elevated upon testosterone withdrawal.[53] Evidence suggests that the Fas pathway may also play a role in germ cell apoptosis as both Fas ligand and Fas receptor are expressed at elevated levels in dying germ cells. Androgens regulate prostate development and differentiation in the embryo and the adult and are believed to be a risk factor for prostate cancer in humans. Treatment of prostate cells with dihydrotestoterone induces changes in cell morphology and regulates a large number of genes involved in small molecule transport, membrane signal transduction, intracellular signal transduction, and metabolism.[54]

Other important aspects of AR function relate to physiological differences in signaling by different androgens.[55] In particular, dihydrotestosterone is a more potent androgen than testosterone, leading to greater stabilization of the receptor and more efficient signaling. Local conversion of testosterone to dihydrotestosterone to enhance androgen action is discussed in another section of this chapter.

Glucocorticoid Receptor

Glucocorticoids are often referred to as *stress hormones* because they are synthesized and secreted in response to pain and emotional trauma, caloric restriction, agonistic social encounters, and general anxiety. Mice homozygous for a null mutation in the GR (i.e., GR knockouts) die around the time of birth, indicating that the GR and glucocorticoids are essential for life.[56] In humans, there are at least nine GR isoforms produced from a single gene. Whereas GRα and GRβ are produced by alternative splicing at the 3´ end of GR mRNA, GR-A, GR-B, GR-C1-3, and GR-D1-3

are produced by alternative translation initiation at the 5′ end of GR mRNA.[57] Glucocorticoids alter the physiology of numerous tissues throughout the body during periods of acute stress. For example, the inhibitory effects of stress on reproduction are mediated by glucocorticoids at all levels of the hypothalamic–pituitary–gonadal axis. These hormones also influence brain function and behavior. Glucocorticoids mobilize energy stores by inducing the degradation of proteins to free amino acids in muscle, lipolysis in adipose tissue, and gluconeogenesis in the liver. Glucocorticoids play a role in the immune and vascular systems, where they induce programmed cell death in lymphocytes and block inflammatory responses. Although these changes are adaptive in the short term, chronic stress accompanied by prolonged glucocorticoid secretion is pathological.

In addition to mediating stress responses, glucocorticoids play an essential part in normal physiology. For example, glucocorticoids regulate the expression of numerous genes involved in energy homeostasis. The expression of phosphoenolpyruvate carboxykinase *(PEPCK)*, is induced by glucocorticoids and is enhanced by the glucocorticoid-dependent increase in the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1) in the liver.[58,59] It has been reported that glucocorticoids also increase pyruvate dehydrogenase kinase 4 levels,[60] which phosphorylates and inactivates the pyruvate dehydrogenase complex (PDC). In turn, PDC inactivation promotes gluconeogenesis by conserving three-carbon substrates. Glucocorticoids regulate the expression of genes related to other physiological processes as well. For example, the well-known anti-inflammatory effects of glucocorticoids are mediated in part by induction of the gene for inhibitor of nuclear factor kappa B alpha (IκBα), which keeps the proinflammatory transcription factor nuclear factor kappa B (NF-κB) in an inactive state in the cytoplasm.

Mineralocorticoid Receptor

Aldosterone, the major circulating mineralocorticoid, specifically binds to and activates the MR. In contrast, cortisol (or corticosterone in rodents) binds to and activates both the MR and GR. To prevent inappropriate MR activation by glucocorticoids, 11β-hydroxysteroid dehydrogenase converts cortisol (or corticosterone) to inactive metabolites in cells containing MR (discussed in detail in Chapter 4). One critical function of aldosterone is to regulate electrolyte balance and blood pressure. Aldosterone's primary target tissue is the distal tubule of the kidney, where it promotes the retention of Na^+ and the elimination of K^+ during urine formation. Retention of Na^+ leads to absorption of water from the distal tubules, an increase in blood volume, and maintenance of blood pressure. Mineralocorticoid insufficiency causes death by Na^+ and water loss, a fall in blood volume, and circulatory shock. Accordingly, MR knockout mice also die of Na^+ and water loss.[61] On the other hand, mineralocorticoid excess contributes to hypertension and pathologies associated with high blood pressure. Additional targets of aldosterone include the heart, brain, smooth muscle, endothelial cells, and adipose tissue, although less is known regarding the cellular and molecular mechanisms of mineralocorticoids in these tissues.

Work has identified several mineralocorticoid-induced genes involved in Na^+ resorption in tight epithelia.[62,63] Aldosterone, for example, increases both the mRNA and protein of serum- and glucocorticoid-regulated kinase (Sgk), activating the phosphatidylinositol-3-OH kinase (PI3K) pathway, stabilizing the epithelial Na^+ channel (ENaC) in an open state as well as increasing the number of ENaCs in the apical membrane. It is also important to note that aldosterone induces the α subunit of ENaC and the α_1 subunit of the sodium/potassium ATPase pump (Na+/K+-ATPase) by the classic transcriptional mechanism attributed to steroid hormones, which is critical for generating the electrochemical gradient necessary for Na^+ resorption at the apical membrane. Aldosterone also induces the expression of the Na+/H+ transporter and numerous other genes that may play a role in electrolyte balance. The role of genes repressed by aldosterone, however, remains unclear.[63] Given this background on the function of different steroid receptors and a few genes regulated by these receptors, we will now focus on commonalities of steroid hormone action that were most likely present in the ancestral steroid receptor.

General Factors That Influence Steroid Hormone Action

Although numerous factors affect signaling by steroid hormones *in vivo*, this chapter focuses on some of the more important and well-characterized regulatory mechanisms. Foremost among the factors determining whether a cell responds to circulating steroid hormones is the intracellular availability of a given steroid and its receptor. Intracellular enzymes metabolize active steroids to inactive forms or vice versa, providing preemptory control over steroid hormone action (see Chapter 4). The number of receptors within a cell also determines its responsiveness to hormone, with regulation of receptor expression at all levels from transcription to protein degradation. When sufficient hormone and receptor are present in a cell, the ligand binds and activates the monomeric receptor (Fig. 5.1). The ligand-bound receptor undergoes a conformational change, dissociates from regulatory heat shock proteins, and is phosphorylated. Cytoplasmic receptors translocate to the nucleus, where they bind as homodimers to specific DNA sequences called *hormone-responsive elements*. The MR, GR, PR, and AR are predominantly cytoplasmic in the absence of ligand. In contrast, ERα and ERβ are localized in the nucleus in the absence of ligand and do not require nuclear translocation for their transcriptional activity. Within the nucleus, the ligand–receptor complex interacts with coactivator and corepressor proteins that remodel chromatin or contact the basal transcription machinery, thereby increasing or decreasing the probability of initiation of transcription. Although we discuss basic regulatory mechanisms at each step in the dynamic process just outlined, the reader is referred to primary research and review articles that treat each mechanism in greater detail.

Hormone Availability

Intracellular enzymes can activate or inactivate steroid hormones to produce tissue-specific or even cell-specific patterns of hormone responsiveness. Aromatase P450, which converts androgens to estrogens, is expressed in the gonads, brain, adipose tissue, skin, and placenta.[64] In most female mammals, the circulating levels of estrogen varies during the

menstrual or estrous cycle. At the nadir of the cycle, circulating estrogen levels are usually below the concentration necessary to activate ER. In contrast, estrogen levels at the peak of the cycle are generally high enough to activate the ER. Consequently, the synthesis and secretion of estrogens from the ovary into general circulation is the main mechanism controlling estrogen action in females. Whereas males have plasma levels of estrogens that are below the concentration necessary to activate ER, they still require estrogen for normal reproductive function and behavior, as demonstrated by ERα knockout mice.[65,66] Therefore, it would seem that the expression of male copulatory behavior in a variety of vertebrates depends on the local conversion of testosterone to 17β-estradiol by aromatase and subsequent activation of ER within the medial preoptic area of the brain.[67,68]

Similarly, localized expression of 5α-reductase, which converts testosterone to dihydrotestosterone, plays a crucial role in regulating androgen action.[69] This reaction is particularly important because dihydrotestosterone binds to the AR with higher affinity than does testosterone and is essential for full virilization of the external genitalia during male development. Interestingly, female knockout mice for type 1 5α-reductase show significant impairment of parturition, suggesting that androgens also play an important role in female reproductive physiology.[70] In general accord with this finding, female knockout mice for AR have smaller litter sizes than wild-type littermates.[46]

In yet another example of steroid-metabolizing enzymes regulating steroid hormone action, two isoforms of 11β-hydroxysteroid dehydrogenase (11β-HSD) oppose each other's action and regulate local levels of active and inactive glucocorticoids (see Chapter 4).[71] Whereas 11β-HSD type 1 is a reductase that converts weak glucocorticoids (i.e., cortisone and 11-deoxycorticosterone) into potent glucocorticoids (i.e., cortisol and corticosterone), 11β-HSD type 2 is a dehydrogenase that catalyzes the reverse reaction to inactivate the more potent glucocorticoids. Clinical and experimental evidence suggests that the relative activity of these enzymes in particular tissues may play an important role in normal glucocorticoid physiology and pathophysiology. These examples illustrate how local biosynthesis or inactivation of steroid hormones can facilitate or inhibit steroid hormone action in a tissue- or cell-specific manner.

Receptor Expression

All other factors being equal, the level of expression of steroid receptors is a major factor regulating steroid hormone action. Whereas certain cells or tissues are insensitive to steroid hormones because they lack the appropriate receptors, other cells and tissues express large numbers of receptors and are exquisitely sensitive to low concentrations of hormones. In addition, hormone responsiveness is often modulated by up- or down-regulation of steroid receptors. One of the best-characterized mechanisms of sensitization is induction of PR gene expression by estrogens. During the ovarian cycle in the rat, 17β-estradiol concentrations slowly rise as ovulation approaches, and 4 hours before ovulation, progesterone concentrations surge.[72] These changes in hormone levels lead to biochemical changes in neurons of the ventromedial hypothalamus that facilitate the display of female sexual behavior. The surge in 17β-estradiol activates the ER and increases transcription and subsequent translation of the PR gene, thereby sensitizing the ventromedial hypothalamus to the subsequent surge in progesterone.[72,73] Essentially the same pattern and mechanism of PR induction by estrogens occurs in stromal cells of the uterus, priming these cells for decidualization in response to progesterone and the implanting blastocyst.[74] Another uterine cell type, the luminal epithelium, displays the opposite pattern of PR expression during the reproductive cycle.[75] Uterine epithelial cells express PR constitutively and rapidly down-regulate PR in response to estrogens. However, the mechanism responsible for this decrease in PR protein is currently unknown. It is also unclear whether PR down-regulation leads to hormone desensitization in these cells.

Changes in mRNA stability provide a second mechanism for regulating the levels of receptor expression in tissues. The preovulatory surge in luteinizing hormone (LH) decreases levels of ERβ mRNA in granulosa cells of rat preovulatory follicles.[76] This decrease in ERβ mRNA is due to a decrease in the half-life of the message from approximately 18 to 5 hours, rather than a decrease in transcription of the ERβ gene. Moreover, the LH-induced decline in steady-state levels of ERβ mRNA is mimicked by forskolin, indicating that LH signaling occurs through the activation of adenylyl cyclase and the generation of cyclic AMP (cAMP). Interestingly, a similar effect was also seen with the protein kinase C (PKC) activator phorbol myristoyl acetate (PMA). The stability of ERα mRNA is also regulated in the Michigan Cancer Foundation-7 (MCF-7) breast cancer cell immortalized line. In these cells, 17β-estradiol decreases the half-life of ERα mRNA from 4 hours to 40 minutes by increasing the ribonuclease activity associated with polyribosomes.[77] In contrast, 17β-estradiol up-regulates ERα mRNA in the endometrium by selectively increasing message half-life from 9 to 24 hours.[78] These studies demonstrate hormone- and tissue-specific effects on the stability of steroid receptor mRNA.

Another well-established mechanism for steroid receptor regulation occurs at the protein level. Treatment with a given steroid generally results in a time-dependent decrease in the level of its cognate receptor in a process called *homologous down-regulation*. Accordingly, a decrease in receptor levels usually results in desensitization to subsequent treatment with the same steroid. Receptor degradation occurs by targeting of the receptor into the ubiquitin–proteasome pathway. The effects of proteasome inhibition on steroid hormone action clearly need further study because there are paradoxical effects on receptor levels and receptor function. Whereas GR accumulation by proteasome inhibition leads to increased transcriptional activity of the receptor, proteasome inhibition of ER and PR degradation results in a decrease in hormone-induced transcription.[79,80] In sum, regulation of steroid receptor expression occurs at multiple levels from gene transcription to mRNA stability to degradation of the mature protein (Fig. 5.5).

Hormone Binding and Changes in Receptor Conformation

Various lines of evidence show that ligand-induced changes in steroid receptor conformation play a central role in transduction of the hormonal signal into a transcriptional response. Early studies, for example, found that trypsin digestion produced different patterns of cleavage for inactive and active

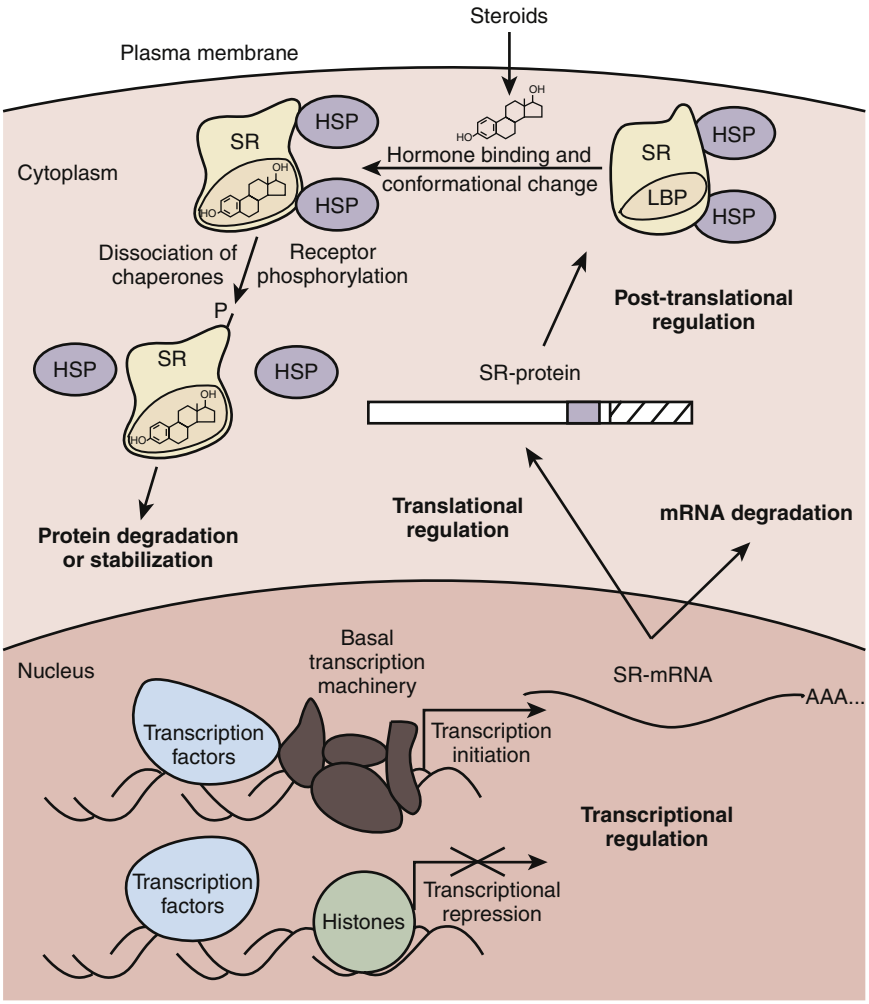


FIGURE 5.5 Schematic diagram showing levels of regulation of steroid receptor expression. AAA, polyadenylated mRNA tail; HSP, heat shock protein; LBP, ligand-binding pocket; mRNA, messenger RNA; SR, steroid receptor.

(i.e., ligand-bound) receptors, suggesting that binding of ligand to receptor caused conformational changes that either concealed or revealed trypsin cleavage sites. Other experiments found that protein to protein interactions between steroid receptors and heat shock proteins were disrupted by the addition of ligand. This result could also be explained by ligand-induced conformational changes that disturb putative contact sites between these proteins. Release of molecular chaperones is critical for receptor activation. Recent work verified inferences about receptor conformation by directly examining the crystal structure of the ligand-binding domain of the AR, ER, PR, and GR, either alone or in complex with ligand.[11,81-83] In particular, these studies showed that the ligand-binding domain is composed of 12 alpha helixes and 4 beta strands folded into a three-layered sandwich (Fig. 5.6). It was also shown that steroid hormones bind within a hydrophobic pocket in the core of the ligand-binding domain and that there are substantial structural differences between ligand-free and ligand-bound receptors.

One of these structural differences provides a basis for the ligand-induced increase in AF-2 activity. The C-terminal helix 12 moves like a trap door to close behind the ligand as it enters the hormone-binding pocket. The new position of helix 12, which contains AF-2, allows the receptor to interact with proteins called *coactivators* that are required for ligand-dependent transcription (Fig. 5.6). Although hormone antagonists bind the same site as agonists, they induce a different receptor conformation (Fig. 5.6). In general, antagonists have a bulky side chain that sticks out of the ligand-binding pocket and prevents movement of helix 12 into a position favorable for coactivator recruitment. Conformational changes are also important for a number of other aspects of steroid hormone action, including cooperativity for hormone binding and receptor dimerization. *Cooperativity* simply means that binding of hormone to a monomeric receptor facilitates binding of hormone to a second receptor, presumably through receptor dimerization.

Steroids have been found to have opposing effects in different tissues in postmenopausal women undergoing hormone replacement therapy (i.e., reduced risk of colorectal cancer and hip fractures, but increased risk of coronary heart disease, pulmonary embolism, stroke, and breast cancer).[84] The finding that combined estrogen and progestin therapy can have beneficial effects in some tissues and harmful effects in others highlights the importance of designing new ligands that dissociate these effects. Selective ER modulators (SERMs) are compounds that have been developed to retain the beneficial effects of estrogens while eliminating unwanted side effects. Raloxifene and tamoxifen are two of the best-characterized ligands in this regard, having been crystalized in complex with ERα.[85-87] Raloxifene acts as an estrogen agonist in bone by increasing bone mineral density and decreasing

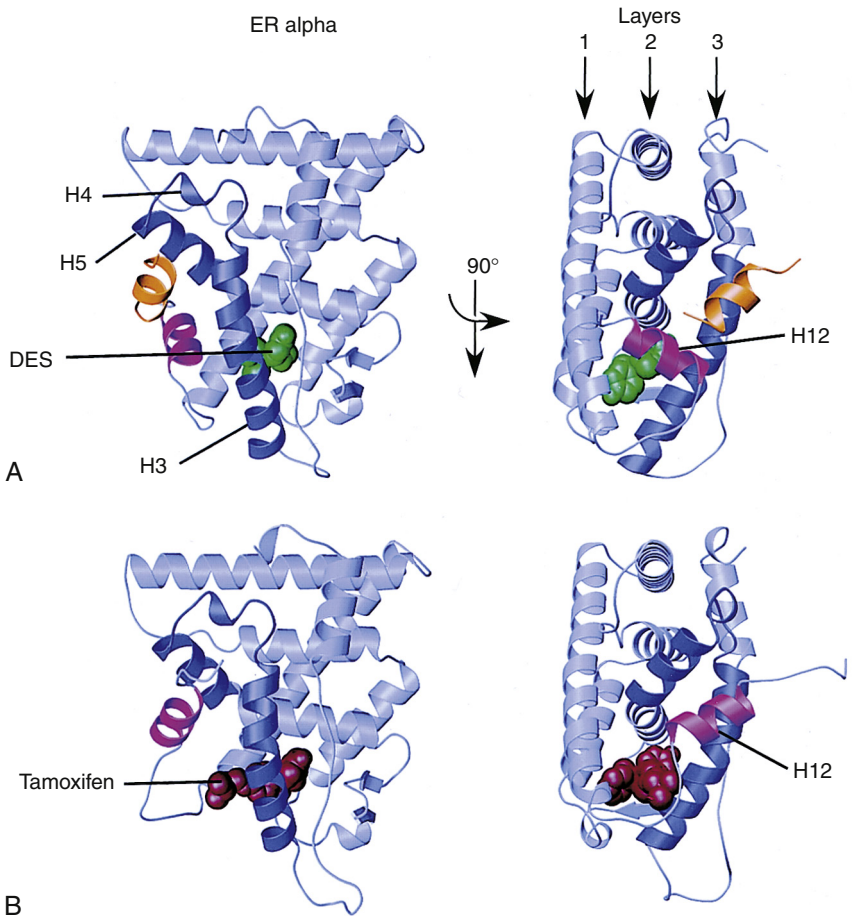


FIGURE 5.6 **A,** Structure of the ERα ligand-binding domain (LBD) bound to diethylstilbestrol (DES; *green*) and interacting with a peptide from the GR-interacting protein (GRIP1) nuclear receptor box II *(orange)*. **B,** Structure of the ERα LBD bound to tamoxifen *(red)*. Note that the position of helix 12 differs between the receptor bound to agonist (DES) and antagonist (tamoxifen). H, helix. *(From Shiau AK, Barstad PM, Loria L, et al. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927–937, 1998, with permission from Elsevier).*

the risk of vertebral fractures in women with osteoporosis. In contrast, raloxifene acts as an estrogen antagonist in the breast and uterus. Tamoxifen, on the other hand, antagonizes estrogen action in the breast, but acts as an agonist in the uterus. Tamoxifen is therefore an effective treatment for breast cancer, but it increases the risk of endometrial cancer.

It has been hypothesized that differences in receptor conformation induced by different SERMs are responsible for the tissue-specific effects of these compounds. For example, raloxifene and tamoxifen may elicit different interactions between a particular ER subtype (i.e., ERα) and tissue-specific coactivators and corepressors. Alternatively, a given SERM could have opposing effects on different ER subtypes (i.e., ERα versus ERβ), which are differentially expressed in estrogen-responsive tissues. The compound tetrahydrocannabinol is just such a ligand, activating ERα by allowing interaction with the coactivator GR-interacting protein 1 (GRIP1) or transcriptional intermediary factor 2 (TIF2) and inhibiting ERβ by preventing interaction with the same coactivator.[83] Progress is also being made with selective progesterone receptor and AR modulators.[88,89] Although such compounds hold much promise, there is still a tremendous amount of research to be done. Ultimately, it will be essential to examine structural changes induced by a variety of ligands, ranging from pure and partial agonists to full antagonists as well as selective steroid modulators, to determine whether they work by similar or different mechanisms (i.e., by altering the position of helix 12 or changing cooperativity in hormone binding and receptor dimerization).

Recent structural studies have revealed that minor changes in DNA sequences itself can alter the structure of the GR DNA-binding domain, suggesting that DNA itself can act as an allosteric modulator of GR structure and function.[90] The structure of an entire steroid receptor, rather than that of isolated domains, is needed to fully understand how structural changes induced by ligand and DNA affects steroid hormone action.

Receptor Phosphorylation

Steroid receptors are proteins modified by phosphorylation at multiple sites. These modifications have distinct effects on the function of different receptors, often in a cell- or promoter-specific manner. Studies mapping phosphorylation sites have shown that steroid receptors are generally phosphorylated at specific serine residues adjacent to proline residues, although particular threonine and tyrosine residues can also be phosphorylated. Although some residues appear to be basally phosphorylated, others are modified in response to ligand binding or activation of other signaling pathways; these include cyclin-dependent kinases, mitogen-activated protein (MAP) kinases, and stress-activated kinases. For example, GR is basally phosphorylated in the synthesis (S) phase and hyperphosphorylated at the growth again/mitotic (G2/M) phase during the cell cycle, implicating cyclin-dependent kinases.[91] It is interesting to note that GR can be phosphorylated in response to glucocorticoid treatment during the S phase, but not the G2/M phase. This pattern of

GR phosphorylation is correlated with the ability of glucocorticoids to activate gene expression at different stages of the cell cycle. The phosphorylation status of the mouse GR has been shown to have a promoter-specific effect in transient transfection assays.[92] Mutation of phosphorylation sites reduced transcriptional activity on a minimal promoter containing simple glucocorticoid response elements, but not on the more complex mouse mammary tumor virus promoter.

Whereas the overall pattern of receptor phosphorylation is critical for receptor function, the contribution of site-specific phosphorylation of nuclear receptors is beginning to be appreciated. The ligand-dependent phosphorylation of serine 404 of GR expands the transcriptional profile of the receptor.[93] Additionally, phosphorylation of serine 134 of GR, which is ligand-independent, acts as a sensor of cellular stress and modulates glucocorticoid-mediated signaling.[94] Mutation of just one serine at position 118 of the ER reduced transcriptional activity by as much as 75%, but this effect was also cell- and promoter-specific.[95] This particular residue can be phosphorylated by MAP kinase *in vitro*. Additional studies have shown that *in vivo* activation of MAP kinase by growth factors leads to phosphorylation of serine 118 and potentiation of transcription via recruitment of a specific coactivator to AF1.[96] Phosphorylation of ER at other sites has been reported to alter receptor dimerization and DNA binding.[95] A phosphoserine at position 650 in the hinge region of the AR appears to be required for full transcriptional activity of this receptor.[97] Cross-talk between the PR and the neurotransmitter dopamine plays a physiological role in the control of sexual behavior in female mice: PR and sexual receptivity can be activated by dopamine in the absence of progesterone.[98] Like the ER, the PR can be phosphorylated in a ligand-independent manner at serine 294 by activation of MAP kinases.[99] Phosphorylation at this residue targets the PR for degradation by the proteasome, but paradoxically enhances transcriptional activity of the receptor.[100]

In summary, various kinases can phosphorylate steroid receptors via ligand-dependent and ligand-independent mechanisms. In turn, receptor phosphorylation can alter steroid hormone action. Given the general importance of these modifications, it is somewhat surprising that protein phosphatases have not been more widely studied for their effects on steroid hormone action.

Interaction with DNA

Steroid receptors enhance or repress gene transcription by forming homodimers that interact with specific DNA sequences in the promoter of target genes.[101] The core motif for hormone response elements consists of two six–base-pair sequences arranged as a palindrome with a three–base-pair spacer (i.e., the consensus estrogen-responsive element is AGGTCAnnnTGACCT, where *n* can be any nucleotide). One receptor in each homodimer contacts each half site. As described earlier, three amino acids in the DNA-binding domain are responsible for specific interactions between the ER and the consensus estrogen-responsive element versus the GR and the consensus glucocorticoid-responsive element (i.e., GGTACAnnnTGTTCT). However, hormone-specific patterns of gene regulation cannot be totally explained by this mechanism because the GR, MR, AR, and PR have similar consensus response elements, as determined by *in vitro* studies.

One explanation for specific regulation of gene expression by glucocorticoids, mineralocorticoids, androgens, and progestins is that natural hormone response elements diverge significantly from the glucocorticoid-response element consensus sequence described earlier. Although nucleotide variants generally lower the affinity of steroid receptors for the hormone response element, they increase binding specificity. In fact, a study of many different hormone response elements *in vitro* indicates that receptors discriminate among nucleotides in the spacer region and in the sequences flanking the six–base-pair motifs that directly interact with the receptor.[18] In other words, each steroid receptor responds in a unique way to different hormone response elements. Studies analyzing ERα and ERβ activity on different natural estrogen response elements support the hypothesis that steroid receptors interact with DNA in an allosteric manner,[102,103] which was later confirmed by crystallography of GR.[90] In turn, DNA-induced variation in receptor structure can influence the recruitment of coactivators to the receptor. Studies of hormone response elements from androgen-sensitive genes suggest another mechanism for steroid receptor specificity; the AR binds direct repeats of the six–base-pair motif, rather than the inverted palindrome typical of other steroid receptors.[104] Additional studies have shown that the distance of the hormone response element from the promoter influences transcriptional activity of steroid receptors.[105] In short, steroid receptor–DNA interactions contribute significantly to the specificity of steroid hormone action, as well as to variation in hormone responsiveness among genes.

Interaction with Coactivators and Corepressors

Within the last decade, it has become clear that the ability of steroid receptors to induce or inhibit gene transcription depends on interactions with proteins called *coactivators* and *corepressors*.[4,106] Steroid receptor recruitment of these proteins serves as a molecular switch regulating chromatin organization at target genes. Coactivators such as the Cyclic AMP Response Element Binding Protein (CREB)-binding protein and p300 have intrinsic histone acetylase activity. Covalent attachment of acetyl groups to specific lysine residues modifies nucleosome function and increases the access of basal transcription factors to promoters (Fig. 5.1). Conversely, low levels of histone acetylation are associated with tightly wound nucleosomes and gene repression (Fig. 5.1). Nuclear receptor corepressor (NCoR) is a protein that interacts with some but not all nuclear receptors to repress transcription. In the case of antagonist-bound ER and PR, NCoR is recruited along with proteins, such as histone deacetylase 3 (HDAC3), that have histone deacetylase activity.

The discovery of additional coregulatory proteins with enzymatic activity expands on the relatively simple model of transcriptional regulation by histone acetylation and deacetylation. These proteins have diverse functions, including methylation, demethylation, phosphorylation, dephosphorylation, poly(ADP-ribosylation), ubiquitination, and sumoylation of steroid receptors, coactivators, corepressors, and histones.[107,108] Overall, the transition between transcriptional repression and activation of a given gene by

steroids appears to result from highly dynamic and context-dependent interactions among these cofactors.

The kinetics of ER-dependent recruitment of coregulatory proteins to promoters has been examined in detail for two estrogen-responsive genes.[109-111] The general picture emerging from these studies is that different cofactors are brought to and released from the promoter in a specific order. Another important observation was that cofactor recruitment and release was cyclical, suggesting that recruitment of a particular cofactor is required for the subsequent recruitment of another cofactor and so on, until the final coregulatory complex is assembled. The final steps in this cycle are formation of the preinitiation complex and transcription by RNA polymerase. Repetition of this cycle suggests that steroid-dependent transcription within a cell would occur in waves and could be rapidly modulated by reversible covalent modifications of coregulatory proteins at each step during cofactor exchange.

Although some coactivators only enhance transcription on chromatin templates, others are able to augment steroid receptor transcriptional activity on naked DNA.[106] These proteins act as molecular bridges spanning the DNA-bound receptor and the basal transcription machinery, thereby stabilizing the preinitiation complex and facilitating transcription initiation. Steroid receptor coactivator 1 (SRC-1) and GRIP1 are two such proteins among a growing family of related nuclear receptor coactivators. These proteins contain multiple leucine-X-X-leucine-leucine (LXXLL, where *X* indicates any amino acid) motifs that are required for specific interactions with AF2 of different steroid receptors. For example, whereas the second LXXLL motif of GRIP1 interacts with the ER and is required to potentiate transcription, the third LXXLL motif of GRIP1 interacts with the GR to potentiate transcription.[11]

Although different coactivators may contribute to receptor-specific patterns of gene expression, there is a certain degree of functional overlap among members of the coactivator family. Recent studies have identified a host of other coactivator proteins (i.e., Vitamin D receptor interacting protein [DRIP], thyroid hormone receptor activating protein [TRAP], and Brahma-related gene 1 [Brg1]) that presumably add to the complexity and specificity of steroid hormone action.[106,112]

Interaction with Other Transcription Factors

Steroid receptors can interact with other transcription factors to facilitate or inhibit their activity. Interestingly, these effects are mediated by direct protein–protein interactions and do not require the DNA-binding domain of steroid receptors. A well-characterized example is the mutually antagonistic interaction between GR and NF-κB. These transcription factors are involved in the regulation of many proinflammatory genes, with glucocorticoids inhibiting gene expression and activators of NF-κB enhancing gene expression.[113] It appears that the GR hinders the transcriptional activity of NF-κB by multiple mechanisms, including direct and indirect interactions with NF-κB. There is some indication that GR–NF-κB interactions can prevent NF-κB from binding κB-responsive elements. In other cases, the GR does not block DNA binding, but interacts with the transactivation domain of the p65 subunit of NF-κB (Fig. 5.1). The GR thereby disrupts NF-κB interactions with the basal transcription machinery. Another hypothesis is that the GR competes for a limited number of coactivators that are required to form a bridge between NF-κB and the basal transcription machinery. Additional studies are required to clarify the relative importance of these mechanisms and whether they contribute to cell- or tissue-specific patterns of glucocorticoid antagonism of NF-κB signaling.

Interactions also occur between steroid receptors and the transcription factor AP-1 (Jun:Fos heterodimers). Whereas glucocorticoids and the GR generally antagonize AP-1 activity, estrogens and the ER can stimulate transcription via interactions with AP-1.[114,115] The mechanism responsible for the opposing effects of glucocorticoids and estrogens is currently unknown, but there is some evidence from transient transfection studies that antagonism between glucocorticoids and estrogens may be mediated by interactions at AP-1–responsive sites.[116] There are two distinct pathways for interaction between the ER and AP-1.[114] In the first, AP-1 stimulates transcription by recruiting CBP/p300 and p160 coactivators, such as GRIP1. The estrogen–ER complex then interacts with GRIP1 by the same AF1 and AF2 contacts as it does at estrogen-responsive elements. In a second pathway, estrogen antagonists bound to ER enhance the activity of AP-1 without physically interacting in the AP-1 complex. The antagonist–ER complex is believed to recruit corepressor proteins, such as NCoR, away from the AP-1 complex, thus relieving repression.

The ligand-bound ER can also interact with the C-terminal DNA-binding domain of the transcription factor Sp1. However, this interaction is independent of the DNA-binding domain of ER because DNA-binding–deficient forms of the ER can stimulate transcription via consensus GC-rich Sp1-binding sites. Taken together, these results indicate that protein–protein interactions are critical for cross-talk between ER and Sp1.[117] In yet another example of cross-talk, signal transducer and activator of transcription 5 (STAT5) and the GR synergistically activate the beta-casein gene.[118] Although steroid receptors interact with numerous transcription factors to enhance or inhibit gene expression in artificial systems, additional work is required to establish the physiological relevance of these mechanisms in whole organisms.

Nongenomic Actions of Steroids

Some effects of steroid hormones are entirely independent of their classic genomic action and are also independent of interactions with other transcription factors.[119] In general, these "nongenomic" effects occur rapidly and sometimes before those required for transcriptional activation. It is interesting to note that estrogen can have rapid, nongenomic effects, even though the ER is primarily nuclear in its localization. Roughly 2% of the cellular ER, however, is located at or near the plasma membrane and has been implicated in nongenomic signaling by estrogens.[120,121] Moreover, these early steroid effects cannot be blocked by inhibitors of transcription, such as actinomycin D, or inhibitors of translation, such as cycloheximide. Although rapid, nongenomic actions of steroids have been recognized for decades, the mechanisms responsible for these effects have only recently begun to be elucidated.[122] In this section, we will review a few examples of nongenomic actions for each class of steroid

hormone. This area of research is currently the most exciting and dynamic in the study of steroid hormone action.

Signaling Via Second Messenger Cascades

Skepticism surrounded many of the early studies of nongenomic effects because pharmacological doses of steroids were used. One of the first studies with physiologically relevant steroid levels showed that intravenous injection of 17β-estradiol produced a rapid increase in uterine cAMP in the presence of an inhibitor of transcription.[123] Subsequent work by others repeated these observations and extended them to show that estrogen-induced generation of cAMP could indirectly stimulate transcription via a cAMP response element.[124] Studies in breast cancer cells have shown that the G-protein–coupled receptor, GPR30, is required for activation of adenylate cyclase by estrogen antagonists.[125] Interestingly, estrogen antagonists could activate GPR30 in cells that lacked the classic ER. However, it is unclear whether the same mechanism is responsible for the rapid effects of estrogen agonists on cAMP levels in the uterus.

Estrogens have also been shown to activate phosphatidylinositol-3-OH kinase (PI3K) in an ER-dependent manner and to modulate cardiovascular function.[126] The ER binds in a ligand-dependent manner to the p85alpha regulatory subunit of PI3K, thereby increasing its activity. Activation of PI3K leads to the activation of protein kinase B (Akt), which directly phosphorylates and activates endothelial nitric oxide synthase (eNOS). Activated eNOS generates NO, causes vasodilation, and decreases vascular leukocyte accumulation. Estrogen-induced inhibition of leukocyte accumulation protects against ischemia–reperfusion injury. Inhibitors of PI3K and eNOS blocked the protective effect of estrogen. Much like the indirect activation of transcription via cAMP, estrogen can activate transcription indirectly by activating the PI3K/Akt pathway.[127] Hafezi-Moghadam and coworkers[128] showed a similar pattern of activation of eNOS by ligand-activated GR. Briefly, dexamethasone stimulated PI3-kinase and Akt, thereby activating eNOS. NO-dependent vasorelaxation led to decreased inflammation and reduced myocardial infarct size after ischemia–reperfusion injury in the mouse heart.

Glucocorticoids have rapid behavioral effects in a variety of vertebrates.[129] Sexual behavior is inhibited in less than 10 minutes by corticosterone in the roughskin newt. In contrast, glucocorticoids enhance lordosis within 5 minutes in female rats. Although the mechanisms involved have not been clearly defined, it appears that glucocorticoids influence neuronal signaling through the *N*-methyl-D-aspartic acid (NMDA) receptor. Corticosterone decreases female preference for male odors in mice. When coadministered with corticosterone, NMDA antagonists blocked this effect.[130] Preincubation of rat hippocampal neurons with glucocorticoids for just 10 minutes prolonged the NMDA-induced rise in intracellular Ca^{2+}.[131] Studies in rats have shown that extracellular aspartate and glutamate (excitatory neurotransmitters) levels increase in the hippocampus shortly after corticosterone administration.[132]

Mineralocorticoids also produce rapid, nongenomic effects that are not blocked by inhibitors of transcription or translation. For example, aldosterone administered at physiologically relevant concentrations (i.e., approximately 0.1 nM) activates the Na+/H+ transporter and increases intracellular pH in renal cells within 3 to 20 minutes.[133] In addition to this rapid effect on transporter activity, aldosterone induces transcription of the Na+/H+ transporter gene by the classic mode of steroid hormone action.[134] Nongenomic and genomic mechanisms of aldosterone action may therefore play important roles in acute and long-term electrolyte balance, respectively. Aldosterone also produces rapid effects on Na+/H+ transporter activity in other cell types, including human mononuclear leukocytes, endothelial cells, and vascular smooth muscle cells. Glucocorticoids were generally unable to activate the Na+/H+ transporter, indicating that the effect is specific for mineralocorticoids. A diacylglycerol-dependent mechanism involving PKC has been implicated in aldosterone activation of the Na+/H+ transporter in some, but not all studies.[135] Intracellular levels of inositol trisphosphate (IP_3) and calcium are rapidly elevated after mineralocorticoid treatment in colon cells. Whereas low calcium in the medium prevented the increase in intracellular calcium, depletion of intracellualar calcium stores by pretreatment with thapsigargin had no effect. Thus, calcium appears to enter from outside the cell in response to aldosterone, rather than being released from intracellular stores. Nevertheless, the MR does not appear to be required for rapid, nongenomic effects on calcium levels, thus implicating a non-classic receptor that has yet to be identified.[136]

Progesterone stimulates motility, Ca^{2+} influx, and the acrosome reaction in spermatozoa.[122,137] These effects are most likely nongenomic because they occur within a few seconds to minutes and because mature sperm have highly condensed chromatin that is transcriptionally inactive.[138] Given that progesterone is found in follicular fluid, in oviductal fluid, and in the extracellular matrix of oocytes around the time of fertilization, progesterone may play a physiological role in the acrosome reaction. In any case, progesterone induces a rapid Ca^{2+} influx in sperm that is blocked by Ca^{2+} chelators or La^{3+}. Thapsigargin, which elicits a rise in cytoplasmic Ca^{2+} by releasing intracellular stores, also triggers the acrosome reaction. Interestingly, PKC appears to be involved in mediating progesterone-induced increase in Ca^{2+}.[139] In conjunction with these changes, progesterone activates phospholipase C, causes rapid hydrolosis of phosphatidylinositol 4,5-biphosphate, and stimulates the synthesis and secretion of platelet-activating factor, which can itself induce the acrosome reaction. There is also evidence that progesterone may activate a distinct membrane receptor that interacts with and activates a gamma-aminobutyric acid GABA(A) receptor/chloride channel. In turn, this channel modulates the acrosome reaction.[137]

Androgens and estrogens rapidly activate the MAP kinase (i.e., sarcoma [Src]/Src homology 2 domain-containing [Shc]/extracellular signal-regulated kinase [ERK]) pathway and block programmed cell death (apoptosis) in a variety of cell types.[140] Androgen action on this signaling pathway is mediated by the classic AR and results from an interaction between the ligand-binding domain and Src homology domain 3 of the protein tyrosine kinase Src. Androgen activation of this pathway is dependent on a conformational change in Src and its subsequent interaction with Shc, which links receptor tyrosine kinases with intracellular signaling proteins. Further studies show that Shc is required for phosphorylation and activation of ERK1/2 in response

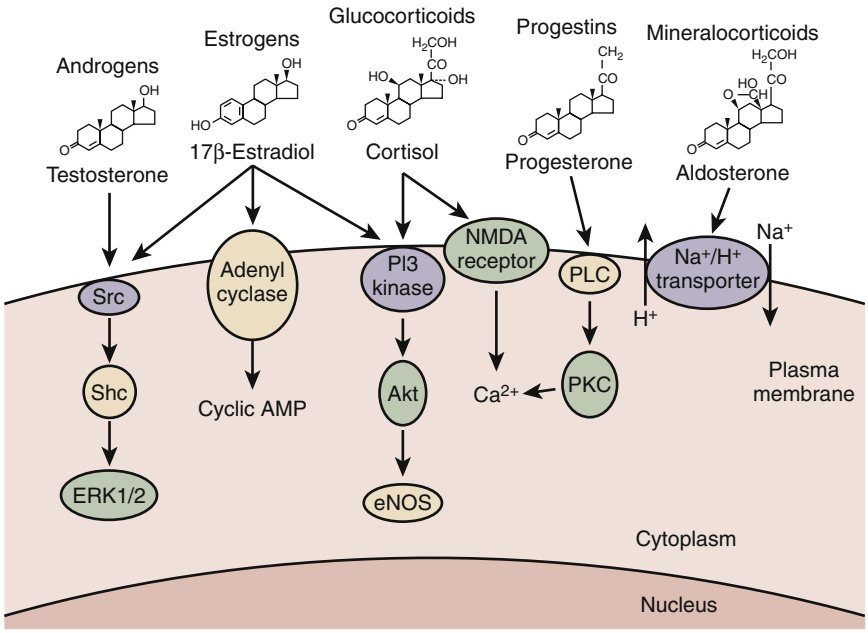


FIGURE 5.7 Schematic diagram showing representative non-genomic effects of steroids. Akt, protein kinase B; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; PI3, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; Shc, Src homology 2 domain; Src, sarcoma-inducing proto-oncogene.

to androgens. Although the events downstream of ERK 1/2 have not been elucidated, evidence is accumulating that the MAP kinase pathways play an important role in cell survival.[140] Estrogens activate the same MAP kinase pathway, but via interaction between ER and the SH2 domain of Src.[140] Based on these results, it was proposed that cross-talk between androgens and estrogens *in vivo* may be mediated in part by AR and ER interactions with Src. Another important finding from that study was that nongenomic and genomic effects mediated by the classic ER can be dissociated using synthetic ligands. Estren did not activate a reporter gene containing estrogen responsive element (ERE), but was able to protect against apoptosis. Conversely, pryazole activated a reporter gene containing EREs, but was unable to protect against apoptosis.

Summary

In conclusion, multiple interactions between steroid hormones and other signaling molecules increase the complexity of steroid hormone action. It has become widely accepted in recent years that physiologically relevant levels of steroids can activate second messenger pathways independently of their classic effects on gene expression (Fig. 5.7). In turn, these second messengers can significantly alter cell, organ, and whole animal physiology. It is important to note that steroid-dependent activation of non-classic pathways can also lead to changes in gene transcription.[124,127] Steroid hormones can therefore have strictly nongenomic effects, genomic effects that are indirectly mediated by second messenger pathways, and genomic effects mediated by steroid receptors that act as ligand-gated transcription factors. Inhibitory and synergistic cross-talk with other transcription factors is another important mode of action for steroid receptors. It has also been shown that steroid receptors can be activated by various factors (e.g., MAP kinases, dopamine) in a ligand-independent manner. As basic mechanisms of steroid hormone action are further elucidated, we will be able to address questions of critical importance for the treatment of disease and for maintenance of general health and well-being. For example, it will be critical to determine whether different mechanisms of steroid hormone action are independent of each other or whether inhibition or activation of one mechanism always affects other mechanisms. It is imperative to obtain such information if we hope to correct abnormal physiological and cellular processes without disrupting normal processes.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 6

Prostaglandins and Other Lipid Mediators in Reproductive Medicine

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The study of prostaglandins had its beginning in reproductive biology. In the 1930s, von Euler first characterized active substances present in semen that induced uterine contractility.[1] The active elements were named *prostaglandins*, based on the erroneous belief that they were produced by the prostate gland. We now know that the copious quantities of prostaglandins in semen are produced by the seminal vesicle gland and that prostaglandin biosynthesis is not limited to the accessory glands of the male reproductive system.

Discovery of the widespread occurrence of prostaglandins, their broad range of biological activities, chemical structures, and biosynthetic pathways, and determination of their relevance to the mechanism of action of nonsteroidal anti-inflammatory drugs (NSAIDs) and aspirin have engaged the interest of generations of reproductive endocrinologists. The general relevance of the field to diverse aspects of biology has prompted an era of intense and broad-based research that few other fields have experienced.[2] The Nobel Prize was awarded to Bergström, Samuelsson, and Vane in 1982 for elucidating the structure and biosynthesis of prostaglandins and for revealing the mechanism of action of NSAIDs.[3]

Today, prostaglandins and related lipid mediators are collectively referred to as *eicosanoids*. Eicosanoids (from *eicosi*, a Greek root meaning "20") are derived enzymatically from 20-carbon polyunsaturated fatty acids, principally arachidonic acid in mammalian species. They are produced by almost all cells in the body in response to hormonal stimulation or mechanical trauma. They evoke a wide array of biological actions in diverse tissues (including the reproductive system) at extremely low concentrations. Tissue specificity in eicosanoid action is afforded by selective expression of biosynthetic enzymes and membrane receptors (and perhaps also nuclear receptors) for eicosanoids. They are short-lived because they are susceptible to spontaneous or metabolical inactivation and act as paracrine or autocrine modulators of cellular function.

In this chapter, we first summarize the salient features of eicosanoid biosynthesis and pharmacology, and then we review the current understanding of their actions in the reproductive system. We also introduce at the end of the chapter other noneicosanoid lipid mediators that are now gaining recognition in reproductive medicine. For a more detailed and comprehensive review of the earlier literature on eicosanoids in reproduction, readers are urged to consult the corresponding chapter in a previous edition of this book.[4]

Biosynthesis of Eicosanoids

Nomenclature

The term *eicosanoid* refers to both prostaglandins and thromboxanes (products of the cyclooxygenase pathway, abbreviated as PG and TX, respectively) and leukotrienes (products of the 5-lipoxygenase pathway, abbreviated as LT). All PGs have a hairpin configuration and contain a cyclopentane ring with two side chains oriented in *trans* positions relative to the cyclopentenone ring. Each group of prostaglandins is allocated a letter (e.g., A, E, F), which denotes particular functional groups at carbons 9 and 11 of the ring structure.

The degree of unsaturation of the side chains is indicated by the subscript numeral after the letter; thus, PGE_1, PGE_2, and PGE_3 have one, two, and three double bonds, respectively. In the case of $PGF_{2\alpha}$, the subscript α denotes the stereochemistry of the 9-hydroxyl group in the cyclopentane ring.

A similar system is used for nomenclature of TXs that have oxygen-bridged six-member rings in place of the five-member ring seen in PGs. All LTs have three conjugated double bonds, and the subscript numeral (e.g., the 4 in LTA_4 and LTC_4) denotes the total number of double bonds in the molecule. LTA_4 is an unstable epoxide and serves as a parent molecule in the biosynthesis of LTB_4 (hydrolysis product), LTC_4, LTD_4, and LTE_4 (peptide derivatives).

Release of Arachidonic Acid From Phospholipid Stores

Eicosanoids are not stored, but rather are synthesized *de novo* when cells are activated by hormonal or mechanical

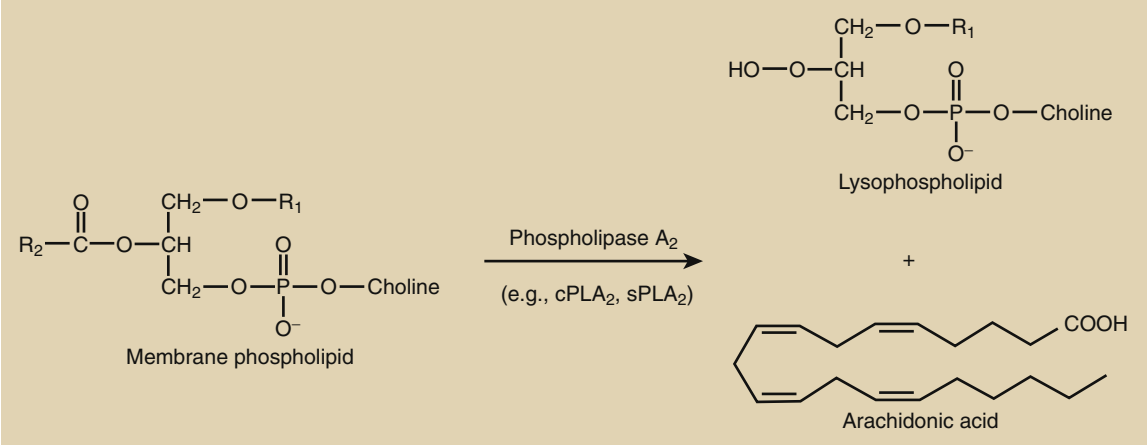


FIGURE 6.1 Release of free arachidonic acid from membrane phospholipid stores is the first step in eicosanoid biosynthesis. Arachidonic acid (R_2-COOH), located at the sn-2 position of phospholipids (phosphatidylcholine, phosphatidylethanolamine, and phospatidylinositol) is mainly cleaved by phospholipase A_2 enzymes when cells are activated. R_1 is often a C16 or C18 saturated or monounsaturated fatty acid in acyl linkage.

stimuli. The first obligatory (and often rate-limiting) step in eicosanoid biosynthesis is the release of arachidonic acid from membrane phospholipid stores (Fig. 6.1).[5,6] This process is exquisitely regulated by a host of enzymes, particularly several types of phospholipase A_2 (PLA_2),[7] and it appears to be coordinated with the induction of downstream eicosanoid biosynthetic enzymes.[6,8-10]

Recent studies suggest that at least three types of PLA_2 are involved in arachidonic acid release during different stages of cell activation.[6] Under basal conditions, the Ca^{2+}-independent PLA_2 ($iPLA_2$) is the dominant phospholipase involved in the liberation of arachidonic acid and other polyunsaturated fatty acids from membrane phospholipids.[6] The phospholipase $iPLA_2$ is primarily involved in membrane remodeling and usually does not cause significant eicosanoid production, because its activity in arachidonic acid release is balanced by acylase enzymes. However, the activity of $iPLA_2$ may become significant when acylase enzymes are down-regulated. For example, mice heterozygous for acyl-coenzyme A synthetase 4 deficiency accumulated high levels of uterine PGs, had abnormal uterus polycysts, and displayed reduced fertility.[11]

When cells are activated, such as by receptor ligation or calcium ionophore stimulation, intracellular calcium levels rise and the Ca^{2+}-dependent cytosolic PLA_2 ($cPLA_2$) becomes involved.[6] The activity of $cPLA_2$ far exceeds that of acylase enzymes, and as a result, there is rapid accumulation of free arachidonic acid to be acted on by either constitutive or inducible eicosanoid-synthesizing enzymes.

Under certain situations of sustained cellular activation, secreted PLA_2 ($sPLA_2$) family members may also participate, further increasing the supply of free arachidonic acid to meet the need of eicosanoid-synthesizing enzymes. Clearly, $cPLA_2$ plays a key role among these enzymes because cells lacking $cPLA_2$ are generally devoid of eicosanoid biosynthesis.[7]

Pathways of Eicosanoid Biosynthesis

After its release from membrane phospholipid stores, arachidonic acid is transformed enzymatically to various eicosanoids through multiple pathways (Fig. 6.2). One pathway is initiated by the enzyme PG H synthase (PGHS, also known as *cyclooxygenase* [COX])[12] and results in the production of PGs, TX (TxA_2), and prostacyclin (PGI_2). These cyclooxygenase-derived products are also referred to as *prostanoids*.

The initial cyclized fatty acid derivative formed in this reaction is PG G_2 (PGG_2). The peroxide moiety at the 15-carbon position of PGG_2 is subsequently reduced to an alcohol group to form PGH_2 as a result of the inherent peroxidase activity of PGHS (Fig. 6.2). Both PGG_2 and PGH_2 are unstable intermediates with very short half-lives.

The conversion of PGH_2 to individual PGs is relatively tissue-specific, depending on the local expression of specific PG-synthesizing enzymes.[12,13] For example, TxA_2 is synthesized in platelets and macrophages, whereas PGI_2 is the dominant COX product of macrovascular endothelial cells.[2,13]

The second major pathway of arachidonic acid metabolism is initiated by lipoxygenases. Like cyclooxygenase, lipoxygenases (LOX) are also dioxygenases that catalyze the insertion of molecular oxygen into arachidonic acid to form a hydroperoxy derivative.[14] The insertion of molecular oxygen can occur at several carbon positions (e.g., 5, 12, and 15) along the fatty acid chain. These determine the specificity of the enzymes, hence, the origin of names such as *5-lipoxygenase* and *12-lipoxygenase*.[14] The enzyme 5-lipoxygenase is of particular importance because its product, 5-hydroperoxy-eicosatetraenoic acid (5-HPETE), serves as a precursor for the LT family of eicosanoids, which are involved in host defense and immediate-type hypersensitivity reactions.[2,15]

Other important eicosanoids formed from lipoxygenase pathways include lipoxins, which are mostly produced by the interaction of neutrophils with other cells in the vascular compartment. They are believed to play a role in the resolution of inflammatory responses.[16]

Arachidonic acid can also be metabolized by an additional pathway, through the activity of cytochrome P450 enzymes.[17] Unlike the cyclooxygenases or lipoxygenases, cytochrome P450 enzymes catalyze the mono oxygenation (insertion of one atom of oxygen from O_2) of arachidonic

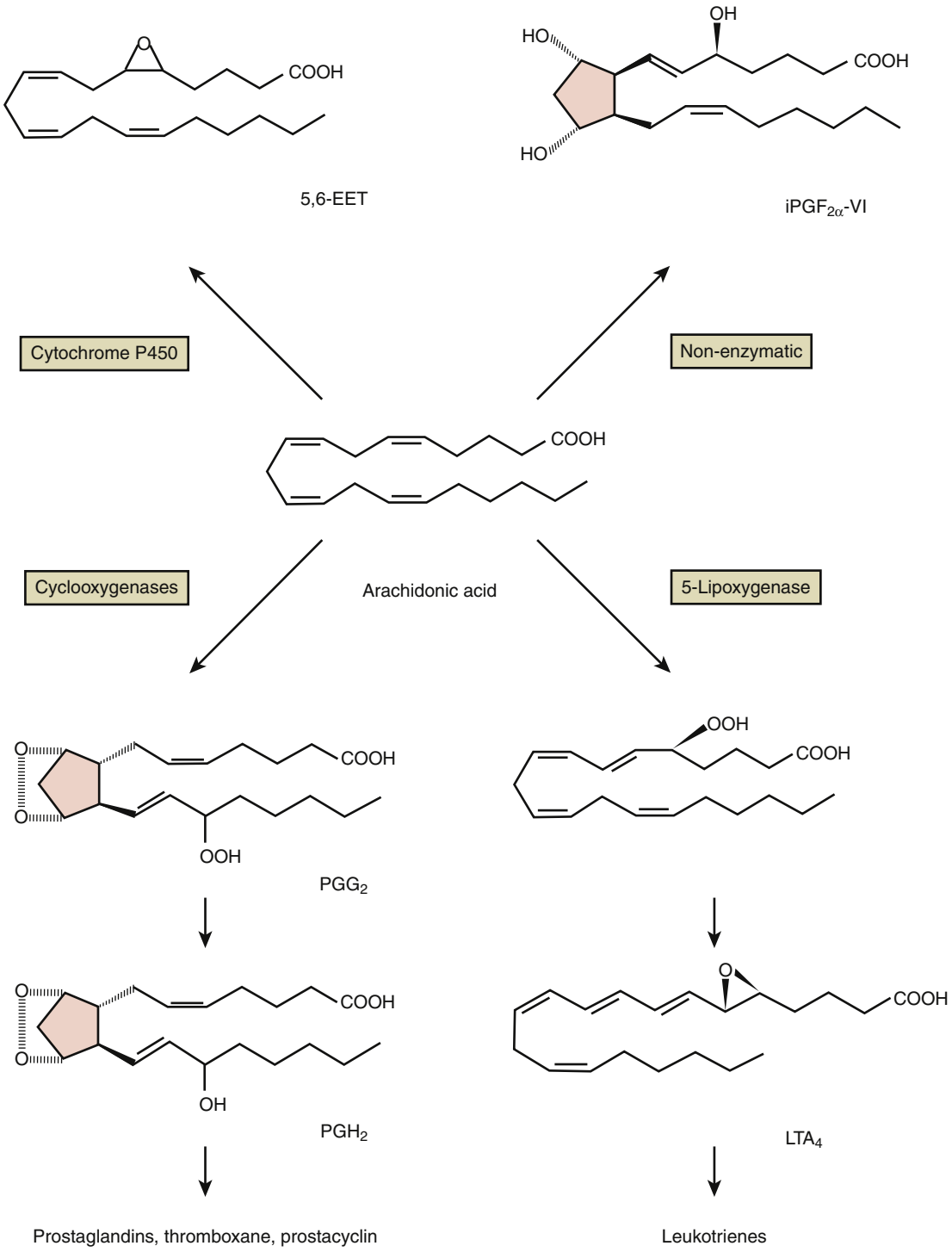


FIGURE 6.2 Major pathways of eicosanoid biosynthesis from arachidonic acid. Arachidonic acid is converted by two forms of cyclooxygenase (formally known as *prostaglandin H synthase*) to PGH_2 and then to prostaglandins, thromboxane, and prostacyclin; by lipoxygenases to leukotrienes (depicted) and other products, such as hydroxy-eicosatetraenoic acids (HETEs) (not shown); by cytochrome P450 enzymes to epoxy-eicosatrienoic acids (EETs; 5,6-EET is shown as an example). Arachidonic acid can also be converted nonenzymatically to isoprostanes through free radical–catalyzed peroxidation and cyclization (there are 64 potential isomers of isoprostanes[22,23]; the structure of isoprostane isomer VI of prostaglandin F2 alpha ($iPGF_{2\alpha}$-VI) is shown as an example). In contrast to the enzymatic pathways, formation of isoprostanes can occur on phospholipids, and release of free arachidonic acid is not a prerequisite.[24]

acid, with hydroxy or epoxy derivatives of arachidonic acid as their major products.[17] Cytochrome P450 products modulate nephron ion transit and renovascular tone.[17-19] Some cytochrome P450–derived epoxy metabolites of arachidonic acid are elevated in women with pregnancy-induced hypertension,[20] and others may have anti-inflammatory activities.[21]

Lastly, peroxidation of arachidonic acid and cyclization to PG-like compounds called *isoprostanes* can also occur nonenzymatically by free radical–catalyzed reactions.[22,23] In

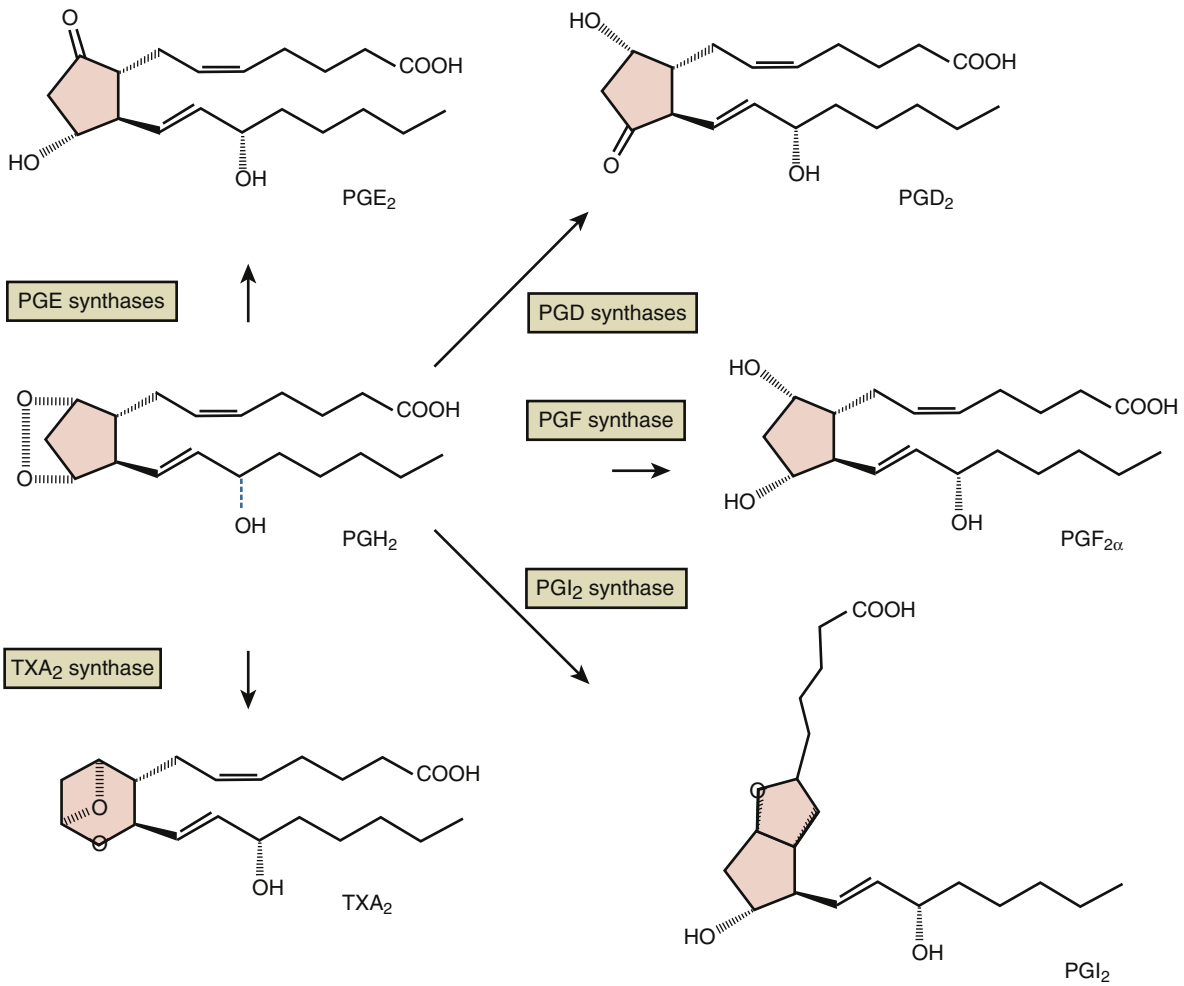


FIGURE 6.3 Biosynthesis and structures of prostanoids from prostaglandin (PG) H_2 (PGH_2). Formation of PGE_2 is catalyzed by PGE synthases, of which at least three have been identified.[8-10,30,31] Formation of PGD_2 is catalyzed by at least three different PGD_2 synthases.[192] Formation of $PGF_{2\alpha}$ is catalyzed by PGF synthase, a member of the aldo-keto reductase family of enzymes. Thromboxane A_2 (TxA_2) and PGI_2 are formed by TxA_2 synthase and PGI_2 synthase, respectively. Both enzymes are cytochrome P450 enzymes associated with microsomal membranes.

contrast to the enzyme-produced eicosanoids, isoprostanes can be formed *in situ* on phospholipids and then released by phospholipases.[24] Isoprostanes may serve as incidental ligands for eicosanoid receptors under certain conditions.[23,25]

More importantly in the clinical setting, urine and plasma isoprostane levels have proven to be reliable markers of lipid peroxidation and oxidant stress in vivo.[22,23] For example, elevated levels of urinary isoprostanes were detected in women with android obesity[26] and in individuals with alcohol-induced liver injury.[27] Both conditions are associated with increased oxidant stress and inflammation, as determined by other independent markers.

Major Products of the Cyclooxygenase Pathway: Prostaglandins, Thromboxanes, and Prostacyclin

Two COX enzymes that initiate the formation of prostaglandins (Fig. 6.2), encoded by separate genes, have been identified in vertebrates.[12,28] One COX enzyme (COX-1) is largely constitutively expressed, whereas the other (COX-2) is more often inducible by cytokines, growth factors, and hormones. This categorization as constitutive COX-1 and inducible COX-2 is useful, but is not always the case (e.g., COX-2 is constitutively expressed in some organs, such as the kidney glomerulus[29]).

The subsequent conversion of PGH_2 to individual prostanoids is relatively tissue-specific and is catalyzed by the corresponding isomerases and synthases (Fig. 6.3).[28] For example, TxA_2 synthase is expressed in platelets and macrophages, PGI_2 synthase is found in endothelial cells, and $PGF_{2\alpha}$ synthase is abundant in the uterus.[2]

There are at least two types of glutathione-dependent PGE_2 synthases.[8-10,30] One PGE_2 synthase, cPGES, is a constitutively expressed cytosolic enzyme functionally coupled to COX-1; the other is a microsomal and inducible enzyme, mPGES-1, which appears to be coupled to COX-2.[8-10,30] Another enzyme, mPGES-2, found in bovine endometrium and other sites, is less well characterized, and its role in PGE_2 synthesis is unclear.[31]

Major Products of the 5-Lipoxygenase Pathway: The Leukotrienes

Products of the 5-lipoxygenase pathway with well-defined biological activities are mainly produced by inflammatory

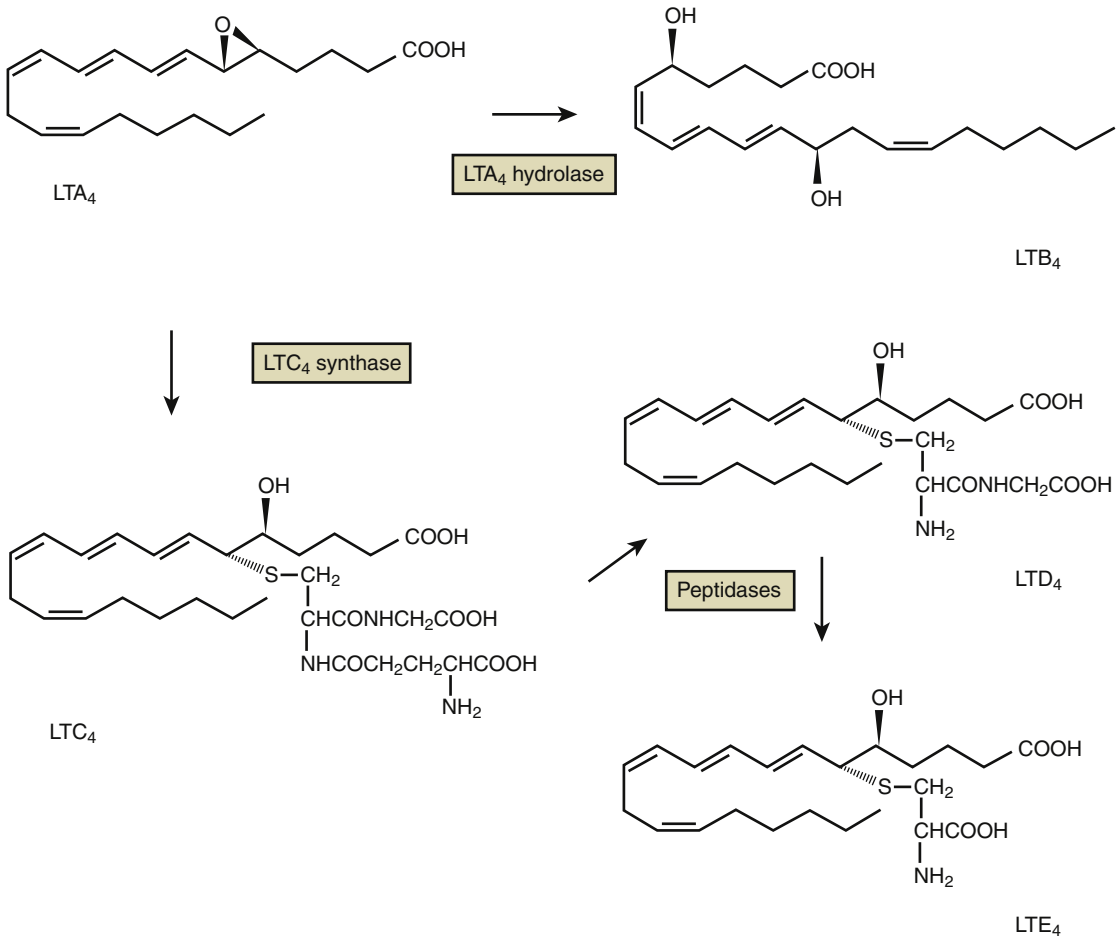


FIGURE 6.4 Structures and biosynthesis of leukotrienes (LTs) from LTA_4. LTA_4 is formed by 5-lipoxygenase and is prone to spontaneous hydrolysis. Hydrolytic attack by LTA_4 hydrolase produces LTB_4, a dihydroxy derivative of arachidonic acid with specific stereochemistry of the hydroxy groups. Conjugation of LTA_4 with glutathione is catalyzed by LTC_4 synthase. Sequential cleavage of the oligopeptide moiety in LTC_4 by extracellular peptidases yields LTD_4 and LTE_4.

cells.[2,32-34] The activation and biochemical cascade of the 5-lipoxygenase (5-LOX) pathway in leukocytes is particularly well understood, because it gives rise to the potent LT family of lipid mediators that have chemotactic or smooth muscle–contracting properties.[2]

Biosynthesis of LTs is initiated by the formation of 5-HPETE from arachidonic acid by 5-LOX in a Ca^{2+}-dependent process that involves translocation of both 5-LOX and $cPLA_2$ to the nuclear envelope.[2,35,36] The activity of 5-LOX also requires the cooperation of an accessory protein, 5-lipoxygenase–activating protein (FLAP), in intact cells.[37]

Some 5-HPETE molecules can escape from the active site of the 5-LOX enzyme and are ultimately reduced by cellular peroxidases to the corresponding 5-hydroxy derivative of arachidonic acid (5-HETE). Other 5-HPETE molecules may be further converted to the labile epoxide known as *leukotriene* A_4 (LTA_4) by a secondary dehydrase activity contained in 5-LOX (Fig. 6.4).[34] LTA_4 undergoes one of several potential transformation routes, depending on the cellular context.

Hydrolytic attack of LTA_4 by LTA_4 hydrolase produces LTB_4[38] a potent chemotactic agent for neutrophils. The conjugation of LTA_4 with glutathione is catalyzed by LTC_4 synthase, thereby forming LTC_4 at the nuclear envelope.[39] Sequential cleavage of the peptide moiety of LTC_4 by extracellular peptidases produces LTD_4 and LTE_4 (Fig. 6.4).

The three cysteinyl LTs, LTC_4, LTD_4, and LTE_4, are the major constituents of the *slow-reacting substance of anaphylaxis* released from antigen-sensitized guinea pig lungs that produced slow and sustained smooth muscle contraction of intestinal smooth muscle strips, observed more than 60 years ago.[32] LTA_4 can also serve as a precursor for the transcellular biosynthesis of lipoxins.[40] A number of biological activities, particularly in the resolution of the inflammatory response, have been proposed for lipoxins.[16]

Lipoxygenases with catalytic oxygenase specificity at different carbon positions of arachidonic acid, such as 12-LOX and 15-LOX, produce the corresponding HETEs as their main end products.[14] The physiological role of these enzymes and their specific products has proven to be elusive—although the murine 15-LOX homologue, which produces both 12-and 15-LOX products, has been implicated in atherogenesis.[41,42] Interestingly, the HPETE product of this enzyme is involved in the initiation and propagation of low-density lipoprotein oxidation, a process known to contribute to the accumulation of fat-laden macrophages in atherosclerotic lesions.[41,42] Products of the same enzyme may also regulate local interleukin-12 production by macrophages in atherosclerotic lesions.[43]

Transport and Metabolism of Eicosanoids

Although eicosanoids are lipid compounds, they do not permeate the cell membrane freely. A PG transporter (PGT), belonging to the organic anion transporter polypeptide family, has been identified and is found in a limited range of cells, where it is subject to humoral and physical stimuli.[44] PGT mediates the cellular uptake of most prostanoids, but not PGI_2.[44] Vascular expression of PGT appears to be responsible for clearing most classes of PGs when these compounds are infused into an animal and enter the pulmonary circulation.[44] On the other hand, multidrug-resistance protein 4 (MRP4) functions as a PG efflux transporter.[45] In the LT pathway, newly synthesized LTC_4 is transported outside of the cells by transporters such as multidrug-resistance protein 1 (MRP1).[46]

A hallmark of eicosanoids is the transient nature of their existence. Products such as PGI_2 and TxA_2 are chemically unstable and are degraded spontaneously through hydrolysis in aqueous solutions, particularly at neutral to acid pH. PGI_2 undergoes hydrolysis to form 6-keto $PGF_{1\alpha}$, a stable and inactive product, whose metabolite, 2,3-dinor-6-keto-$PGF_{1\alpha}$, has been used as a surrogate marker for systemic PGI_2 biosynthesis *in vivo*. Similarly, TxA_2 rapidly undergoes facile hydrolysis to form the inactive product TxB_2, which is subject to beta oxidation. Plasma or urinary TxB_2 metabolites have served as useful indices of TxA_2 formation *in vivo*.

The most important catabolical step for PGs is the conversion of the 15-hydroxy group to a 15-keto group by a NAD(+)-dependent 15-hydroxyprostaglandin dehydrogenase (15-OH-PGDH).[47] 15-OH-PGDH is a cytosolic protein with highest concentrations in the lungs, the placenta, the spleen, and the kidney cortex.[47] Both PGE_2 and $PGF_{2\alpha}$ are excellent substrates for 15-OH-PGDH.[47]

The second notable step in the degradation of PGs is reduction of the double bond at position 13 by $\Delta^{13,14}$-PG reductase, which is highly specific for 15-keto PGs; this enzyme has a tissue distribution similar to that of 15-OH-PGDH.[48] The metabolites then undergo both beta (i.e., carboxyl chain) and omega oxidation, resulting in urinary excretion of the final products.

Pharmacology of Eicosanoids

COX-1, COX-2, Traditional NSAIDs, and Selective COX-2 Inhibitors

The discovery of the COX-2 enzyme and its physiological and pharmacological characterization in the early 1990s hailed a new era in eicosanoid research.[49] COX-2 expression is readily inducible by cytokines, growth factors, and hormonal stimulation.[12] The two COX enzymes, within a given species, share 60% to 65% sequence identity in their primary structures.[12]

Both enzymes are membrane-anchored proteins, localizing primarily to the luminal surface of the endoplasmic reticulum and the nuclear envelope.[12] The crystal structures of COX-1 and COX-2 are remarkably similar, as are their catalytical mechanisms and kinetics. However, two subtle structural and biochemical differences have been noted between the two isozymes. First, the active site of COX-2 is larger and more accommodating than COX-1; this property

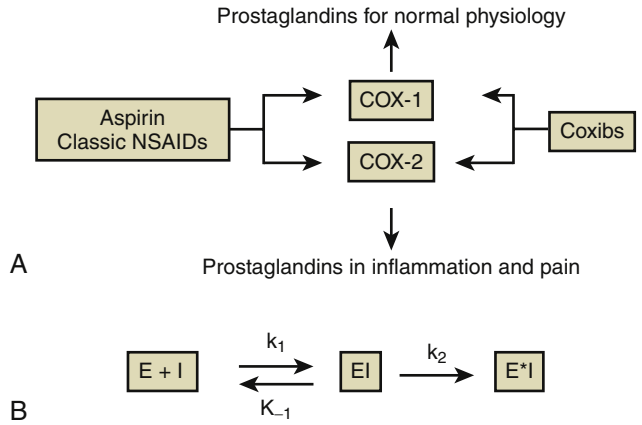


FIGURE 6.5 Mechanism of action of aspirin, traditional nonsteroidal anti-inflammatory drugs (NSAIDs), and coxibs. **A,** Aspirin and traditional NSAIDs inhibit both COX-1 and COX-2, whereas coxibs preferentially inhibit COX-2. **B,** All COX enzyme (E) inhibitors (I) exhibit one of three kinetic models of inhibition: (1) rapid, reversible binding to form EI (e.g., ibuprofen); (2) rapid, lower-affinity reversible binding, followed by time-dependent, higher-affinity, slowly reversible binding (e.g., flurbiprofen and coxibs); (3) rapid, reversible binding, followed by covalent modification (acetylation) of a serine residue in COX (aspirin). The mechanism for the time-dependent inhibition is not well understood, but may involve conformational change of the enzyme (E*I). Coxibs confer their selectivity for COX-2 by causing time-dependent inhibition of COX-2, but not COX-1 enzymes.

was exploited by the pharmaceutical industry for the purpose of developing selective COX-2–specific NSAIDs. Second, the gross kinetics (e.g., K_m, V_{max}) for COX-1 and COX-2 are similar, but COX-2 competes more efficiently with COX-1 in intact cells when the release of arachidonic acid is limiting.[6,28] Thus, when COX-1 and COX-2 are both present in the same cells, catalysis of arachidonic acid (provided exogenously or through activation of cellular lipases) is favored by COX-2.[6] COX-1 appears to function independently only when COX-2 is absent or inhibited.[6,28,50]

Researchers have proposed that this difference in sensitivity to arachidonic acid concentration is related to the sensitivity of their heme groups to lipid peroxide–catalyzed oxidation, and thus activation of the cyclooxygenase enzymes.[51] The concentration of lipid peroxide required to activate COX-1 is 10 times more than that required to activate COX-2 in the presence of low concentrations of arachidonic acid.[51]

Aspirin and traditional NSAIDs (e.g., indomethacin) function as nonselective inhibitors of both COX-1 and COX-2,[12] and their mechanisms of action have been extensively investigated, even before the discovery of COX-2. Long-term use of traditional NSAIDs in managing inflammatory disorders is associated with significant adverse effects, such as gastrointestinal bleeding (Fig. 6.5).[2,12,28,49] COX-2 was initially portrayed as the COX isoform responsible for producing PGs that contribute to the inflammatory process, which offered a rationale for developing selective inhibitors targeted at COX-2.

According to this "outdated" paradigm, COX-1 is responsible for synthesizing the "housekeeping" PGs required for maintaining normal physiological (e.g., mucosal protection in the stomach and normal kidney function). Thus, the adverse

Table 6.1 Summary of Eicosanoid Receptor Types and Properties

Receptor	Cognate Ligand	Property*	Intracellular Signaling
IP	PGI_2	Relaxing	↑ cAMP
TP	TXA_2	Contractile	↑ Ca^{2+}
FP	$PGF_{2\alpha}$	Contractile	↑ Ca^{2+}
DP1	PGD_2	Relaxing	↑ cAMP
DP2 (CRTH2)	PGD_2	Chemoattractant	↑ Ca^{2+}
EP1	PGE_2	Contractile	↑ Ca^{2+}
EP2	PGE_2	Relaxing	↑ cAMP
EP3	PGE_2	Inhibitory	↓ cAMP
EP4	PGE_2	Relaxing	↑ cAMP
BLT1	LTB_4	Leukocyte Restricteds	↑ Ca^{2+} ↓ cAMP
BLT2	LTB_4	Widely distributed	↑ Ca^{2+} ↓ cAMP
CysLT1	LTD_4	Lung SMC, macrophages	↑ Ca^{2+}
CysLT2	LTC_4, LTD_4	Spleen, Purkinje fibers, etc.	↑ Ca^{2+}

*Smooth muscle activities.
cAMP, cyclic AMP; SMC, smooth muscle cells.

effects associated with traditional NSAID use would result from their inhibition of the COX-1 enzyme, whereas their anti-inflammatory efficacy would be attributable to coincidental inhibition of COX-2 (Fig. 6.5). This principle formed the basis for the development and marketing of the selective COX-2 inhibitors known as the coxibs (e.g., celecoxib, rofecoxib, and valdecoxib) for treating the arthritides[2,52]; however, a designation of COX-1 and COX-2 as "good" and "bad" enzymes turned out to be an oversimplification.

COX-1 and COX-2 genes and enzymes are present even in zebrafish[12,53] suggesting that both enzymes have fundamental physiological roles to play. Indeed, both COX-1 and COX-2 gene–disrupted mice display multiple developmental and physiological defects, including abnormalities in steps of reproduction.[54-57] Also, the administration of the selective COX-2 inhibitors to healthy human subjects suppressed systemic prostacyclin biosynthesis.[58] This finding further supports the notion that, in some settings, for example, endothelial cells subjected to hemodynamic shear, COX-2 is constitutively expressed and active, participating in normal physiological processes.

Eicosanoid Receptors

An explosion of knowledge regarding the expression, structure, signaling pathways, and physiological functions of eicosanoid receptors has taken place in the past 20 years.[59,60] The existence of membrane receptors for eicosanoids was supported by early pharmacological evidence[61] and was consistent with the notion that eicosanoids are locally released paracrine mediators acting on self and neighboring cells.

The first eicosanoid receptor to be cloned was the human TX receptor in 1991.[62] There are 13 distinct eicosanoid receptors cloned and characterized, 9 for cyclooxygenase-derived prostanoids and 4 for LTs (Table 6.1).[2,59,60] All 13 eicosanoid receptors characterized so far are rhodopsin-like, seven transmembrane domain–containing, G-protein–coupled receptors.[59,60] The nine G-protein–coupled prostanoid receptors, conserved in mammals from mouse to human, are the TX receptor (TP), the prostacyclin receptor (IP), the $PGF_{2\alpha}$ receptor (FP), two PGD receptors (DP1 and DP2), and four PGE_2 receptors (EP1, EP2, EP3, and EP4). These receptors are encoded by separate genes. In addition, there are several splice variants for the EP3, FP, and TP receptors, which differ only in their C-terminal tails (Fig. 6.6).[59,63-65]

The second PGD receptor, DP2 (also known as CRTH2),[66] induces intracellular calcium mobilization and is a chemoattractant receptor for type 2 T-helper cells.[66] DP2 is also found on human eosinophils where both PGD_2 and 15-deoxy-PGJ_2 (a degradation product of PGD_2) serve as activation ligands at nanomolar concentrations.[67,68]

The remaining eight prostanoid receptors fall into three categories according to their functional properties and the secondary messenger systems to which they couple. IP, DP1, EP2, and EP4 are smooth muscle–"relaxing" receptors that signal predominantly through a G_s-mediated increase in intracellular cyclic AMP (cAMP). The FP, TP, and EP1 receptors form the "contractile" subgroup and transduce their signals through a G_q-mediated increase of intracellular calcium. The EP3 receptor is regarded as an "inhibitory" receptor because it is coupled to G_i and causes inhibition of intracellular cAMP.

Several aspects of prostanoid receptor biology and pharmacology require comment. First, the existence of four subtypes of PGE_2 receptors is quite remarkable because all other prostanoids (except PGD_2) each have only a single receptor. Sequence-based phylogenetic tree analysis of the prostanoid receptors reveals that the four PGE_2 receptors were the first to evolve because homology among these subtypes is less than that among receptors of different classes of prostanoids.[59,60] Thus, the four PGE_2 receptors must have evolved to play distinctive physiological functions, and other prostanoid receptors are likely derived from functionally related PGE_2 receptor subtypes through gene duplication.[60]

Second, although there is high ligand selectivity for each of the prostanoid receptors, cross-activation by prostanoids other than the cognate ligand may occur; coupling to alternative downstream signaling pathways at different concentrations of the same ligand may also take place.[60,69] Third, the multiplicity in prostanoid receptor subtypes and signaling pathways, coupled with their differential tissue expression, explains why the same PG at different concentrations or in different tissues can sometimes produce opposing effects.

Among the four membrane LT receptors characterized to date, two are receptors for LTB_4 (BLT1 and BLT2) and two are for the cysteinyl LTs (CysLTl and CysLT2). The high-affinity ligand-binding BLT1 is primarily expressed on leukocytes,[70] whereas BLT2 has a wider tissue distribution and binds LTB_4 with lower affinity.[71] Both of these receptors signal through intracellular calcium mobilization, as well as through inhibition of adenylyl cyclase.

The existence of distinct cysteinyl LT receptors has been supported by pharmacological evidence.[72] CysLT1 has been the primary target for the development of anti-LT drugs for asthma symptom management. Molecular characterization of CysLTl confirmed it to be a G-protein–coupled receptor, with LTD_4 as its preferred endogenous ligand.[73] CysLTl is expressed on lung smooth muscle cells and tissue macrophages, and activation of the receptor results in an elevation of intracellular calcium.[73]

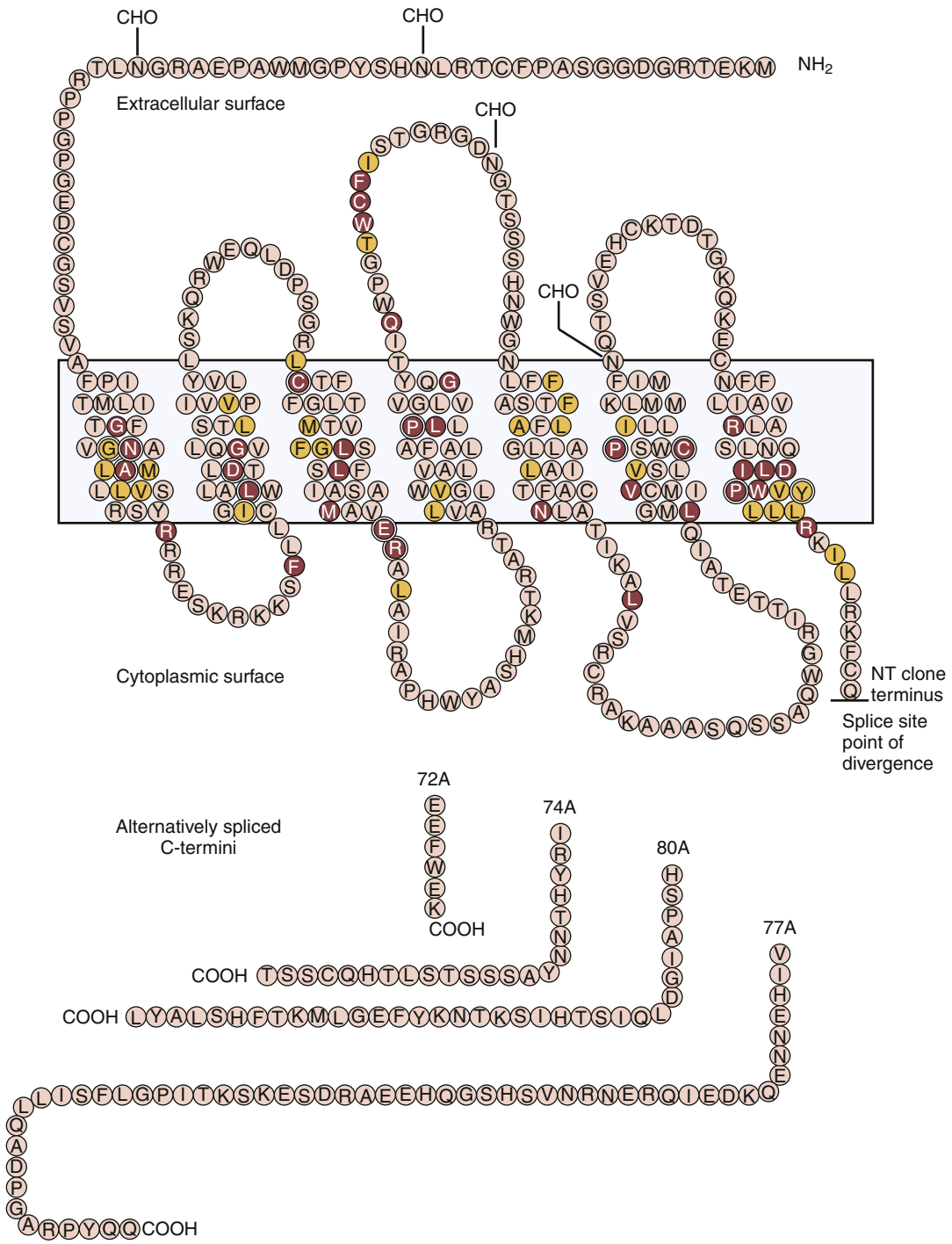


FIGURE 6.6 Schematic representation of five rabbit EP3 receptor splice variants differing only in their intracellular carboxyl termini. The carboxyl-variable tails range from 56–amino acid residues for clone 77A to none for the NT (no-tail) clone. Conserved residues in the EP3 receptor are also identified after EP3 receptor sequences from various species are aligned with those of other prostanoid receptors. Conserved residues are indicated by *yellow circles,* and invariant residues are indicated by *red circles.* Residues with *bulls-eye symbols* are conserved across the entire superfamily of G-protein–coupled receptors; those without this inset are unique to the prostanoid receptors. The amino acid sequences of each splice variant are represented by a one-letter amino acid code. *(From Breyer RM, Bagdassarian CK, Myers SA, et al. Prostanoid receptors: subtypes and signaling.* Annu Rev Pharmacol Toxicol *41:661–690, 2001, with permission.)*

CysLT2, originally defined by pharmacological methods in trachea and pulmonary vein preparations, is expressed in the spleen, Purkinje fibers of the heart, the adrenal gland and the microvasculature of certain organs.[74,75] Both LTD_4 and LTC_4 are equipotent agonists for this receptor, which also increases intracellular calcium when activated.[74] Other CysLT receptors may exist but still await definitive characterization.

Apart from activating plasma membrane receptors, there is considerable interest in the putative functions of eicosanoids as ligands for nuclear receptors, specifically members of the peroxisomal proliferator–activated receptor (PPAR)

Table 6.2 Physiological Roles and Therapeutics of Eicosanoids in Reproduction

Function	COX Enzyme	Eicosanoids	Receptor	Therapeutics
Ovulation	COX-2	PGE_2	EP2	
Luteolysis		$PGF_{2\alpha}$	FP	
Fertilization	COX-2			
Implantation	COX-2	PGI_2	PPAR	
Decidualization	COX-2	PGI_2	PPAR	
Dysmenorrhea	COX-2	$PGE_2/PGF_{2\alpha}/PGI_2$		NSAIDs/coxibs
Ductus arteriosus remodeling	COX-2	PGE_2	EP4	
Preeclampsia		PGI_2/TxA_2		Aspirin?
Cervical ripening				PGE analogs
Preterm labor	COX-2	$PGE_2/PGF_{2\alpha}$		NSAIDs/coxibs
Labor	COX-1	$PGF_{2\alpha}$	FP	
Erectile dysfunction				PGE_1

COX, cyclooxygenase; NSAIDs, nonsteroidal anti-inflammatory drugs.

family. However, unlike the well-accepted functions of the membrane receptors, the involvement and *in vivo* significance of nuclear receptors in eicosanoid signaling remains an evolving concept.[76] Several prostanoids in the PGA and PGD series have been shown to be ligands for PPARα or PPARδ in receptor activation and functional assays (e.g., promotion of fibroblast differentiation to adipocytes); dehydration products of these prostanoids (such as 15-deoxy-$\Delta^{12,14}$-PGJ_2) and products of lipoxygenase pathways, such as 8(S)-HETE and LTB_4, have similar activities.[77-80]

PGI_2 formed from COX-2 has been proposed to act as a natural ligand for PPARδ to mediate processes ranging from colorectal carcinogenesis,[81] to apoptosis,[82] to embryo implantation.[83] However, the relative importance of eicosanoids signaling through nuclear receptors under physiological conditions remains speculative compared to the well-established signaling via eicosanoid G protein receptor-coupling.

Eicosanoids and Reproduction

The role of eicosanoids in reproductive processes is one of the most intensely investigated of their biological functions. In fact, the uterine-contracting property of PGs led to their discovery. The activity of PGs in stimulating uterine contractility and inducing cervical ripening rendered them and their synthetic analogs attractive candidates for labor induction and as abortifacients.[84-86] For example, misoprostol, a PGE_1 analog, has been used, alone or in combination with mifepristone, for first-, second-, and third-trimester abortions.

Traditional NSAIDs, via their capacity to inhibit eicosanoid biosynthesis, block ovulation in several mammalian species,[87] and PGE_2 and $PGF_{2\alpha}$ are well known as effective luteolytic agents within the veterinary community.[88,89] PG biosynthesis is involved in maintaining ductus arteriosus (DA) patency during fetal life, and in anecdotal reports, NSAID use during pregnancy was associated with increased risk of premature DA closure and fetal demise.[90,91] Advances in eicosanoid pathway molecular biology have significantly improved our understanding of the role PGs play in reproduction. Much of this knowledge has emerged from studies using knockout mice. Table 6.2 summarizes the major reproductive steps where eicosanoids are known to play a physiological role or have been shown to have therapeutic relevance.

Gonadotropin Release

Prostaglandins modulate the secretion of luteinizing hormone (LH) and, to some extent, follicle-stimulating hormone (FSH).[92] This is believed to occur through modulation of hypothalamic LH-releasing hormone (LHRH), because PGE_2 stimulates the release of LHRH *in vivo*,[93] but does not directly affect the release of LH from the pituitary.[92-93] Furthermore, immortalized neurons contain transcripts of both COX-1 and COX-2.[94] PGE_2 may also regulate the LHRH pulse generator.[92,94-95]

However, studies of COX-deficient mice have shown that the hypothalamic content of LHRH in COX-1-deficient and COX-2-deficient mice is comparable to that of wild-type mice.[96] This observation suggests that neither COX-1 nor COX-2 plays an indispensable role in LHRH production in mice. The potential effect of COX enzyme deficiency on the pulsatility of LHRH release during the estrous cycle remains to be evaluated.

In contrast to hypothalamic LHRH, pituitary FSH content was significantly elevated in both COX-1– and COX-2–deficient mice.[96] There was also a tendency for increased pituitary LH content in the COX-deficient mice, although the increase was less pronounced.[96] The mechanism for the increase in pituitary FSH content in the COX-deficient mice is not clear. Nevertheless, serum gonadotropin and steroid levels in COX-1– and COX-2–deficient mice were unchanged compared with wild-type mice.[96]

Furthermore, COX-1– and COX-2–deficient mice exhibited estrous cycles and normal mating behavior. Therefore, COX enzymes may not be involved in the regulation of the neuroendocrine axis suggesting that the reproductive abnormalities observed in COX-deficient mice[54-57] must have resulted from defects in peripheral reproductive organs.

Ovulation

An essential role for PGs in ovarian function was established many years ago,[87] and the intricacies of their mechanism of action have been delineated (see Chapter 9). A series of elegant studies established that induction of COX-2 in the preovulatory follicles of the rat ovary was an obligatory downstream event in the LH surge–stimulated ovulation process.[97-100]

In contrast to the COX-1 enzyme, which was constitutively expressed in thecal cells and corpora lutea, LH-induced COX-2 enzyme was present in granulosa cells of the rat preovulatory follicles.[97-100] This observation was later extended to other mammalian species, including the cow and mare.[101] Interestingly, the time required for COX-2 induction in preovulatory follicles after the LH surge differed considerably in various species (2 to 4 hours in the rat, 18 hours in the cow, and 30 hours in the mare), yet induction of COX-2 expression always occurred approximately 10 hours before ovulation in each of these mammals.[101]

LH surge–induced COX-2 expression in the granulosa cells of macaques, a primate species, has also been established using reverse transcriptase–polymerase chain reaction and immunocytochemistry methods.[102] Furthermore, follicular administration of indomethacin in the rhesus monkey was demonstrated to significantly, although not completely, inhibit oocyte release from the ovary.[103]

Thus, in primate, rodent, and domesticated animal species, the induction of COX-2 seems to serve as an "alarm" signal for ovulation.[101,104] Ovulation, initiated by the LH surge that leads to a cascade of proteolytic events that culminate in follicle rupture and release of the ovum, has often been compared to an inflammatory response.[105] In this regard, it is fitting that COX-2, rather than COX-1, should be implicated in this process.

The predominant role of COX-2 in the ovulation process was ratified by studies using COX-1– and COX-2–deficient mice. Mice lacking the COX-2 enzyme had multiple reproductive failures, including ovulation defects, whereas COX-1–deficient mice had impaired parturition, but ovulated normally.[57,96] Biochemical studies showed that exogenous gonadotropins (pregnant mare serum gonadotropin [PMSG] and human chorionic gonadotropin [hCG]) stimulated a fourfold increase in ovarian PGE_2 levels in wild-type and COX-1–deficient mice 8 hours after hormonal challenge; COX-2–deficient mice showed no such increase.[96]

Examination at the cellular level showed that the primary defect responsible for failed ovulation in COX-2–deficient mice was impaired expansion of the cumulus oophorus. This impairment possibly originated from an alteration in the proteoglycan contents of the cumulus oophorus and follicular wall.[96] Whether COX-2 deficiency might also have affected the activation of proteolytic enzymes that participate in the breakdown of the follicular wall is not yet known. Indomethacin treatment, however, did cause oocyte retention but did not prevent follicular rupture in the rhesus monkey ovary.[103]

The critical role of COX-2 in ovulation has also been corroborated by pharmacological studies using coxibs as selective inhibitors of this enzyme in wild-type animals. Administration of celecoxib to mice[106] and NS-398 to rats[107] decreased, but did not abolish, ovulation in the treated animals. NS-398 also reduced PGE_2 synthesis in isolated LH-stimulated rat preovulatory follicles *in vitro*.

The degree of inhibition of PGE_2 synthesis by NS-398 in this assay was similar to that achieved by indomethacin, supporting the conclusion that COX-2 mediated the previously observed ovulation blockade by traditional NSAIDs.[87] Finally, delayed follicular rupture in humans by the selective COX-2 inhibitor rofecoxib was observed in a randomized double-blind study,[108] supporting a link between NSAID use and reversible female infertility originating from ovulation interference.[108]

Several lines of evidence suggest that PGE_2 is the principal COX-2 product that mediates the ovulation process.[109] First, ovarian PGE_2 production is increased in response to the LH surge. Second, application of exogenous PGE_2 to gonadotropin-primed, COX-2–deficient mice corrected the anovulatory phenotype.[96] Administration of $PGF_{2\alpha}$ in the same experiment also slightly improved the ovulation outcome, but this was most likely attributable to cross-activation of PGE receptors by $PGF_{2\alpha}$ because no ovulatory abnormality was detected in FP-deficient mice.[110]

Third, a moderate reduction in the ovulation rate has been detected in two independently generated EP2-deficient mouse lines,[111,112] but no apparent ovulation defect has been seen in other prostanoid receptor–mutant mice. EP2-deficient mice had severely compromised fertility, which was attributed partially to an ovulation defect and predominantly to fertilization failure.[111,112]

A role for EP2 in ovulation and fertilization is supported by its specific expression in the cumulus cells of the preovulatory follicles, where it is up-regulated in response to gonadotropin stimulation.[112] The impaired fertilization of EP2-deficient mouse eggs was shown to result from abortive cumulus expansion in the oviduct,[112] a phenomenon also observed in the preovulatory follicles of COX-2–deficient mice.[96] These studies suggested that EP2 may be essential for the late-stage events of ovulation by inducing ordered cumulus expansion after ovulation to ensure successful fertilization; however, it is relevant but not indispensable for follicular rupture and ova release.

Contrary to these initial findings, another follow-up study of adult COX-2–deficient and EP2-deficient mice concluded that the ovulatory process, *not* follicular growth, oocyte maturation, or fertilization, is primarily affected by these deficiencies.[113] This study also showed that severely compromised ovulation, seen in adult COX-2–deficient and EP2-deficient mice, was not manifested in immature (3-week-old) mice with those same deficiencies. This finding suggests that the process of ovulation may be more dependent on PGs in adult mice.[113]

If EP2 is the receptor subtype physiologically responsible for initiating PGE_2-induced ovulation in follicles, then the incomplete penetration of the ovulation phenotype in EP2-deficient mice could indicate compensatory responses from other EP receptor subtypes in this process. In addition to EP2, EP4 messenger RNA (mRNA) and protein are detected in mouse and monkey oocytes.[109] More recent data have implicated multiple EP receptors in regulating proteolysis in the primate periovulatory follicle.[114]

Corpus Luteum Function and Luteolysis

Ovarian PGE_2 levels were slightly suppressed in both COX-1–and COX-2–mutant mice 24 hours after hormonal stimulation.[96] This discovery suggested that, whereas COX-2–derived PGs are responsible for ovulation, both isozymes may contribute to PG production during formation of the corpora lutea. However, neither COX isoform is indispensable for granulosa cell development or corpora lutea formation, because ovarian morphology and steroid hormone production in COX-deficient mice were similar to those of

wild-type mice 24 to 30 hours after gonadotropin stimulation.[96] There was also no apparent difference in estrous cyclicity between wild-type, COX-1–deficient, and COX-2–deficient mice.[96]

The role of $PGF_{2\alpha}$ as a physiological luteolysin has been well established in many nonprimate mammalian species that depend on the presence of the uterus for corpus luteum regression. Indeed, this finding proved to be a major breakthrough in the livestock industry, where $PGF_{2\alpha}$ and its synthetic analogs are routinely used to regulate the breeding of domesticated animals.

Although FP is expressed in the corpora lutea of mice during a normal estrous cycle, no change was found in the estrous cycle or in the number of corpora lutea in homozygous FP-deficient mice.[110] On the other hand, FP-deficient pregnant mice showed a parturition defect due to lack of luteolysis before the onset of labor.[110] Thus, $PGF_{2\alpha}$ acts as a luteolysin in pregnant mice, but not in nonpregnant, normally cycling female mice.

The involvement of PGs in the luteolysis of primate species is not as well defined (see Chapter 9). Unlike in other mammals, the primate corpus luteum undergoes luteolysis in the absence of the uterus. There is, however, evidence to suggest that locally produced PGs in the ovary may act as mediators to promote luteolysis. For example, $PGF_{2\alpha}$ is produced by the human corpus luteum, and specific receptors for $PGF_{2\alpha}$ are located in human luteal tissue.[115] High doses of $PGF_{2\alpha}$ injected directly into human corpus luteum *in vivo* also caused premature regression of the corpus luteum and shortening of cycle length.[116]

On the other hand, results from PG synthesis–inhibitor experiments in the rhesus monkey have been equivocal.[117,118] Also, it is not known whether PG levels change significantly in the primate ovary or corpus luteum during the luteal phase.[119] Thus, although there is considerable evidence that PGs of ovarian or luteal origin may participate in the induction of corpus luteum regression in primates, further studies will be required to conclusively resolve this question.[119]

Fertilization, Implantation, and Decidualization

The role of PGs in oocyte maturation and fertilization is supported by the phenotype of COX-2–deficient mice (see Chapter 9).[57] In addition to an ovulation defect, the few ovulated eggs from COX-2–deficient female mice recovered after PMSG and hCG challenge were totally incapable of being fertilized.[57] In accordance with this observation, immunostaining detected COX-2 enzyme in wild-type mouse cumulus cells enclosing ovulated eggs.[57] Thus, local COX-2–derived PGE_2 in the cumulus cells may function as an autocrine factor to activate EP receptors and induce cumulus cell expansion necessary for successful fertilization.

COX-1, on the other hand, is not required for embryo implantation and uterine decidualization.[57] Because COX-2–deficient mice are defective in ovulation and fertilization, the implantation and decidualization processes in these animals could be investigated only by embryo transfer (from wild-type mice) and by a procedure involving intraluminal infusion of oil to induce artificial decidualization.[57,83,120]

Initial published studies showed that 50% of the transferred embryos were successfully implanted in the uteri of wild-type mice at day 5 after pseudopregnancy, but fewer than 2% of the embryos implanted in the uteri of COX-2–deficient mice were successfully transferred.[57] Likewise, induction of artificial decidualization in wild-type mice caused a sixteenfold increase in uterine wet weight, but did not produce any appreciable weight gain in COX-2–deficient mouse uteri.[57] The failure of implantation and decidualization in COX-2–deficient mice is not secondary to steroid hormone insufficiency from ovarian dysfunction, because supplementation of progesterone and estradiol did not rescue the phenotype.[57]

A specific role for COX-2 (but not COX-1) in these processes is also supported by the dynamic regulation of COX-2 expression in mouse uterus. Although COX-2 is normally expressed minimally in the uterus, it is robustly induced at 2 hours after artificial decidualization and its expression returns to basal levels within 8 hours. COX-1, in contrast, is constitutively expressed in the mouse uterus and barely changes its expression during decidualization.[57]

A critical role for COX-2 in implantation was disputed in a separate study conducted by Cheng and Stewart.[120] Their investigation into the role of COX-2 in embryo implantation and uterine decidualization concluded that COX-2 has a role in mediating the initial uterine decidual response, but is *not* essential for embryo implantation, sustained decidual growth, or embryo development throughout the remainder of pregnancy.[120]

PGI_2 may be the eicosanoid that mediates COX-2–dependent embryo implantation and uterine decidualization.[57,83] Researchers have proposed that the effect of PGI_2 in these processes is mediated by the nuclear receptor PPARδ. PGI_2 is the most abundant prostanoid formed at implantation and decidualization sites, and PGI_2 synthase transcriptsare also present and up-regulated at the implantation sites between day 5 and day 8 of pregnancy.[57,83]

The finding in earlier studies that two PGI_2 analogs (carbaprostacyclin [cPGI] and iloprost) activated PPARδ[121] and that IP-deficient mice had normal fertility[122] led to the hypothesis that endogenous PGI_2 mediates embryo implantation and decidualization through PPARδ.[57,83] The coordinated uterine expression of PPARδ and COX-2 during decidualization supports this contention. The hypothesis was also supported by the finding that PGI_2 agonists such as cPGI and iloprost (as well as a classic PPARδ-selective agonist, L-165,041) restored implantation and decidualization in COX-2–deficient mice.[57,83] Moreover, PPARδ was found to be essential for spontaneous and preimplantation PGI_2-stimulated embryo development and blastocyst hatching.[123] The implantation of cultured embryos was enhanced by PPARδ activation.

Nevertheless, many questions remain to be answered about the validity of this hypothesis. First, varied phenotypes of PPARδ-deficient mice have been reported.[124] Second, cPGI and iloprost have been shown to activate PPARδ,[57,83,121] but one cannot assume with certainty that endogenous PGI_2 will do the same. Indeed, cicaprost, another PGI_2 agonist for membrane IP, was not an activator of PPARδ,[121] suggesting that caution should be exercised when extrapolating data from PGI_2 analogs. Third, it is possible that implantation and decidualization could be mediated

by COX-2 and PPARδ as separate and redundant pathways. To further muddy the waters, more recent studies suggest that IP receptors play an important role in preimplantation embryo development and mediate the embryo's response to exogenous PGI_2.[125] A PPARδ agonist had no effect in IP receptor knockout mice, suggesting that the nuclear receptor pathway may be downstream of IP activation. Clearly, more research is needed on this topic. Even if the PGI_2-PPARδ hypothesis is accepted at face value, the mechanism and downstream events of this signaling pathway in the uterus remain to be established. No specific defect in the expression of implantation-related genes or steroid responsiveness has been found in the uterus of COX-2–deficient mice.[57] The direct causes of impaired implantation and decidualization failure in these animals are still unknown. Clearly, however, other eicosanoids are needed for the implanted embryos to develop to term. Thus, administration of cPGI was shown to improve implantation and decidualization in COX-2–deficient mice, but the subsequent growth of the implanted embryos in these mice was not comparable to that implanted in the wild-type mice.[57,83] Interestingly, coadministration of cPGI with PGE_2 markedly improved embryonic and decidual growth in COX-2–deficient mice.[83]

Menstruation and Dysmenorrhea

Abnormal PG biosynthesis is involved in the pathogenesis of human dysmenorrhea, a gynecological disorder that is defined as the occurrence of pain associated with menstruation (for a more detailed discussion of this topic, please see Chapter 27). Elevated levels of $PGF_{2\alpha}$ and PGE_2 occur in the endometrium and menstrual fluid of women who have dysmenorrhea, with an elevated ratio of $PGF_{2\alpha}$ to PGE_2.

A markedly increased capacity for PGI_2 synthesis has been detected in endometriosis tissue. This elevation is considered to cause hyperalgesia and to contribute significantly to dysmenorrhea in endometriosis.[126] Furthermore, intrauterine administration of $PGF_{2\alpha}$ induces uterine contractility and dysmenorrhea-like pain.[4]

Cumulative data of clinical trials have indicated that 80% of patients with significant primary dysmenorrhea experience adequate pain relief with the use of traditional NSAIDs.[127] Both celecoxib and valdecoxib, two COX-2–selective inhibitors, have also been used for the treatment of primary dysmenorrhea.[128,129] However, valdecoxib was removed from clinical use in 2005 because of an association with elevated risk of Stevens-Johnson syndrome and cardiovascular hazard, whereas celecoxib remains in use.

Parturition and NSAID Use in Preterm Labor

There has been a great deal of interest in the role that PGs play in term and preterm labor (see Chapters 12 and 28). Aspirin-like drugs were shown to delay parturition in humans more than 30 years ago,[130] and NSAIDs are also well known to be effective agents in attenuating the progression of term and preterm labor in various species.[131,132] PGE_2 and $PGF_{2\alpha}$, produced by both maternal and fetal tissues during pregnancy,[133] stimulate uterine contractions *in vitro* and *in vivo*; they also promote the coordinated inflammatory response that results in dilation and thinning of the cervix.[133] Studies with COX-1– and prostanoid receptor–deficient mice have provided additional insight into the role and mechanism of action of PGs in the process of parturition and preterm labor.

Although FP-deficient female mice grew and developed normally and became pregnant as expected, they did not deliver fetuses normally at term.[110] This complication was due entirely to impaired parturition. Fetuses could be rescued from FP-deficient mothers by cesarean operation before or at the expected term and survive normally. Uterine induction of the oxytocin receptor at term, an event believed to trigger parturition, did not occur in the FP-deficient mouse uterus.[110]

Additionally, FP-deficient mice did not experience a decline in maternal plasma progesterone level close to term; this decline usually occurs before parturition in mammals. It was quite revealing, however, that ovariectomy just before term completely normalized the parturition defect in FP-deficient mice.[110] Thus, ovariectomy of gravid FP-deficient mice decreased plasma progesterone levels, induced uterine oxytocin receptor expression, and initiated the onset of labor within 24 hours.[110] These observations suggest that $PGF_{2\alpha}$ induces labor through its luteolytic action in the ovary in rodents; its uterotonic action in the myometrium apparently is not essential for parturition.

A similar parturition defect was characterized in COX-1–deficient mice, and administration of $PGF_{2\alpha}$ was sufficient to correct this defect.[134] Thus, COX-1 is essential for the normal onset and progression of term labor and cannot be compensated for by unimpaired COX-2 activity. Remarkably, ovarian $PGF_{2\alpha}$ levels could be blocked by 85% in a COX-1 knockdown mouse model, and residual PG was still sufficient to promote timely luteolysis for initiation of labor.[135] There is still uncertainty regarding the contribution of fetal $PGF_{2\alpha}$ to this process. In one experiment involving reciprocal blastocyst transfers between wild-type and COX-1–deficient mice, maternal COX-1 expression was found to be both sufficient and necessary for the normal onset of labor.[134]

However, in another study, COX-1–deficient females carrying wild-type fetuses delivered their pups on schedule and with normal survival rates,[136] suggesting that maternally derived $PGF_{2\alpha}$ is dispensable. The idea that fetal $PGF_{2\alpha}$ contributes to parturition is also supported by another study by Cook and coworkers who found that increased mouse placental $PGF_{2\alpha}$ biosynthesis, catalyzed at least in part by up-regulated COX-2, is associated with uterine activation and the timing of birth.[137]

It is notable that studies of FP-deficient mice suggested that the primary target of $PGF_{2\alpha}$ action with regard to parturition is the corpus luteum, yet COX-1 mRNA levels in the ovary were quite low and relatively unchanged during pregnancy.[110] This contrasted with a fortyfold induction of COX-1 in the uterus in late gestation.[110] In addition, PGF synthase and a $PGF_{2\alpha}$-metabolizing enzyme, 15-OH-PGDH, were also time-dependently regulated in the pregnant mouse uterus, with a dramatic increase in PGFS and a decrease in 15-OH-PGDH toward late gestation.[138] Thus, primarily uterine-derived $PGF_{2\alpha}$ apparently activates ovarian FP and determines the timing of labor.

The lack of a parturition phenotype in PG receptor–deficient mice other than FP knockout mice suggests that PGs are less important in promoting cervical ripening and

myometrial contraction in the mouse. This hypothesis is supported by the finding that the luteolytic effect alone of $PGF_{2\alpha}$ made its function indispensable for murine parturition. This role contrasts greatly with what is believed to occur in human labor. Various PGs are produced in human myometrium with prostacyclin being the highest.[139] Recently, PGI_2 was paradoxically shown to prime pregnant human myometrium for an enhanced contractile response in parturition via induction of genes encoding for contractile proteins via the IP receptor.[140,141] Receptor sites for EP1, EP3, and FP are also present in human myometrial tissue,[142] as are the enzymes that generate PGs, including COX-2 and PGE synthase isoforms.[143]

Increased PG production is also associated with cervical ripening and a topically applied PG product, containing either dinoprostone or misoprostol, is the most popular means to soften and dilate the cervix in human labor induction.[144] Local administration of PGs to the cervix in pregnant women results in clinical, histological, and biochemical changes that are consistent with those observed during physiological cervical ripening. The mechanism of action of PGs in human cervical ripening is not known, but may include induction of major degrading enzymes and alterations in the expression of genes encoding proteins that modify proteoglycan function. The lack of a phenotype in COX and PG receptor knockout mice in cervical ripening and uterotonic responses may indicate a species difference, or it may suggest compensation in these tissues between the two COX enzymes.

Although COX-1 is involved in term labor, COX-2 induction is likely to be primarily responsible for PG-mediated preterm labor. COX-2, but not COX-1, is induced during inflammation-mediated preterm labor elicited by lipopolysaccharide (LPS) administration.[145] In a murine model, the COX-2–selective inhibitor SC-236 was found to be more effective than the COX-1–selective inhibitor SC-560 in stopping LPS-prompted preterm labor and increasing uterine PG synthesis.[145] Furthermore, COX-1–deficient mice, which showed delay in the onset of term labor, exhibited no delay in the onset of preterm labor after LPS treatment.[145]

Although it is likely that COX-2–derived $PGF_{2\alpha}$ acted as a luteolysin in the mouse model of LPS-induced preterm labor, it is not known whether the uterotonic effect of $PGF_{2\alpha}$ and other COX-2–derived PGs may also have played a role in this setting. The latter possibility may be particularly relevant to human preterm labor, because indomethacin, an inhibitor of both COX-1 and COX-2, has been used successfully in treating human preterm labor, both when given systemically and when delivered locally through the vaginal route.[146]

The clinical utility of indomethacin as a tocolytical agent, however, is tempered by concerns over fetal and neonatal complications, such as constriction of the ductus arteriosus.[146] The adverse effects associated with indomethacin use in preterm labor have been mainly linked to inhibition of COX-2 based on mouse studies using selective COX-1 and COX-2 inhibitors.[147] COX-1–selective inhibitors, should they be developed clinically, would be expected to produce fewer adverse effects than traditional NSAIDs when used as tocolytical agents.[147] Studies in animals and pregnant women comparing indomethacin and coxibs produced mixed results when fetal ductus blood flow was used as a measure of potential adverse effects. [148]

Ductus Arteriosus Remodeling

As mentioned earlier in the chapter, one of the complications of using NSAIDs such as indomethacin to treat preterm labor is the induction of premature closure of the fetal DA.[90,149] The DA is a large fetal vessel that shunts deoxygenated blood away from the pulmonary circulation to the descending aorta and to the umbilicoplacental circulation, where oxygenation takes place. In neonates, rapid remodeling of the DA leads to its closure after adaptation of spontaneous breathing in newborn infants. Although patency of the DA *in utero* is essential for proper fetal growth and development, failure of the DA to close after birth, called *persistent patent DA*, compromises postnatal health by causing circulatory complications such as pulmonary hypertension and congestive heart failure.[149]

Prostaglandins are intimately involved in DA function and its perinatal remodeling, and details of their mechanism of action are emerging. The finding that indomethacin administration induced premature DA closure in fetuses[90,149] suggested that fetal PGs are essential for maintaining DA patency. However, mice lacking both COX-1 and COX-2, unable to make any PGs, die postnatally with patent DA,[136,150] indicating that fetal-derived PGs are not necessary for maintaining DA patency *in utero*. Such PGs *do*, however, play an indispensable role in DA remodeling after birth.

How does one reconcile this phenotype with the results of pharmacological inhibition studies? Researchers have proposed that PGE_2 in the fetal circulation (supplied in part by the placenta) maintains dilation of the DA *in utero*, and that COX-2 in the DA produces constrictor PGs that are important for DA contraction after birth.[150] Thus, indomethacin-induced premature DA closure in fetuses may have reflected inhibition of dilatory PGE_2 synthesis in the placenta without sufficient inhibition of ductal COX-2 to attenuate DA contraction. Although COX-1 deficiency alone did not affect perinatal DA remodeling, it was found to exacerbate the phenotype of patent DA on the background of COX-2 deficiency.[150]

In other studies, mice deficient in the smooth muscle relaxant receptor EP4[151,152] or the PGE_2-metabolizing enzyme 15-OH-PGDH[153] also failed to survive postnatally due to patent DA. EP4 expression in DA and 15-OH-PGDH expression in the fetal lung increase dramatically just before birth, supporting the conclusion that these proteins play an important role in perinatal DA remodeling.

The phenotypes of EP4-deficient and 15-OH-PGDH–deficient mice, together with the results of pharmacological studies, support alternative models for the DA remodeling process.[152-154] Signals through EP4 have two essential roles in DA patency and remodeling, namely, vascular dilation and intimal cushion formation (ICF). The vascular smooth muscle–relaxing ability of PGE_2 acting via EP4 maintains patency during fetal life. When PGE_2 levels plummet at birth due to rapid and efficient metabolism by late gestation–induced 15-OH-PGDH activity, the vasodilatory role is curtailed and functional DA closure is triggered rapidly by an increase in oxygen tension. ICF occludes the vascular lumen and results in permanent closure after birth. ICF within the DA is a result of an increase in vascular smooth muscle cell migration and proliferation and the production

of hyaluronic acid (HA) under the endothelial layer, along with decreased elastin fiber assembly. PGE_2 via EP4 signaling during late gestation controls a gene, *HAS2*, that regulates HA synthesis and ICF.[154]

Pregnancy-Induced Hypertension and Preeclampsia

The possible involvement of PGs in pregnancy-induced hypertension (PIH) and preeclampsia and the potential therapeutic efficacy of low-dose aspirin in their prophylactic treatment have received considerable attention. PIH and preeclampsia occur in 10% of pregnancies and are recognized as important, prevalent sources of risk to both mother and fetus (see Chapter 28).

Although the exact cause of the disease is unknown, dysregulated production of PGI_2 and TxA_2 has been postulated as one of many potential etiological factors.[155] Decreased urinary PGI_2 metabolites are found to precede the development of PIH,[156] and increased TxA_2 metabolite excretion occurs in patients with severe preeclampsia.[157,158]

Such changes may predispose to vasoconstriction of small arteries, activation of platelets, and uteroplacental insufficiency—clinical outcomes that are associated with PIH and preeclampsia. In a murine model of enhanced TxA_2 activity, transgenic overexpression of TP in the vasculature resulted in intrauterine growth retardation that was rescued by timed suppression of TxA_2 synthesis with indomethacin.[159]

Many clinical trials have been conducted to evaluate the use of low-dose aspirin in the prevention of preeclampsia. Although some randomized studies indicated a beneficial effect for women at increased risk, most large-scale clinical trials did not establish a positive effect of low-dose aspirin use on reducing the incidence of preeclampsia or on improving perinatal outcomes in pregnant women at high risk for preeclampsia.[160-162]

In some studies, neither maternal serum TxB_2 concentrations at enrollment nor their subsequent reduction by low-dose aspirin intake were correlated with adverse pregnancy outcomes[163,164] but this may reflect poor study design as serum thromboxane does not adequately reflect augmented thromboxane biosynthesis. It is possible that aspirin trials failed because an increase in TX production is not the initial anomaly. Less controversial than the role of ASA in improving fetal outcomes in preeclampsia is the time-dependent hypotensive effect of low dose aspirin in a variety of hypertensive populations, including women with preeclampsia.

Eicosanoids in Male Reproduction

Although PGs are critically involved in multiple steps of female reproduction, as previously discussed, their physiological role in male reproduction is not well understood—although it appears to be less remarkable, based on gene knockout studies. No discernible male reproductive phenotype has been noted in any of the COX-deficient or prostanoid receptor–deficient mice.[54,55]

On the other hand, a number of studies have described androgen-dependent regulation and distinctive tissue distribution patterns of COX-1 and COX-2 enzymes, as well as PG-synthesizing enzymes, in the male reproductive tracts of rodents and humans.[165-168] Specific functions have also been documented for COX-2–derived PGD_2 and PGE_2 in mediating cytokine production in Leydig cells and in regulating apoptosis in the rat epididymis.[169,170] Although they are not definitive, such studies do suggest that PGs may be synthesized and functional in a regulated manner in the male reproductive organs, even though their physiological roles in these sites are dispensable.

In the future, a better understanding of the eicosanoid network in the male reproductive system may lead to new PG-based therapies or provide mechanistic insight about existing therapies. For example, highly specific expression of COX-2 has been found in the distal end of the rat vas deferens.[165] Because the distal vas comprises an extensive submucosal venous plexus connected to the penile corpora cavernosa, PGs from the vas may play a role in erection.[165] In this context, it is notable that intracavernosal injection of PGE_1 has been used clinically as an effective therapy for erectile dysfunction in man.[171,172] While PGs were originally isolated in large amounts in male seminal vesicles, their role at this site has remained enigmatic. The recent finding that ovarian PGs may act as sperm guidance factors via an insulin and Forkhead box class O (FOXO) transcription factor pathway[173] could indicate a synergistic role with male accessory gland-derived PGs.

Other LIPID Mediators: Lysophosphatidic Acid and Sphingosine-1-Phosphate

Besides eicosanoids, other lipid mediators have been implicated in reproductive function, in particular, lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P). First, we briefly examine the features, synthesis, and signal transduction of LPA, and then we turn our attention to S1P.

The lipid mediator LPA is a lipid-signaling molecule as well as an intermediate in the *de novo* biosynthetic pathway of phospholipids, consisting of a glycerol backbone, a phosphate head group, and a long-chain fatty acid (usually oleic acid or palmitic acid), most commonly in acyl linkage (Fig. 6.1).[174] LPA generation is complex, proceeding by at least two pathways: conversion from lysophospholipids or via phosphatidic acid. Several phospholipase activities are necessary, including phospholipase A_1 (PLA_1)/PLA_2 plus lysophospholipase D (lysoPLD) and phospholipase D(PLD). Additional extracellular phospholipases, such as secretory PLA_2 (sPLA$_2$-IIA), membrane-associated PA-selective PLA_1 (mPA-PLA_1), and lecithin–cholesterol acyltransferase (LCAT), can also be involved.[175] LPA signaling is mediated primarily by six members of the G-protein–coupled receptor family, currently referred to as LPA_1, LPA_2, LPA_3, LPA_4, LPA_5, and LPA_6.[176]

The lipid mediator S1P is a bioactive sphingolipid derived from the abundant phospholipid sphingomyelin. Sphingomyelinases generate ceramide, which is cleaved by ceramidases to sphingosine, followed by phosphorylation by sphingosine kinases to S1P (Fig. 6.7).[177] S1P signals extracellularly via transmembrane receptors but also has intracellular targets.[178,179] There are five S1P receptors, also members of the GPCR family, referred to as $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$.[180] The S1P stimulation of these receptors elicits a battery of downstream effects, including inhibition of cAMP and activation of mitogen-activated protein kinases, phospholipase C, and PI3 kinase to evoke a broad range of cellular activities.[180]

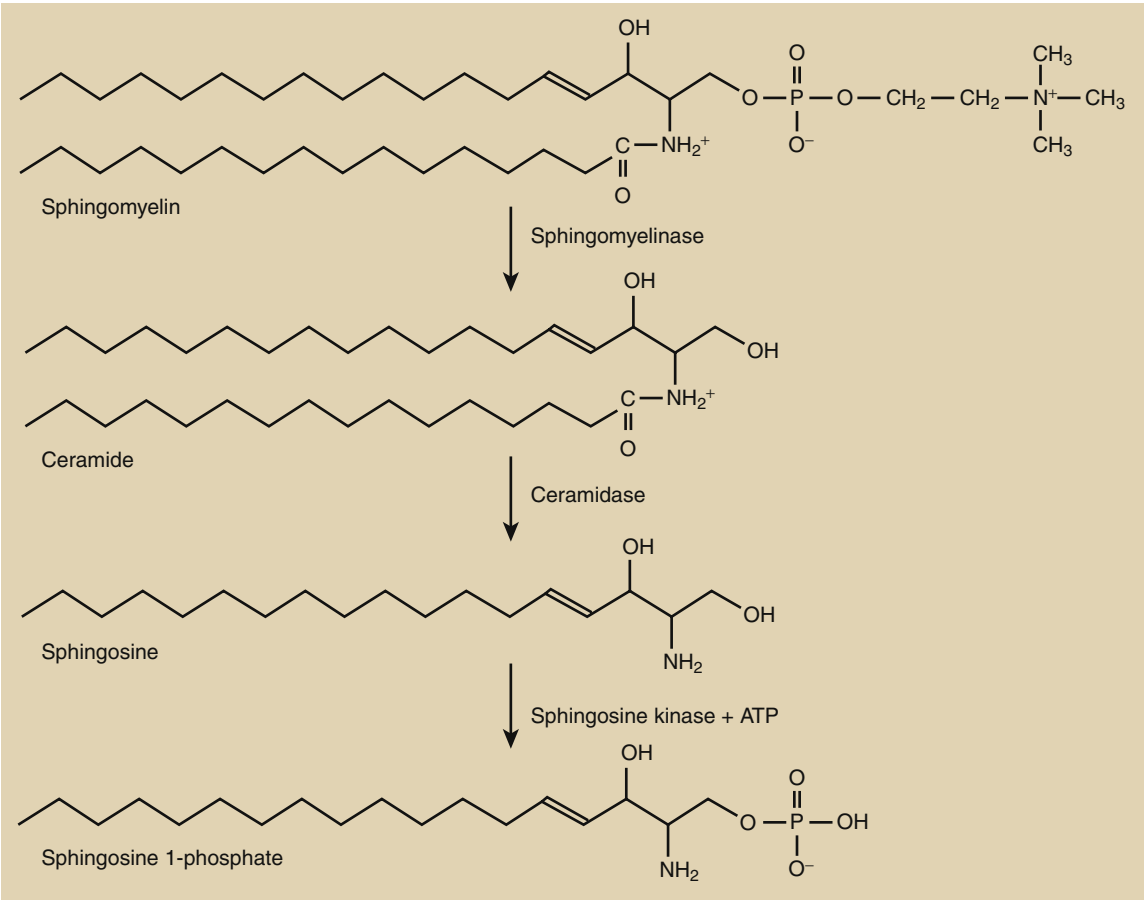


FIGURE 6.7 Structures and biosynthesis of a representative sphingosine-1-phosphate (S1P) lipid mediator from sphingomyelin. Sphingomyelinase removes the phosphorylcholine head group to yield ceramide. Ceramidase cleaves the amide bond removing one aliphatic chain to generate sphingosine. In the presence of ATP, sphingosine kinase will phosphorylate sphingosine to generate S1P.

LPA and S1P in Reproductive Function

The effect of LPA on the female reproductive system was recognized as early as 1980, when a series of LPA molecules with variable fatty acyl side chain lengths and degrees of saturation were shown to stimulate rat uterine smooth muscle contraction.[181] Most of the LPA receptor genes have been disrupted in mice, with some affecting reproductive function. LPA_3 (also known as *Edg7*) exhibits a female reproductive phenotype.[182,183] LPA_3 mRNA has been detected in oviduct, placenta, and uterus, but not in ovary and oocytes with expression highest early in pregnancy, at approximately embryonic day 3.5 (E3.5) in mice. LPA_3 is regulated positively by progesterone and negatively by estrogen.[182,183] LPA_3-deficient females produce small litters and show a prolongation of pregnancy by approximately 1.5 days (normal gestation, 19.5 days).[182] The mice show no obvious defects in ovulation, ovum transport, or blastocyst development. However, the defects were related to delayed implantation and altered crowding or positioning of embryos, which led to delayed embryonic development and death, accounting for the reduced litter size. The observed phenotypes were the result of maternal LPA_3 signaling and were not due to embryo LPA_3 signaling.[182] Strikingly, the phenotypes were similar to those seen with $cPLA_2$ female knockout mice and rodents treated with indomethacin.[184-186] Remarkably, LPA_3-deficient female uteri had markedly reduced COX-2 expression and PGE_2/PGI_2 levels at E3.5, thus linking LPA signaling to PG biosynthesis and fertility control.[182] Overall, this particular study raises speculation that therapeutic manipulation of LPA_3 signaling could influence the low implantation rate that is a major drawback during infertility treatments using assisted reproductive technologies.

LPA_1, LPA_2, and LPA_3 are differentially expressed in male testes with the latter two in the basal regions of seminiferous tubules, primarily in immature germ cells (spermatogonia and spermatocytes) and LPA_1 showing stage-specific expression in germ cells.[187] Triple knockout mice exhibit male reproductive defects including alterations in mating behavior and heterogeneic spermatogenic disruption resulting in sterility.[187] Many questions remain to be answered on the mechanisms for these effects.

Of the "newer" LPA receptors LPA_4, LPA_5, and LPA_6, there is no evidence for the involvement of these signaling pathways in reproductive function. LPA_4 has been detected in female ovaries, uterus, and placenta, but only one of three research groups reported a reduced litter size in mice deficient for LPA_4 but this was apparently attributable to other nonreproductive mechanisms.[188]

Clues to the roles of S1P in reproductive function have come from various sources. Based on expression studies of the five receptor subtypes that bind S1P, it is known that only three ($S1P_1$, $S1P_2$, and $S1P_3$) show widespread

distribution in mice, with evidence in gonadal tissues and in the uterus during decidualization (E4.5-7.5).[180,189] $S1P_1$ and $S1P_2$ colocalize with COX-2 at the maternal/fetal interface throughout pregnancy, suggesting a link between sphingolipid and PG signaling and indicating that S1P coordinates uterine mesometrial angiogenesis during implantation.[189]

Because $S1P_1$-deficient mice die in mid-embryogenesis as a result of complications of vasculogenesis, it has not been possible to decipher a role for this particular signaling pathway in reproductive function.[180] Both $S1P_2$- and $S1P_3$-null mice show no obvious phenotypes, with the exception of slightly smaller litter sizes.[176,180] However, when both rceptors are knocked out there is infertility.[176] The specific roles of each S1P receptor subtype in female reproductive function will still require more study.

A role for S1P in the preservation of female fertility is being recognized.[190] Programmed cell death (apoptosis) is an established paradigm in the mammalian female germline. Ceramide generated by membrane cleavage of sphingomyelin by sphingomyelinase or via *de novo* biosynthesis by ceramide synthase is translocated from cumulus cells to adjacent oocytes to induce germ cell apoptosis.[191] This is prevented by S1P, a ceramide metabolite within the same pathway or by acid sphingomyelinase (ASM) deficiency. The therapeutic management of infertility by S1P in premature menopause and in female patients with cancer appears promising, but awaits further study.[190]

Sphingolipids also seem to play a role in male germ cell apoptosis.[192,193] Ceramide induces an early apoptotic pathway event in male germ cells that is partially suppressed by S1P. However, although maintenance of normal sphingomyelin levels in testes and normal sperm motility are dependent on ASM, testicular ceramide production and the ability of germ cells to undergo apoptosis do not require ASM.

Summary

Prostaglandins and other lipid mediators are involved in many steps of the human reproductive process. Detailed understanding of their mechanisms of action in these steps has been greatly facilitated by the characterization of two COX enzymes, individual PG, LPA, and S1P receptors, and by the engineering of COX, PG, and LPA receptor gene–disrupted animal models. These studies have shown some distinct roles played by each of the two COX enzymes and by specific PG and LPA receptor pathways in mammalian reproduction.

Despite the tremendous progress made so far, much remains to be learned, particularly with regard to the molecular events downstream of PG, LPA, and S1P receptor activation in each of the reproductive steps where PGs, LPA, and S1P were found to be essential. Another challenge is to integrate the findings in animal models into the study of PGs, LPA, and S1P in human reproductive endocrinology. Continued interest and research in the field may lead to new PG-based and other lipid mediator therapies for reproductive disorders (such as endometriosis and preterm labor) and the reduction of adverse effects of NSAID use in women of reproductive age.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 7

Growth Factors and Reproduction

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Beginning in early embryonic development, multiple growth factor pathways are essential for viability. In particular, transforming growth factor beta (TGFβ) family signaling pathways are important in growth and differentiation, as well as for regulation of embryonic stem cells (ESCs). For example, activins maintain human ESCs in their totipotent state, whereas bone morphogenetic proteins (BMPs) cause differentiation of human ESCs. Throughout reproduction, TGFβ family members function along with other growth factor signaling cascades (e.g., wingless-related (WNT), epidermal growth factor [EGF], steel-factor receptor [KIT], etc.) to permit the maintenance of the germline, development of the reproductive tracts, production of gametes, and creation of the next generation (Fig. 7.1). In this review, we will focus on major growth factor signaling in the mammalian reproductive tract beginning with the development of primordial germ cells (PGCs), and we will primarily concentrate on the roles of the TGFβ family.

Ovarian Development

Growth Factors in Primordial Germ Cell Formation and Migration

Future components of the mammalian ovary and testis develop long before a distinct bipotential organ can be discerned (Fig. 7.2). Growth factors are key for maintenance of the germline and their migration to the genital ridge. At the onset of gastrulation, PGC progenitors are induced in the epiblast in response to instructive signals from BMPs. This has been defined with great help through the development of mouse models[1-4] and is summarized in Table 7.1. BMP4 and BMP8B secreted from the extraembryonic ectoderm, and BMP2 secreted from the visceral endoderm, are required for the early discrimination of PGC precursors from somatic cells of the embryo. These BMPs signal in a dosage-dependent manner to the epiblast cells through a BMP receptor cascade, which involves phosphorylation of the BMP SMADs, SMAD1 and SMAD5, that along with their common SMAD partner, SMAD4, have been shown to function in this pathway. BMP2, BMP4, and BMP8B act on the pluripotent epiblast cells in the posterior of the embryo between embryonic day (E) 5.5 and E6.0 to induce their competency to become PGC precursors. At ~E6.25, six epiblast cells adjacent to the extraembryonic ectoderm express the BMP-regulated and PGC specification genes, PR domain containing 1 *(PRDM1*; also called *BLIMP1)* and *PRDM14*, and become committed to be true PGC precursors, the first such fate-committed cells of the embryo.[5] By E7.5, 40 founder PGCs are established.

Once PGC specification has occurred at E7.5, PGCs express markers of pluripotency including *POU5F1*, octomer-binding transcription factor 4 *(OCT4)*, SRY-box containing gene 2 *(SOX2)*, *NANOG*, developmental pluripotency-associated 3 *(DPPA3) (Stella)*, and *ALPL* (alkaline phosphatase). Between E7.5 and E8.5, the PGCs demonstrate major chromatin changes, increasing the levels of trimethylation of lysine 27 on histone H3 (H3K27me3) and erasing the dimethylation (H3K9me2) marks,[6] patterns that resemble the chromatin patterns of pluripotent stem cells. The H3K27me3 marks appear to repress the somatic cell gene expression program similar to ES cells. These changes cause PGC arrest at the G2 stage of the cell cycle, transiently causing the PGCs to become transcriptionally silent as they migrate from the base of the yolk sac along the hindgut to the genital ridge.[6]

An extracellular matrix gradient is important for PGC migration, and if too much matrix is deposited, PGCs show reduced migration. For example, suppression of TGFβ signaling by removing the TGFβ type 1 receptor, *Tgfbr1*, leads to enhanced migration due to reduction in the levels of TGFβ-induced collagen type 1 in the extracellular matrix.[7] Alternatively, *in vitro* migration assays demonstrate that KIT ligand, a growth factor that binds to KIT on PGCs, functions as an effective chemoattractant for PGC migration into the genital ridge. Consistent with the pleiotropic roles of the KIT ligand-KIT pathway, these proteins not only function in PGC migration but also aid in PGC survival and proliferation. The PI3K/AKT and SRC kinase pathways are also involved downstream of KIT in the functioning of the PGCs.[8]

Ovary Specification

PGCs proliferate during their migration to the genital ridge and after colonization. At this time, the gonad is not sexually differentiated and is termed "bi-potential" or "indifferent." BMP signaling within the bipotential gonad is necessary to develop a niche for migrating PGCs. Deletion of the BMP type 1 receptor, *Bmpr1a/Alk3*, in the genital ridge leads to death of somatic cells within the mesonephric mesenchyme and reduced levels of *Kitl* in the coelomic epithelium, ultimately decreasing the number, as well as

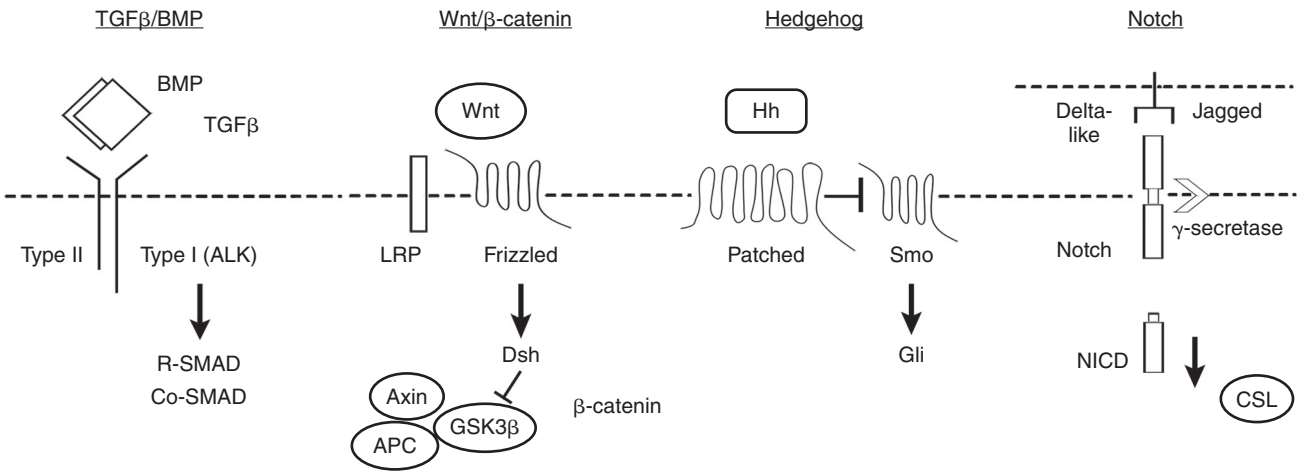


FIGURE 7.1 Canonical signaling for four major developmental pathways in ovarian and uterine function. **TGFβ /BMP:** TGFβ family members are secreted as dimeric proteins that bind to cell surface type 2 and type 1 serine threonine kinase receptors, which phosphorylate the intracellular SMAD transcription factors (R-SMAD). Once phosphorylated, the R-SMADs form a complex with a common SMAD (co-SMAD) and accumulate in the nucleus to regulate target genes. **WNT/β-catenin:** In the absence of WNT signaling, β-catenin is degraded by a complex including glycogen synthase kinase 3 (GSK3), axin and adenomatous polyposis coli (APC).[13] In its presence, WNT binds to a Frizzled receptor and coreceptor (low density lipoprotein receptor-related protein [LRP]) and β-catenin is stabilized by inactivation of the GSK3β complex by the phosphoprotein Disheveled (Dsh). β-catenin then accumulates in the cytosol and translocates to the nucleus as part of a transcriptional complex that includes T-cell factor/lymphoid enhancer binding factor (TCF/LEF) family members (not shown). **Hedgehog**: Hedgehog binds to the transmembrane receptor, Patched, resulting in the derepression of the Smoothened (Smo) receptor and activation of the glioma associated oncogene (GLI) transcription factors. **Notch:** The membrane-bound Notch receptor binds to its ligands, Delta-like and Jagged, localized on adjacent cell membranes. This actives the γ-secretase complex that cleaves the Notch intracellular domain (NICD). The NICD then translocates to the nucleus and interacts with the Epstein-Barr virus latency C promoter binding factor, suppressor of hairless/Lag1 (CSL) transcription factor family. Core components of each pathway are shown, without additional positive and negative regulatory inputs.

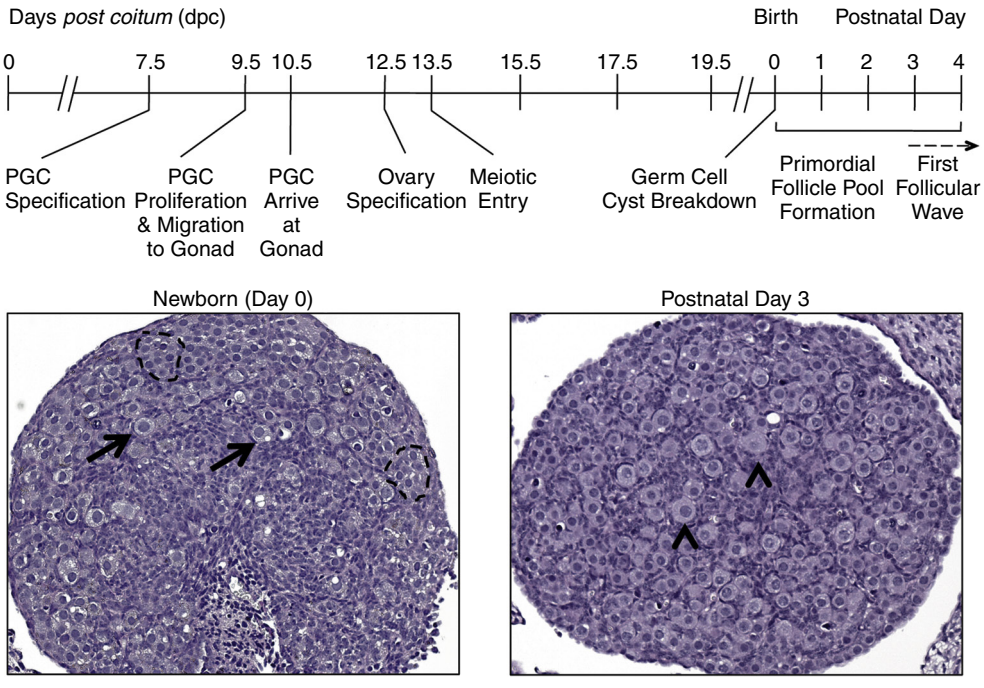


FIGURE 7.2 Timeline for female germline development from fertilization to birth in wild-type mice. Days post coitum (dpc) are shown in the top panel with key landmarks shown below. *Left lower panel,* histology of a newborn mouse ovary (postnatal day 0). Oocytes are organized into germ-cell-cysts *(dashed lines)* containing multiple oocytes, with a few forming primordial follicles *(arrows). Right lower panel,* Histology section of a postnatal day 3 mouse ovary. Most germ-cell-cysts have undergone breakdown to form primordial follicles, which in turn activate to form primary follicles *(arrowheads).*

migration, of the PGCs.[9] In mice, PGC proliferation ceases around E10.5-11.5, corresponding to 8 to 9 weeks of gestation in humans. *Bmp7* is required for germ cell proliferation, and *Bmp7* null mice have reduced numbers of germ cells after E11.5.[10] Activin may substitute for BMP7 in human embryos, since BMP7 is not expressed in the human ovary and BMP signaling alternatively promotes germ cell apoptosis.[11] Activin increases germ cell survival and proliferation in human ovaries prior to the entry of PGCs into meiosis.[12]

Differentiation of the bipotential gonad to form an ovary requires multiple signaling inputs, including that from the WNT-β-catenin pathway. Partial sex reversal is seen in XX

mice null for *Wnt4*, the WNT pathway activator protein *R-spondin 1 (Rspo1)*, or follistatin *(Fst)*, an antagonist of activin and BMP signaling. Loss of function *RSPO1* in XX patients results in complete female-to-male sex reversal while duplication of the chromosome region containing *WNT4* and *RSPO1* causes XY male-to-female sex reversal. Similarly, XY male-to-female sex reversal is observed in mice with a stable overexpression of β-catenin. β-catenin appears to be a central component to ovary specification, integrating signaling from the WNT and TGFβ family. Activation of WNT signaling allows stabilization and nuclear translocation of β-catenin, which interacts with a number of transcription factors of the TCF-LEF family to promote specific gene transcription.[13] Increased levels of β-catenin prevent the expression of SOX9, a transcription factor that promotes testes development by inducing Sertoli cell differentiation. β-catenin also increases expression of *Fst*, which shows a female-specific expression pattern at 11.5 dpc. *Wnt4*-null or *Fst*-null ovaries develop a coelomic vessel similar to that found in testes, and germ cells are lost by birth. *Wnt4*-null ovaries show an up-regulation of *Inhbb* (activin B) and *Fst*-null ovaries also express *Inhbb*. Genetic crosses between *Wnt4*-null or *Fst*-null mice to *Inhbb*-null mice prevents development of the coelomic vessel and rescues normal ovary development, indicating that *Inhbb* expression (via suppression by Wnt4) or activity (via antagonism through follistatin) must be limited to maintain ovary specification.[14-16] *Bmp2* is also expressed in a female-specific pattern after E11.5, though *Bmp2*-null mice die prior to this time period, and its function in the developing ovary has yet to be determined.

Male germ cells arrest in mitosis during embryogenesis and do not enter meiosis until after birth. In contrast, female germ cells enter meiosis shortly after ovary specification, which occurs around E13.5 in the mouse. Meiotic entry requires signaling inputs that are both intrinsic and extrinsic to the germ cells. Meiotic initiation at E13.5 in the mouse occurs in an anterior to posterior wave in the ovary from retinoic acid (RA) that is produced from the mesonephros. RA in the female ovary induces expression of *Stra8 (stimulated by retinoic acid gene 8)* in the germ cells. Germ cells from female mice null for *Stra8* do not enter into meiosis and do not express the meiotic markers *Dmc1* and *Spo11*. *Stra8* expression in the male is prevented by expression of *Cyp26b1*, which degrades RA. Loss of *Cyp26b1* in the testes results in a parallel increase in *Stra8* expression in germ cells as seen in normal ovary development and provides further support for the importance of RA signaling as a regulator of *Stra8* and meiotic onset. Additional genes are likely employed to maintain *Stra8* suppression in male germ cells prior to birth and their entry into meiosis, including *Nanos2*, an RNA-binding protein,[17] and *Nodal*, a member of the TGFβ family.[18]

Formation of the Ovarian Reserve

During embryonic development, oocytes within the ovary are found as clusters, or germ cell cysts (Fig. 7.2). These clusters form by both aggregation as well as clonal division.[19,20] Meiotic progression in oocytes ceases at the diplotene stage of prophase I when germ cells are enclosed by somatic cells into individual follicles called primordial follicles. Oocytes in primordial follicles remain arrested until released to complete meiosis I during ovulation and form the "ovarian reserve", which is thought to ultimately determine reproductive lifespan. Germ cell cyst breakdown occurs around birth in mice or during the second trimester in humans. In mice, the majority of germ cells within the cysts will die, with an estimated one-third remaining as primordial follicles.[21] Massive germ cell loss also occurs in humans, with an estimated 1 million oocytes surviving at birth from approximately 6 million in the fetal human ovary.

The mechanism for how oocytes become enclosed in primordial follicles has remained elusive, though multiple signaling inputs are likely required, particular those factors that mediate the interaction of oocytes with germ cells (Fig. 7.3). Signaling pathways that have been implicated in germ cell-cyst breakdown are kit ligand (KITL), nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), Notch-Jagged, and anti-müllerian hormone (AMH).[22] Improper germ cell cyst breakdown can manifest as multiple oocytes within follicles (called *polyovular follicles*), though their biological effects on fertility are not certain. Mice with mutations in the TGFβ family, such as ovarian activin βA *(Inhba)* deficiency, inhibin alpha subunit overexpression, and knockout mice for oocyte-expressed *Bmp15*, often show increases in polyovular formation. In addition, polyovular follicle formation can be generated by exposure of neonatal mice to estrogen and estrogen-like compounds, which may suppress ovarian activin expression, thus disrupting the regulation of germ cell cyst breakdown.[23-25]

The origin of the somatic cells that surround each primordial follicle and eventually become granulosa cells of the follicle has been controversial. Sertoli cells and granulosa cells may arise from ingressing cells of the coelomic epithelium during gonad development, though the timing of their specification may be different.[26,27] In the testis, regulation of the transcription factor *Sox9* by the testis-determining factor, sex determining region on chromosome Y (SRY) is required for Sertoli cell differentiation. In the ovary, forkhead box 2 (FOXL2) acts to maintain granulosa cell differentiation, in

Table 7.1 Phenotypes for Mice with Mutations in PGC Pathway Components (Ordered Based on Temporal Function During Embryogenesis)

Gene Symbol	Phenotype; Findings
Bmp2	Embryonic lethal; reduced PGC
Bmp4	Embryonic lethal; no PGC
Bmp8b	Viable; male infertility; reduced PGC
Smad1	Embryonic lethal; reduced PGC
Smad5	Embryonic lethal; reduced PGC
Smad4	Embryonic lethal; absent PGC
Prdm1 (Blimp1)	Embryonic lethal; PGC specification defect
Prdm14	Infertility; PGC specification defect
Pou5f1 (Oct4)	Pluripotency marker
Nanog	Pluripotency marker
Sox2	Pluripotency marker
Kitl	Variable phenotypes depending on mutation; PGC migration defect
Kit	Variable phenotypes depending on mutation; PGC migration defect
Tgfbr1 (ALK5)	Embryonic lethal; enhanced PGC migration

The full version of this table, including references, can be found on the companion Expert Consult Web site at www.expertconsultbook.com.

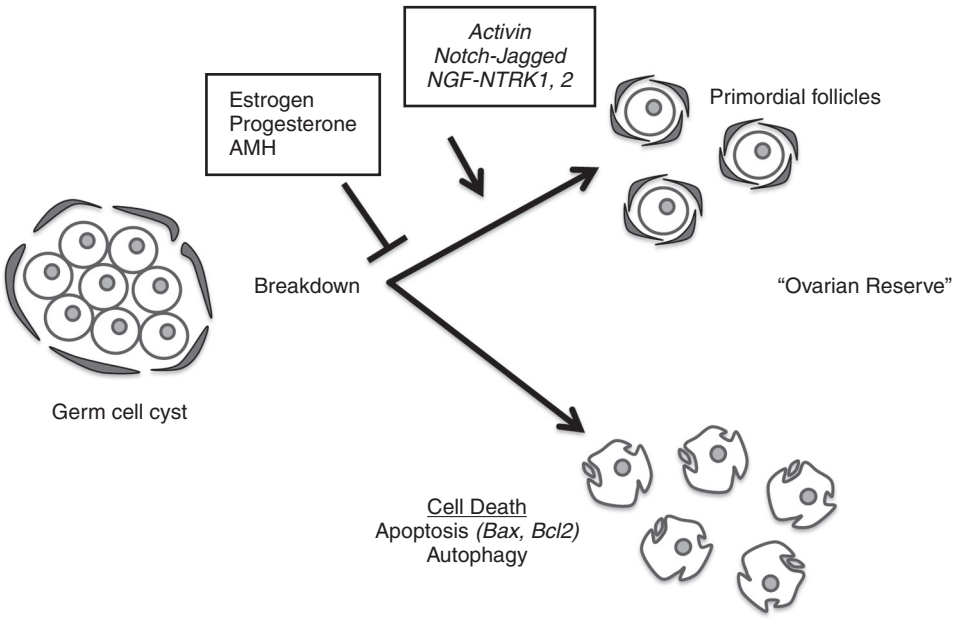


FIGURE 7.3 Key pathways in mice regulating germ cell cyst breakdown to form the ovarian reserve. Oocytes in germ-cell-cysts *(left)* undergo breakdown to either form primordial follicles *(upper pathway)* or die *(lower pathway)*. The number of resting primordial follicles makes up the "ovarian reserve." Key signal transduction pathways that regulate these processes are shown.

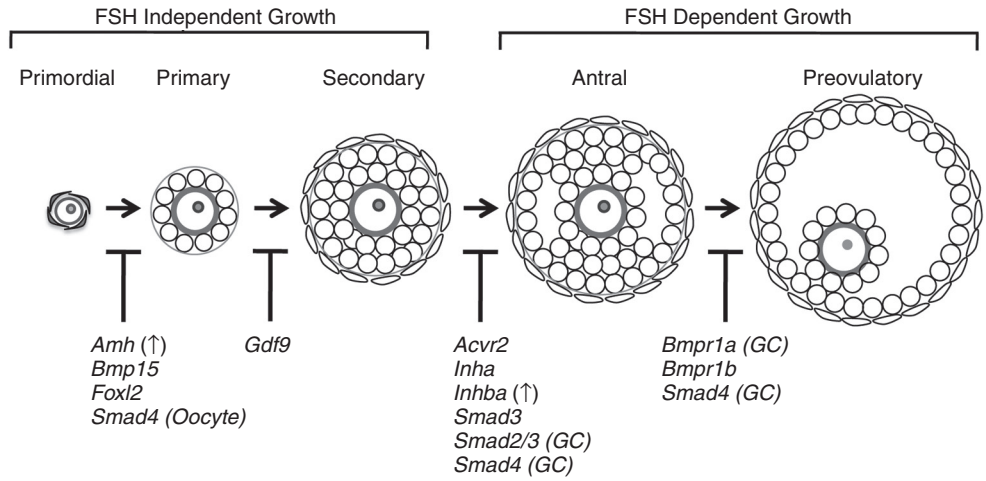


FIGURE 7.4 Schematic of follicle development. Resting follicles (primordial) are recruited into the growing pool and initially form "primary" follicles as granulosa cells form a cuboidal epithelium around the oocyte and proliferate. Multiple layers of GCs and acquisition of a thecal cell layer characterize "secondary follicles." An antrum then forms, and the follicle undergoes further differentiation to the preovulatory or "Graafian" stage under the influence of pituitary gonadotropins. Blocks in follicle development for various mouse knockout models are shown as discussed in the text and tables. Arrows in parentheses indicate a more rapid transition when the indicated genes are deleted.

part by suppressing expression of *Sox9*.[28] Mutations in the *FOXL2* genes are associated with several human diseases. The *FOXL2* gene is mutated in women with blepharophimosis ptosis epicanthus inversus syndrome (BPES), which in one form is associated with primary ovarian failure. Subsequently, it was shown that mice null for *Foxl2* were sterile with small ovaries and defects in primordial follicle assembly and growth (Fig. 7.4). FOXL2 interacts with different transcription factors, including SMAD2 and SMAD3, to control gene transcription in non-ovarian cell types, such as regulating *FSHB*, *FST*, and gonadotropin releasing hormone receptor synthesis in anterior pituitary cells.[29-31] In the developing mouse ovary, BMP2 and FOXL2 also cooperatively up-regulate *Fst* gene expression.[32] A somatic missense mutation in *FOXL2* is associated with almost all adult granulosa cell tumors,[33] a rare class of ovarian tumors. This mutation causes an amino acid substitution in the DNA-binding domain of FOXL2, though its functional consequence has not been fully clarified.

Ovarian Folliculogenesis

Ovarian Follicle Development

Once formed, quiescent primordial follicles supply all the oocytes during the reproductive lifespan of the female. This source of oocytes appears to be nonrenewable in mammals, although germ-like stem cells that can be differentiated into oocytes in culture have been reported.[34] Following their initial recruitment, a cohort of primordial follicles

Table 7.2 Mouse Models for Ligands in the TGFβ Family

Mutant Gene	Reproductive Phenotype	Fertility Status
Amh	Increased primordial follicle activation	Normal
Bmp6	Reduced ovulation	Subfertile
Bmp7	Reduced primordial germ cells	N.A.
Bmp15	Decreased ovulation and fertilization rates	Normal to subfertile
Gdf9	Follicular arrest at the primary follicle stage	Infertile
Fst	Loss of oocytes by birth	N.A.
Fst (Conditional)	Reduced growing follicles, ovulation and fertilization defects	Subfertile to infertile
Inha	Infertility, abnormal follicle growth, sex-cord stromal tumors	Infertile
Inhba (Conditional)	Increased polyovular follicles	Subfertile
Inhbb	Lactation defects	Infertile
Inhba Inhbb (Conditional)	Increased numbers of antral follicles and accumulation of corpora lutea	Infertile
Tgfb2	Increased oocytes at birth	N.A.*

*N.A., not available due to embryonic or perinatal lethality
The full version of this table, including references, can be found on the companion Expert Consult Web site at www.expertconsultbook.com.

undergoes a defined number of developmental follicular stages (Fig. 7.4).[35] The phosphoinositide-3-kinase (PI3K) regulates activation of oocytes within primordial follicles. Activation of PI3K signaling though deletion of the negative regulatory protein, *Pten*, or loss of an inhibitory transcription factor, *Foxo3*, results in global activation of primordial follicles and sterility in mice. The requirement of PI3K signaling in oocytes may be limited to the primordial follicle stage, because conditional deletion in mice of *Pten* from the primary stage onward has no affect on reproduction. Other oocyte-specific transcription factor cascades regulate early oocyte development, including *Nobox*, *Sohlh1*, *Sohlh2*, and *Figla*, which initiate an oocyte-specific gene expression pattern. Mutations in *NOBOX* and *FIGLA*, or their downstream targets, such as *GDF9* and *BMP15*, have been further implicated in the development of primary ovarian insufficiency in women.[36]

Following primordial follicle activation, pregranulosa cells undergo a squamous to cuboidal transition, forming the "primary follicles" (Fig. 7.4). Granulosa cell proliferation continues, and a second somatic cell layer composed of thecal cells recruited from the ovarian stroma, forms adjacent to the basement membrane surrounding the outermost layer of granulosa cells, resulting in a population of "secondary follicles." The oocyte-specific TGFβ family member, growth and differentiation factor 9 *(Gdf9)* is required at this stage, and mice null for *Gdf9* arrest at the primary to secondary stage and do not develop a thecal layer (Fig. 7.4); (Table 7.2). Thecal cells are required for steroidogenesis, as they produce androgens necessary for production of estrogen, and may provide additional growth factors and nutrients necessary for follicle growth.[37] The follicle arrest in *Gdf9*-null follicles is due to overproduction of inhibin α *(Inha)*—one of the subunits required to make the protein hormone inhibin, an antagonist of activin. Ovaries from *Inha-Gdf9* double knockout (DKO) mice develop multilayer follicles and form a morphologically distinct layer of cells that appear to be thecal cells. However, the thecal cells in *Inha-Gdf9* DKO ovaries never fully differentiate and are nonfunctional due to lack of expression of key genes, such as *17 alpha-hydroxylase (Cyp17)*, the luteinizing hormone (LH) receptor *(Lhcgr)*, and *Kit*.[38]

An additional TGFβ family member, anti-müllerian hormone (AMH) is also important as regulator of early follicle development, particularly for inhibition of primordial follicle activation (Table 7.2). AMH was discovered based on its function in sex determination and its ability to cause regression of the Müllerian duct during male reproductive tract development (see following section in this chapter, "Regression of the Müllerian Ducts"). In the ovary, AMH is expressed from granulosa cells of growing follicles until their development becomes dependent upon follicle-stimulating hormone at the later antral stages. Mice null for AMH initially have normal sized litters compared to wild-type mice, but demonstrate an accelerated loss of primordial follicles associated with an increasing number of growing follicles. This suggests that AMH negatively regulates primordial follicle activation.[39] In women, serum levels of AMH correlate with the number of antral follicles, and both decline with age. Thus, AMH has become a marker of ovarian reserve as well as reproductive disease in women. Its clinical utility is currently controversial but expanding in scope for use in monitoring ovarian reserve, response to *in vitro* fertilization protocols, granulosa cell tumor recurrence, and ovarian diseases such as primary ovarian insufficiency and polycystic ovary syndrome.[40,41]

Coordination of growth, and communication between cell types in the follicle is essential for follicle development to occur.[42] Multiple signaling pathways have critical inputs, including WNT-β-catenin, steroid hormone signaling, and kit ligand-kit signaling.[1,43] Preantral follicle growth is largely thought to be driven by intraovarian factors independent of input from FSH (Fig. 7.4). Mice null for the FSHβ subunit *(Fshb)* are infertile and do not form antral follicles. A similar block in follicle development is also seen in mice null for the activin binding receptor, *Acvr2a*, which have suppressed FSH production due to loss of activin signaling in the pituitary. Exogenous administration of gonadotropins in *Fshb*-null females results in ovulation, thus "rescuing" the phenotype[44], and demonstrates the switch from gonadotropin-independence to dependence at the final stages of follicle growth (Fig. 7.4).

Activin is a homodimer of β subunits derived from one of four genes in mammals, primarily *Inhba* or *Inhbb*, which generate activin A (βA:βA homodimers), activin B (βB:βB), or activin AB (βA:βB). Inhibin is an antagonist of activin and is formed by the dimerization of the *Inhba* or *Inhbb* gene products with a dissimilar alpha subunit, a product of the *Inha* gene (α:βA, inhibin A or α:βB forming inhibin B). Activin and inhibin were discovered based on their ability to stimulate (activin) or inhibit (inhibin) the secretion of FSH from the pituitary.[45] They are now known to be potent regulatory proteins with paracrine, autocrine, and endocrine functions in embryonic and adult tissue homeostasis, including the ovary. Inhibin antagonizes activin by competition for activin's binding receptors in conjunction with betaglycan,

Table 7.3 Phenotypes of Mice with Ovarian Deletion of the SMAD Transcription Factors

Gene	Cre	Cell-Specific Deletion	Reproductive Phenotype	Fertility Status
Smad1	Amhr2cre	Granulosa cell	None	Normal
Smad2	Amhr2cre	Granulosa cell	None	Normal
Smad3	Amhr2cre	Granulosa cell	None	Normal
Smad4	Amhr2cre	Granulosa cell	Reduced antral follicles and ovulation rates, cumulus cell defects, increased luteinization, increased progesterone	Subfertile, then 50% infertile by 6 months
Smad4	Gdf9-icre	Oocyte	Reduced litter sizes	Subfertile
Smad4	Zp3-cre	Oocyte	None	Normal
Smad5	Amhr2cre	Granulosa cell	None	Normal
Smad8	Knockout	Global	None	Normal
Smad2 Smad3	Amhr2cre	Granulosa cell	Reduced antral follicles and ovulation rates, cumulus cell defects	Subfertile, then infertile at 5 months
Smad1 Smad8	Amhr2cre	Granulosa cell	None	Normal
Smad5 Smad8	Amhr2cre	Granulosa cell	None	Normal
Smad1 Smad5	Amhr2cre	Granulosa cell	Granulosa cell tumors	Subfertile, then infertile at 4-6 months
Smad1 Smad5 Smad8	Amhr2cre	Granulosa cell	Granulosa cell tumors	Subfertile, then infertile at 4-6 months

The full version of this table, including references, can be found on the companion Expert Consult Web site at www.expertconsultbook.com.

a co-receptor.[46,47] Activin signaling can also be inhibited by follistatin, a high-affinity binding protein that prevents activin from binding to its receptors.

The activin subunits, *Inhba* and *Inhbb*, are expressed primarily in granulosa cells, though the receptors localize to thecal cells, granulosa cells, and oocytes.[48] Intraovarian activin promotes cell proliferation, and may modulate FSH action and steroidogenesis. *Inhba*-null mice and *Fst*-null die shortly after birth with craniofacial defects.[49-51] *Inhbb*-null female mice give birth to normal-sized litters, but offspring fail to thrive because the mothers have lactation defects.[52] Tissue-specific ("conditional") knockouts of *Inhba* and *Fst* have been generated in granulosa cells (Table 7.3). Stepwise removal of both activin subunit genes (*i.e.*, conditional deletion of *Inhba* concurrent with global loss of *Inhbb*) results in female sterility.[53] Activin-deficient mice have increased antral follicles and defective ovulation, suggesting that activin's role at the preantral stage is either limited or redundant with other factors. Strikingly, activin-deficient ovaries display an increasing number of corpora lutea with age and increases in two hormones of the reproductive axis, FSH and progesterone. Corpora lutea, the temporary endocrine structures that produce progesterone necessary for pregnancy to occur, fail to regress in activin-deficient ovaries, supporting a role for activin in the very terminal stage of follicle development. One key gene that is regulated by the TGFβ family, *Ctgf* (connective tissue growth factor), is down-regulated in activin-deficient ovaries.[53] Conditional deletion of *Ctgf* also results in impaired ovulation and increases in the number of corpora lutea as the activin-deficient ovary.[54]

Follistatin and inhibin are two antagonists of activin. Conditional deletion of follistatin in granulosa cells results in infertility in female mice (Table 7.2).[55] Deletion of the inhibin alpha subunit *(Inha)* also results in infertility and defects in follicle growth, but importantly causes the development of sex-cord stromal tumors (Table 7.2).[56,57] Activin-production from these tumors causes a cancer cachexia-like death in early adult *Inha*-null male and female mice.[58] Tumor development in *Inha*-null females can be slowed by removing *Acvr2a*, by deleting one of activin's downstream transcription factors (*Smad3*; discussed later in the chapter), or by overexpressing follistatin. These models support a growth-promoting role for activin in granulosa cells and sex-cord stromal tumor development.

SMAD2 and SMAD3 are the principle transcription factors that relay activin, TGFβ, and GDF9 signaling. Significant redundancy between these proteins likely exists during granulosa cell development, because single conditional knockouts of either *Smad2* or *Smad3* do not show reproductive defects (Table 7.3). However, *Smad2 Smad3* conditional DKO mice have reduced female fertility and fecundity, reduced numbers of antral-stage follicles, and contain follicles that have undergone premature luteinization. Furthermore, cumulus expansion—a process of extracellular matrix formation and loss of oocyte-cumulus cell connections that occurs during ovulation—is altered (discussed later in the chapter).[59] The phenotype of the *Smad2 Smad3* DKO is similar to that found for granulosa cell-specific *Smad4* conditional knockouts,[60] though the latter also showed increased atretic preantral follicles, suggesting that a SMAD4-dependent non-SMAD2-3 pathway may be important for preventing atresia in the preantral follicle stage.

BMPs are also expressed during preantral and antral stage follicle development from oocytes (*Bmp6* and *Bmp15*), granulosa cells *(Bmp2)*, and thecal cells (*Bmp4* and *Bmp7*). *Bmp6*-null mice have mild changes in their fecundity and BMP6 may regulate some aspects of ovulation.[61] BMP15 plays critical roles in sheep fecundity (discussed later in the chapter), and may interact with GDF9, as mice null for *Bmp15* containing one allele of *Gdf9* have reduced litter sizes compared to *Bmp15*-null mice.[62] Conditional deletion of two of the BMP type 1 receptors, *Bmpr1a* and *Bmpr1b*, in the ovary results in granulosa cell tumor development, as does deletion of the downstream BMP signaling SMADs, *Smad1* and *Smad5*. The histologic, hormone, and gene expression profile of the *Smad1 Smad5* DKO mice is similar to the juvenile form of human granulosa cell tumors.[63] *Smad1 Smad5* DKO mice die from metastatic disease, but do not develop the cancer cachexia-like syndrome of

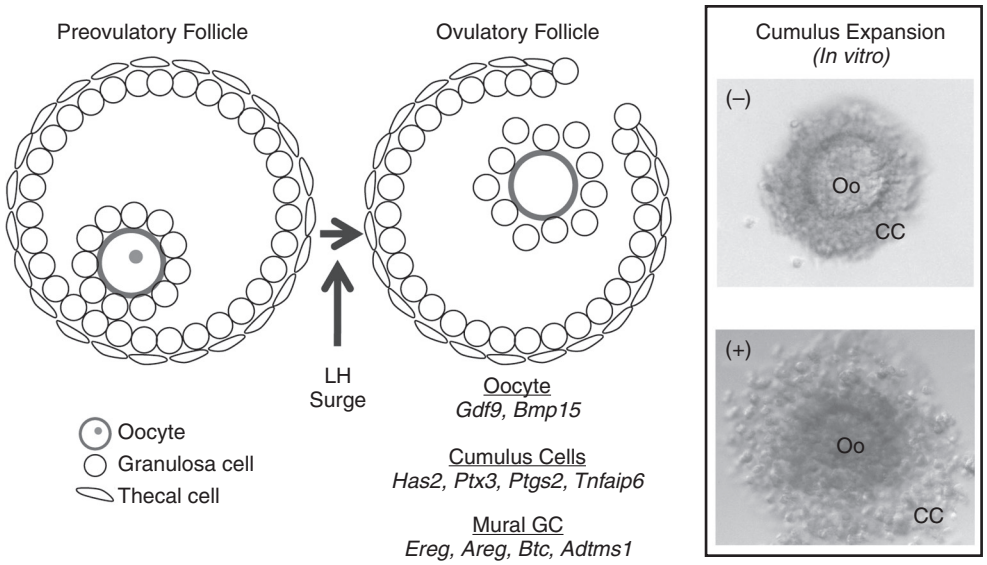


FIGURE 7.5 Ovulation and cumulus expansion in the mouse. Prior to the LH surge, preovulatory follicles contains an oocyte surrounded by cumulus cells and separated from the mural granulosa cells by a fluid filled cavity called an antrum. After a surge of luteinizing hormone (LH), the cumulus cells undergo expansion and become embedded in a hyaluronan-rich matrix, and the cumulus-oocyte complex is extruded from the follicle. An example of cumulus expansion *in vitro* using intact mouse cumulus-oocyte complexes and FSH treatment is shown in the right panels. Genes known to play critical roles in cumulus expansion are indicated underneath the cell types that express them. Oo, oocyte; CC, cumulus cells.

Inha-null mice, suggesting that activin is not overexpressed from the *Smad1 Smad5* DKO tumors. However, activated phosphorylated SMAD2 and SMAD3 is found in *Smad1 Smad5* DKO or *Inha* null tumors, as well as human juvenile granulosa cell tumors samples, suggesting that an active SMAD2/3 pathway plays a conserved role in development or progression of granulosa cell tumors.

Cumulus Expansion and Ovulation

Preantral follicle growth is followed by an antral follicle stage, which is defined by the presence of a fluid fill cavity, the antrum (Fig. 7.4). Under the influence of pituitary gonadotropins, antral follicles are selected to undergo final differentiation, ovulation, and subsequently, corpus luteum formation. Because of the presence of an antrum, preovulatory follicles contain two independent populations of granulosa cells: mural granulosa cells line the follicle wall and are closest to the theca and vasculature, while cumulus cells surround the oocyte and are linked initially with the oocyte for their functions (Fig. 7.5). The growth factor contributions of the mural and cumulus cell and their relevance to ovulation are most apparent at the time of the LH surge. The LH receptor *(Lhcgr)* is preferentially expressed only in mural granulosa cells, allowing them to respond to the LH surge, which initiates a cascade of events leading to oocyte meiotic resumption, cumulus expansion, follicle rupture, and terminal differentiation of the remaining granulosa and thecal cells to create the corpus luteum. Cumulus cell expansion, which is the production of a hyaluronan-rich extracellular matrix surrounding the oocyte, is initiated by the LH surge and is required for normal ovulation and fertilization. However, since the cumulus granulosa cells lack LHCGR and yet respond to the LH, this indicates an indirect effect of the LH surge on their fate.

How does the LH surge lead to induction of target genes in cumulus cells that are critical for cumulus expansion? Conti and colleagues have shown that the LH surge causes a rapid increase in epidermal growth factor (EGF)-like family members, amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC) specifically in mural granulosa cells of preovulatory follicles (Fig. 7.5).[64] These ligands are synthesized as membrane proteins and released from the cell surface into the antral fluid by proteolytic cleavage. These EGF-like proteins activate the EGF receptor (EGFR) on cumulus cells to exert the indirect effect of the LH surge to stimulate cumulus expansion and oocyte maturation. The effects of these EGF-like factors on cumulus expansion occurs through up-regulation of the genes encoding hyaluronan synthase 2 *(Has2)*, tumor necrosis factor-induced protein 6 *(Tnfaip6)*, pentraxin 3 *(Ptx3)*, and prostaglandin synthase 2 *(Ptgs2)*, whose products are essential for formation and stabilization of the extracellular matrix of the cumulus cells (discussed later in the chapter). Transcripts for *Areg*, *Ereg*, and *Btc* are also detected in cumulus oocyte complexes after the surge, suggesting an autocrine regulatory loop maintains high EGF-like growth factor expression in cumulus cells. These data are not specific to mice since amphiregulin is abundant in human follicular fluid from patients undergoing *in vitro* fertilization.[65,66]

Whereas EGF-like factors play an important role in cumulus expansion and oocyte maturation, oocyte-secreted growth factors are also essential for cumulus expansion. *In vitro*, expansion of isolated cumulus oocyte complexes can occur with the addition of EGF (as well as FSH and cyclic adenosine monophosphate [AMP] analogs). The oocyte is required for expansion since oocytectomized cumulus oocyte complexes fail to expand when only FSH or EGF is added to the culture medium.[42] Expansion of OOX complexes are rescued by co-culture with denuded oocytes or conditioned

medium from denuded oocytes, indicating the presence of oocyte growth factors are also involved in the expansion. As mentioned earlier, follicular development in *Gdf9* KO mice is arrested at the primary follicle stage. However, our group has shown that recombinant GDF9 up-regulates *Has2*, *Ptgs2*, *Ptger2*, *Tnfaip6*, and *Ptx3* in granulosa cell culture systems.[67,68] Injection of mouse oocytes with *Gdf9* si-RNA, but not *Bmp15* si-RNA, suppresses *Has2* and *Ptgs2* expression, and cumulus expansion when oocytectomized cumulus complexes are incubated with knockdown oocytes.[69]

The cumulus cell matrix contains an essential meshwork of hyaluranon and proteins. Hyaluronan chains from the structural backbone of the cumulus matrix, and these chains are stabilized through interactions with other matrix proteins. TNFAIP6 catalyzes the formation of covalent cross-links between hyaluronan and the heavy chain of serum-derived inter-α-trypsin inhibitor (IαI), and PTX3 stabilizes the cumulus matrix through interactions with IαI. PTGS2 synthesizes PGE2 and prostaglandin EP2 receptor (PTGER2), appears to function upstream of TNFAIP6. Although *Has2* ovary-specific knockouts have not been generated, the importance of the regulation of the other EGF/GDF9/BMP15 regulated gene products *in vivo* in cumulus expansion is obvious. Targeted disruption of *Ptgs2*, the prostaglandin E receptor 2 *(Ptger2)*, *Tnfaip2*, or *Ptx3* results in abnormal or absent cumulus expansion and extreme subfertility while *Tnfaip6*-null females are sterile. Knockdown of *Has2* in cultured cumulus oocyte complexes suppresses cumulus expansion. IαI biosynthesis in the liver requires alpha-1-microglobulin-bikunin light chain (encoded by *Ambp*) for proper assembly and secretion, and *Ambp*-null females have impaired cumulus expansion and subfertility. Thus, oocyte-secreted growth factors (GDF9 and BMP15) and EGF-like growth factors (AREG, EREG, and BTC) combine to induce an array of genes that are necessary for mammalian cumulus expansion and fertility.

Mutations in mice, sheep, and humans have been important in identifying key functions of GDF9, BMP15, and their downstream gene products in cumulus expansion. GDF9 signals through a heterodimeric complex of type 1 and a type 2 receptors to phosphorylate SMAD2/3 and complex with SMAD4 and conditional deletion of both *Smad2* and *Smad3* or *Smad4* in granulosa cells using *Amhr2-cre* disrupts cumulus expansion *in vivo* and suppresses fertility (as mentioned above).[59,60] Naturally occurring mutations in sheep *BMP15* and *GDF9* or the BMP type 1 receptor, *BMPR1B/ALK6*, modify ovulation rate in sheep (Table 7.4). In most cases, mutations in *BMP15* or *GDF9* increase ovulation rate when heterozygous, but homozygous null animals are sterile. Ovaries from homozygous mutant sheep appear similar to mouse *Gdf9-null* ovaries, with follicles arrested at the primary stage. Partial immuno-neutralization against GDF9, BMP15 or in combination in sheep will increase ovulation rate and the number of lambs, indicating that both GDF9 and BMP15 regulate this process.[70] Genetic variants have been identified in women with premature ovarian failure (*BMP15* or *GDF9*) or dizygotic twinning *(GDF9)*.

While there is convincing evidence that GDF9 is sufficient to function as a cumulus expansion-enabling factor (CEEF), there is, however, controversy as to whether GDF9 is the sole CEEF.[71] BMP15 has also been implicated in the regulation of cumulus expansion and upregulates expression of EGF-like growth factors in cumulus cells *in vitro*.[72] Although the fertility defects in *Bmp15*$^{-/-}$ mice are subtle and *Gdf9*$^{+/-}$ are phenotypically normal, *Gdf9*$^{+/-}$ *Bmp15*$^{-/-}$ double mutant mice on a 129SvEV inbred background are infertile with impaired cumulus expansion.[62] Furthermore, coculture of *Gdf9*$^{+/-}$ *Bmp15*$^{-/-}$ oocytes with OOX cumulus complexes fails to enable cumulus expansion in the presence of FSH and the cumulus cells have less activation of MAPK, suggesting BMP15 acts synergistically with GDF9 as a CEEF.[73] BMP15 is believed to signal through the type 1 activin receptor-like kinase, ALK6 (also known as BMPR1B) to activate SMAD1/5/8 pathways.[74] *Bmpr1b*$^{-/-}$ female mice are infertile secondary to defects in cumulus expansion, despite a paradoxical increase in *Ptgs2*.[75] Finally, BMP15 is found in the follicular fluid of patients undergoing *in vitro* fertilization (IVF) and is associated with increased fertilization and embryo development.[76] Although a direct link with BMP15 and cumulus expansion in human cumulus oocyte complexes was not made, other studies have associated higher levels of *HAS2* and *PTGS2* in human cumulus cells with higher quality embryos.[77,78] As both *BMP15* and *GDF9* are expressed in human cumulus oocyte complexes,[79] the relative contribution of each factor in human cumulus expansion and developmental potential remains to be determined. Studies in *Bmp15*$^{-/-}$ and *Gdf9*$^{+/-}$ *Bmp15*$^{-/-}$ mice, however, suggest both growth factors influence developmental competence of oocytes following fertilization.[73,80]

Nuclear receptor interacting protein 1 (NRIP1), also known as receptor interacting protein 140 (RIP140), functions as a coregulator of the nuclear receptor superfamily. *Nrip1*$^{-/-}$ female mice are also infertile secondary to anovulation; however, the unruptured follicles in these mice go on to form corpora lutea with trapped oocytes.[81] More detailed histological analysis of *Nrip*-null ovaries demonstrated defects in cumulus expansion,

Table 7.4 Mutations Affecting Ovulation Rate and Prolificacy Sheep BMP15 and GDF9

Gene	Mutation	Breeds	Protein Domain	Mutation Type
GDF9	FecG^{H}	Belclare, Cambridge	Mature	Substitution
	FecG^{E}	Santa Inês*	Mature	Substitution
BMP15	FecX^{B}	Belclare	Mature	Substitution
	FecX^{I}	Inverdale	Mature	Substitution
	FecX^{H}	Hanna	Mature	Premature stop
	FecX^{G}	Galway	Prepro	Substitution; premature stop
	FecX^{L}	Lacaune	Mature	Substitution
	FecX^{R}	Rasa Aragonesa		Premature stop
BMPR1B	FecB	Booroola,† Merlino, Hu, Han, Garole, Javenese Thin-tail	Kinase domain	Substitution

*Heterozygotes have normal ovulation rates, but rates increase in homozygous sheep.
†Heterozygotes and homozygotes have increased ovulation rates with the heterozygotes midway between homozygotes and non-carriers.
The full version of this table, including references, can be found on the companion Expert Consult Web site at www.expertconsultbook.com.

Table 7.5 Mouse Models with Defects in the Periovulatory Period

Mutant Gene	Reproductive Phenotype	Fertility Status
Luteinizing hormone β *(Lhb)*	Block in folliculogenesis; early antral stage	Infertile
Luteinizing hormone/choriogonadotropin receptor *(Lhcgr)*	Block in folliculogenesis; early antral stage	Infertile
Prostaglandin-endoperoxide synthase 2 *(Ptgs2)*	Defects in ovulation, cumulus expansion, and implantation	Mostly infertile
Prostaglandin E receptor 2, subtype EP2 *(Ptger2)*	Defects in cumulus expansion; decreased fertilization	Subfertile
Pentraxin 3 *(Ptx3)*	Defects in cumulus-oocyte complex integrity and ovulation	Subfertile
Tumor necrosis factor alpha induced protein 6 *(Tnfaip6)*	Impaired cumulus matrix formation resulting in failed cumulus-oocyte complex expansion	Infertile
CCAAT/enhancer-binding protein β *(Cebpb)*	Reduced ovulation; fail to form corpus luteum	Infertile
Nuclear receptor interacting protein (*Nrip1*; RIP40)	Ovulation defect; ovaries accumulate luteinized and unruptured follicles	Infertile
Progesterone receptor (*Pgr*; PR)	Anovulation; corpora lutea absent	Infertile
Alpha 1 microglobulin/bikunin *(Ambp)*	Defects in ovulation and cumulus-oocyte complex integrity	Subfertile
Amphiregulin *(Areg)*	Suppressed cumulus expansion, delayed meiotic reentry	Subfertile
Bone morphogenetic protein 15 *(Bmp15)*	Defects in cumulus expansion and ovulation	Subfertile
Bone morphogenetic protein receptor, type 1B *(Bmpr1b)*	Defects in estrous cyclicity, cumulus expansion, and endometrial gland development	Subfertile
Epiregulin ($Ereg^{wa2/wa2}$; hypomorph)	Suppressed cumulus expansion, defective meiotic resumption, ovulation defect	Subfertile
MAD homolog 4 *(Smad4)* (cKO)	Premature luteinization; decreased ovulation with trapped oocytes	Subfertile

The full version of this table, including references, can be found on the companion Expert Consult Web site at www.expertconsultbook.com.

suggesting NRIP1 also is an important mediator of this process, as well as ovulation. Gene expression profiling showed that following gonadotropin stimulation *Nrip*-null mice fail to induce many genes already discussed that are important for cumulus expansion, including EGF-like growth factors (*Areg*, *Ereg*, and *Btc*), *Has2*, *Ptgs2*, *Tnfaip6*, *Cspg2* (versican); whereas many genes involved in cell to cell adhesion are up-regulated (and might, therefore, impede expansion). *Nrip1* is most dramatically up-regulated in mural granulosa cells following PMSG administration (to mimic FSH), whereas *Cebpb* increases after PMSG plus hCG administration.[81-83] Because both FSH and LH activate cAMP/PKA/CREB pathways to influence gene expression, the increase in NRIP1 and C/EBPβ in the periovulatory period may function to modulate transcription of the many target genes with cAMP response elements.

The timing of luteinization is important for normal reproductive function and the fact that it occurs following ovulation supports a role for oocyte-derived inhibitors that prevent premature luteinization. As mentioned, oocyte-secreted factors (OSFs) oppose FSH signaling to define two distinct granulosa cell populations in the antral follicle. Specifically, OSFs signal through SMAD2/3 to oppose FSH induction of ovulatory *(Lhcgr)* and steroidogenic *(Cyp11a1)* markers in the cumulus cells close to the oocyte,[84] suggesting a TGFβ family member, such as GDF9, may be an important inhibitor of luteinization. Support for this is seen in mice with conditional deletion of the common SMAD, *Smad4*, in granulosa cells of preantral follicles.[60] Although *Smad4* cKO ovaries exhibit a number of follicular defects (discussed earlier in the chapter), including impaired cumulus expansion and ovulation, there were a number of small and large follicles with luteinizing cells surrounding oocytes, and these findings were accentuated after exogenous gonadotropin (PMSG) administration only in 3-week-old animals. Molecularly, in contrast to control mice, granulosa cells from PMSG-stimulated *Smad4* cKO mice had significantly higher levels of genes related to steroidogenesis and luteinization, including *Lhcgr*, *Cyp11a1*, *Hsd17b7*, *Star*, secreted frizzled protein *(Sfrp4)*, and the prostaglandin F2α receptor *(Ptgfr)*. While it is also possible that the loss of SMAD4 prevents granulosa cells from responding appropriately to BMP/TGFβ/activin signals from the surrounding stroma that might also inhibit luteinization, Eppig and colleagues provide convincing evidence that OSF-activation of SMAD signaling is key to preventing premature differentiation of granulosa cells.[84]

While we have tried to highlight critical mediators of ovulation, cumulus expansion, and luteinization, many additional factors are involved in these processes in the periovulatory follicle. Mutant mouse models illustrating the diverse number of factors that regulate these events are summarized in Table 7.5.

Formation of the Reproductive Tracts

In mammals, the sex of an organism is determined by two connected mechanisms: first, the chromosomal sex causes the indifferent gonad to acquire a specific gonadal sex (primary sex differentiation), which in turn, is responsible for determining the phenotypic sex (secondary sex differentiation). Individuals carrying a Y chromosome are considered genetically males; the *Sry* gene is located on the Y chromosome and has been shown to be essential for the gonad to differentiate into testes.[85-87] The production of male-specific hormones and growth factors by the testes causes the expression of characteristics typical of males.

Before sexual differentiation, mammalian embryos of both sexes have an indifferent gonad and two pairs of reproductive ducts: the wolffian (mesonephric) ducts and the müllerian (paramesonephric) ducts. The male and female reproductive tracts and the kidneys primarily develop from the intermediate mesoderm. In humans, the wolffian ducts first appear at Carnegie stage 10 (equivalent to E9 in mouse) as mesenchymal ridges that segregate from the intermediate mesoderm and undergo mesenchymal to epithelial transition to form two epithelial canalized ducts. The wolffian ducts give rise to the vas deferens and the seminiferous

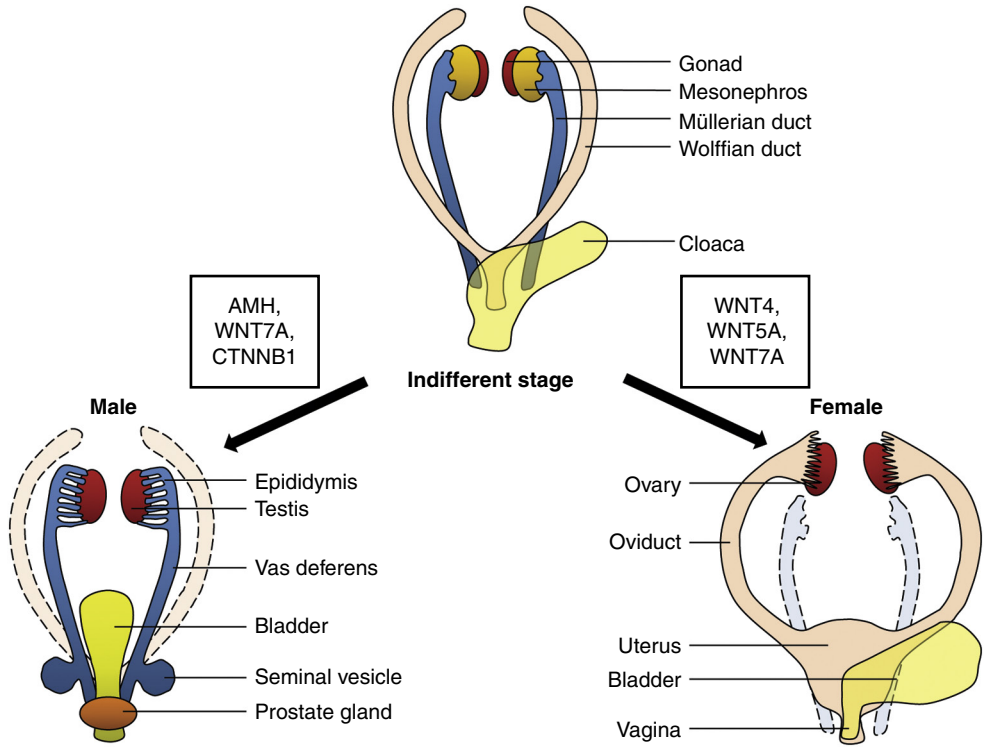


FIGURE 7.6 Sexual differentiation of the reproductive system. At the indifferent stage, male and female embryos have bipotential gonads, and both müllerian and wolffian ducts. Depending on the presence or absence of the Sry gene in males and females respectively, the gonads and ducts differentiate into male or female reproductive organs. The expression of *Wnt7a* in the müllerian duct induces the production of AMH receptor (AMHR2) and makes the müllerian duct responsive to AMH. When Sry is expressed, the indifferent gonad becomes testis, which produces AMH and induces the regression of the müllerian duct. The testis also allows the maintenance of the wolffian duct through the production of testosterone. When Sry is not expressed, the müllerian duct is maintained and develops into the female reproductive tract; this process requires the expression of different members of the Wnt signaling pathway. At the same time, since the indifferent gonad became ovary, testosterone is not produced and the wolffian duct is not maintained.

tubules of the male reproductive system. The anlages of the müllerian ducts form from an invagination of the coelomic epithelium through the mesonephros at Carnegie stage 16 (E11.5 in mouse) extend to contact the wollfian ducts, and finally elongate toward the cloaca (dependent on the presence of the wolffian ducts). At E15.5, the müllerian ducts differentiate into the oviducts, uterus, and upper vagina (Fig. 7.6).[88-90]

In the past 15 years, the use of knockout mice has identified the main regulators of reproductive tract formation (Table 7.6). For example, female and male mice homozygous null for the pair-box gene *Pax2* completely lack the genital tracts. Mice null for *Emx2* show initial formation of the wolffian ducts but subsequent degeneration at E11.5, while in *Emx2* null females, no müllerian ducts are observed at E13. Lastly, female mice lacking the *Lim1* transcription factor that survive past E10 completely lack müllerian duct derivatives.

Table 7.6 Main Genes Involved in the Development of the Reproductive Tracts

Gene	Phenotype
Pax2	Null mice show neonatal lethality; males and females lack kidneys, ureters, and genital tracts
Emx2	Null mice show neonatal lethality; females and males lack kidneys, ureters, gonads, and genital tracts
Lim1	Null mice show embryonic lethality; females lack müllerian duct

The full version of this table, including references, can be found on the companion Expert Consult Web site at www.expertconsultbook.com.

Regression of the Müllerian Ducts

In both female and male mice, the müllerian ducts form at E11.5, but they regress in males during the secondary sex differentiation between E13 and E14. Anti-müllerian hormone (AMH), a TGFβ superfamily member, has been shown to be responsible for the regression of the müllerian ducts in males.[91-94] AMH is secreted by Sertoli cells of the fetal testis while its receptor (AMHR2) is expressed in mice in the mesenchyme of the müllerian duct at ~E13.5. In females, because the gonad differentiates into ovaries and no AMH-producing Sertoli cells are formed, the müllerian ducts are not stimulated to regress and are therefore maintained. AMH is both necessary and sufficient for the regression of the müllerian ducts; male mice homozygous for a mutant allele of *Amh* or its type 2 receptor *(Amhr2)* have a normally developed male reproductive tract but also bear a uterus and oviducts (Table 7.7). These mice have functional sperm but most are infertile due to the hindering of the sperm transfer by the female reproductive organs. Moreover, when *Amh* is overexpressed in female mice, the derivatives

Table 7.7 Genes Involved in Müllerian Duct Regression and Development

Gene	Phenotype
Amh	Null males have a complete male reproductive tract and functional sperm, but also uterus and oviducts; females are fertile with enlarged ovaries. Female mice overexpressing Amh show blind vagina and no uterus or oviducts
Amhr2	Null males have a normal male reproductive tract, but also uterus and oviducts
Alk2, Alk3	Alk2 and Alk3 null mice show embryonic lethality; conditional ablation of Alk2 and Alk3 in the müllerian duct mesenchyme (Alk2-Alk3 double conditional knockout mice) causes duct retention in males
Smad1, Smad5, Smad8	Smad1 and Smad5 null mice show embryonic lethality; Smad8 null mice are viable and fertile. Deletion of Smad5, Smad1-Smad5 or Smad5-Smad8 in the müllerian duct mesenchyme causes partial müllerian duct retention. Smad1-Smad5-Smad8 triple conditional knockout mice show complete müllerian duct retention
Wnt7a	Null mice show partial perinatal lethality; null males fail to undergo müllerian duct regression. Females are infertile due to abnormal development of uterus and oviduct
Wnt4	Null mice show neonatal lethality; null females are masculinized: they have wolffian duct and lack müllerian duct
Wnt5a	Null mice show perinatal lethality; females show caudal truncation of the reproductive tract and defective uterine glandular development
Ctnnb1	Null mice show embryonic lethality; male mice with a conditional deletion of Ctnnb1 in the müllerian duct mesenchyme show müllerian duct retention

The full version of this table, including references, can be found on the companion Expert Consult Web site at www.expertconsultbook.com.

of the müllerian ducts are absent. Considering the importance of this protein for achieving correct sexual differentiation, it is not surprising to find that its expression is subject to tight regulation; some of the genes upstream of AMH and responsible for its regulation show how AMH is connected to the testis-determining pathway. SRY is required for the activation of SRY-box containing gene *(Sox9)* expression, and SOX9 and steroidogenic factor 1 (SF1) have been shown to cooperate in the regulation of *Amh* transcription.[95] In addition, it has been suggested that Wilms tumor homolog (WT1) synergizes with SF1 to activate the transcription of *Amh*, and dosage sensitive sex-adrenal hypoplasia congenital (DSS-AHC) critical region on the X chromosome gene 1 (DAX1) antagonizes this synergy.[96]

When expression of either of the type 1 receptors, *Acvr1 (Alk2)* or *Bmpr1a (Alk3)*, is conditionally ablated, the müllerian duct mesenchyme does not regress in male mice, a phenotype similar to *Amh* and *Amhr2* null mice; this indicates that the two receptors act redundantly to transduce the AMH signal.[97] In addition, the three BMP-specific SMADs (SMAD1, SMAD5, and SMAD8) can activate the regression of the müllerian ducts to varying degrees[97] and mice with loss of these genes in combination retain müllerian duct derivatives.

Several pieces of data have been produced in support of the hypothesis that AMH signaling interacts with the wingless-related mouse mammary tumor virus (MMTV) integration site (WNT) pathway to regulate the regression of the müllerian ducts (Table 7.7).[98] WNT signaling seems to play a role both upstream and downstream to AMH. In particular, WNT7A, which is also involved in the development of the müllerian ducts, is required in the duct epithelium for the expression of *Amhr2* in the surrounding mesenchyme of the müllerian duct. Thus, WNT7A makes the müllerian duct responsive to AMH, and, accordingly, the müllerian duct fails to regress in male mice carrying a null mutation in the *Wnt7a* gene. In the canonical WNT pathway, the binding of WNT ligands to the cognate receptors causes the nuclear accumulation of β-catenin (CTNNB1); β-catenin forms a complex with cofactors to promote the activation of downstream target genes.[13] When *Ctnnb1* is conditionally ablated in the mesenchyme of the müllerian ducts of male mice, there is no regression of the ducts. The lack of CTNNB1 in the mesenchyme does not compromise the expression of *Amhr2* in those cells, indicating that WNT7A signaling is not affected and that another WNT is responsible for CTNNB1 activation in the mesenchyme during duct regression. WNT4 is a good candidate since AMH has been shown to be necessary and sufficient for the expression of *Wnt4* in the duct mesenchyme of male mice during its regression. However, males without WNT4 in the mesenchyme of the müllerian ducts do not show any defect in duct regression. It is noteworthy that these results have been obtained by using a conditional knockout model *(Amhr2-Cre)* that may have a *cre* recombinase activity not sufficiently high to recombine the *Wnt4* floxed allele to an efficient extent.

Müllerian Duct Development

The female reproductive tract in mammals includes the oviducts (fallopian tubes), uterus, cervix, and the vagina.[99] Different mammalian species exhibit significant variations in the morphology of the reproductive tract that are primarily due to differences during the development of the müllerian duct, the embryonic structural precursor of the mature female reproductive tract. Besides these morphological differences, the processes that enable the continuation of the mammalian species all rely on the functionality of this group of organs: the fertilization of the oocytes by spermatozoa occurs in the oviducts; the implantation of the embryo and its development take place in the uterus (with the exception of the egg-laying mammals); and the fetus moves through the cervix and vagina to finally be delivered.[90]

The absence of SRY in females sets a permissive environment for the gonads to become ovaries (primary sex differentiation; see section above "Ovary differentiation"); because the ovaries do not produce testosterone or AMH, the wolffian ducts are not supported in their development and thus regress. At the same time, the absence of AMH allows the müllerian ducts to be maintained. WNT4 has been shown to be essential for the formation of the müllerian ducts, and both male and female mice null for WNT4 lack müllerian ducts.[100] *Wnt4* is expressed in the mesonephros at E9.5-11 but not in the mesonephric tubules or wolffian ducts. *Wnt4* is also observed in the mesenchyme of the indifferent gonad at E11; during primary sex differentiation, expression of *Wnt4* decreases in the male gonad but is

maintained in the female gonad. The müllerian ducts form at E12 from a region of the coelomic epithelium positive for *Wnt4* expression, and *Wnt4* is expressed in mesenchymal cells surrounding the müllerian ducts throughout fetal life.

Wnt4 null mice die shortly after birth due to kidney failure.[101] In the absence of WNT4, newborn female mice show masculinization of the gonad, absence of the müllerian duct, and a fully developed wolffian duct, indicative of a reversal of sexual phenotype.[100] Absence of the müllerian duct can be due to either a block in the formation or to a male-specific regression taking place in the sexually reverted females. To identify the cause of the müllerian duct absence, an analysis of *Wnt4* null mice at an earlier time point was performed, which revealed that WNT4 is responsible for the initial phases of the formation of the müllerian duct in both sexes. In the absence of WNT4, female mice also exhibit a failure in the suppression of Leydig cell development and gonadal steroidogenesis. The presence of these male-specific processes in females leads to the maintenance of the wolffian ducts.

WNT7A is another member of the WNT family that plays a central role in the development of the female reproductive tract.[102] *Wnt7a* is expressed along the length of the müllerian duct epithelium in males and females when the duct forms at E12.5. It continues to be expressed throughout life in the derivatives of the müllerian duct in females; in males, *Wnt7a* expression disappears when the müllerian duct regresses. Female and male mice homozygous for a mutant *Wnt7a* allele are sterile. The female sterility is caused by defective differentiation of the müllerian duct derivatives; there are no oviducts and the uterus is thinner than in controls due to a reduction of the epithelial and stromal compartments, a severe defect in uterine gland formation, and a hyperplastic myometrium. A closer investigation of the reproductive tract reveals that the oviducts and the uterus of mutant mice are affected by partial posteriorization: the oviducts show features typical of the uterus, and the uterus has characteristics of the vagina. Accordingly, the expression of *Hoxa10* and *Hoxa11*, which have been shown to be required for the correct anterior-posterior patterning of the reproductive tract, shows postnatal reduction.[103] Thus, after WNT4 initiates the process of ductal formation, WNT7A is required to regulate the morphogenesis of ductal derivatives.

WNT5A is a third member of the WNT family that is highly expressed in the uterus and plays a role in both its development and postnatal growth.[104] The female reproductive tract is immature at birth and undergoes a process of differentiation during the first 2 weeks of postnatal life. Interactions between the epithelial and mesenchymal compartments are of central importance in the regulation of uterine differentiation. In particular, the epithelium is developmentally plastic, and its differentiation is stimulated by signals generated in the stroma. *Wnt5a* is highly expressed in the uterine mesenchyme and regulates stromal-epithelial crosstalk during uterine growth. Mice null for *Wnt5a* die at birth with defects in anterior-posterior body axis development.[105] The posterior structures of the reproductive tract (cervix and vagina) are absent in newborn *Wnt5a* mutants, and the uterine horns show significant reduction in size. When reproductive tracts from newborn *Wnt5a* null mice are grafted into wild-type adults, the uterus fails to develop glands, suggesting that WNT7A and WNT5A play nonredundant roles in uterine gland formation.

Role of Growth Factors in Uterine Biology

Regulation of Uterine Receptivity

Successful reproduction ensures species survival. In mammals, the female zygote is released from the ovary during ovulation and is fertilized by sperm in the reproductive tract. Upon fertilization, the zygote starts a process of cellular division and differentiation that leads to the formation of an implantation competent structure, the blastocyst, made up of two distinct populations of cells: the inner cell mass and the trophoblast. To successfully continue and complete its development, the embryo needs to rely on a supporting structure that provides nourishment and protection for the whole gestational period. In mammals, this structure is the uterus, and, as a first step, the embryo needs to make physical contact with it and start a molecular cross-talk that eventually leads to its implantation. Depending on the species, different strategies of implantation are used: in rats and mice, the luminal epithelium of the uterus undergoes apoptosis to facilitate the invasion of the embryo through the uterine wall; in primates, the luminal epithelium remains intact and the embryo intrudes between the epithelial cells to finally reach the stromal compartment.[106,107] The implantation of the embryo occurs during a limited period of time called the "window of receptivity." A highly regulated synchrony between the development of the embryo and the receptive status of the uterus needs to be accomplished to achieve a successful implantation.

The sexual hormones estrogens (in particular 17β-estradiol, E2) and progestins (in particular progesterone, P4) are produced by the ovaries in cyclic waves and cause profound changes in the uterus during the different phases of the estrous (mice) or menstrual (humans) cycle (Fig. 7.7). P4 and E2 act on the uterus by binding and activating the nuclear receptors PR and ER, respectively. Once activated, the nuclear receptors translocate into the nucleus and promote the transcription of downstream target genes. The receptive status of the uterus changes cyclically and can be classified as prereceptive, receptive, or nonreceptive (refractory). During the prereceptive phase, the uterus is not able to accept the implantation of the embryo but prepares for its arrival. In both humans and mice, an increase in the serum levels of P4 is observed prior to implantation during a phase called "secretory phase;" P4 acts on the endometrium by stimulating its full differentiation and making it a favorable environment for a potentially implanted embryo. During this period, tubular glands and spiral arteries develop, and the endometrium becomes thick and spongy. To achieve the receptive stage, a temporary rise in the levels of E2 is needed. The window of receptivity lasts 24 to 36 hours in mice (day 4 and morning of day 5 of pregnancy) and about 4 days in women (days 20 to 23 of the menstrual cycle). If the implantation of the embryo does not take place, the endometrium is reabsorbed in mice or expelled (menstruation) in humans to complete the estrous/menstrual cycle. After its degeneration, the endometrium undergoes a "proliferative phase" and regenerates under the influence of E2, signaling the beginning of a new cycle.

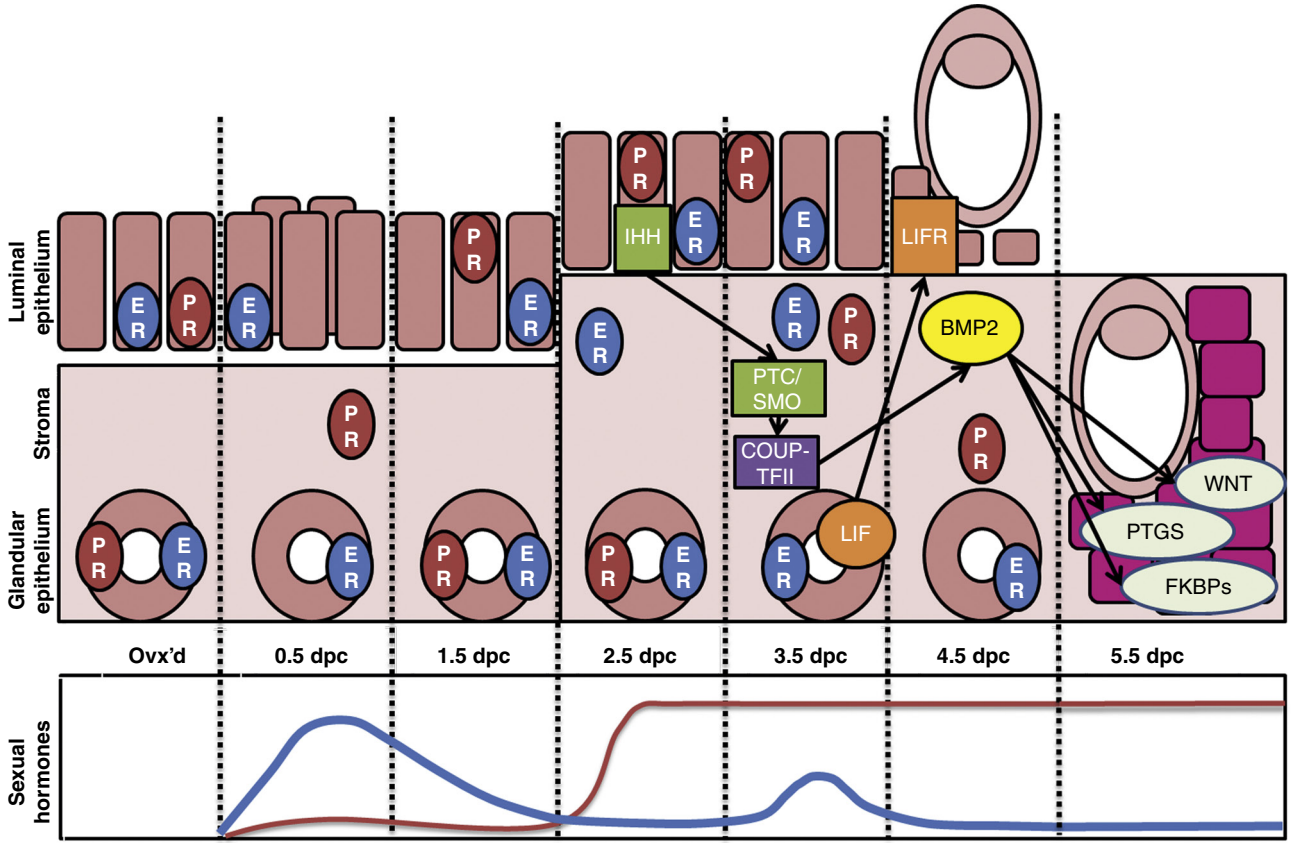


FIGURE 7.7 Molecular regulation of embryo implantation in the mouse. Estrogen and progesterone receptors are differentially expressed in the epithelial and luminal compartment of the uterus before the implantation and control the uterine receptivity through the regulation of several downstream factors. At 2.5 days post coitum (dpc), an increase in the levels of ovarian progesterone (P4) promotes the expression of indian hedgehog *(Ihh)* in the uterine epithelium. IHH acts on the adjacent stromal compartment where it causes the production of COUP-TFII at 3.5 dpc that, in turns, activates the expression of *Bmp2* at 4.5 dpc. A peak in the levels of estrogen (E2) at 3.5 dpc causes leukemia inhibitory factor *(Lif)* to be expressed in the glandular epithelium; LIF signals through its cognate receptor LIFR in the luminal epithelium and triggers the molecular and cellular changes that make the epithelium become receptive to the attachment of the embryo. When the embryo attaches at 4.5 dpc, the stromal cells are stimulated to proliferate and differentiate into decidual cells. BMP2 acts upstream to WNTs, PTGS2, and FKBPs to regulate the decidual reaction in stromal cells.

The molecular mechanisms that regulate the receptivity of the uterus and the implantation of the embryo have been investigated, and several mouse models have been produced to clarify the roles that different factors play during these processes. In this section, we will discuss the main signaling pathways shown to be involved in the regulation of the early stages of pregnancy in mammals.

Implantation of the Embryo

Estrogen and progesterone act on the uterus to trigger the changes that will make it receptive to the implantation of the embryo (Fig. 7.7). One mechanism that has been demonstrated to be required for achieving a receptive state is the inhibition of ER activity in the epithelium mediated by stromal PR. Therefore, to achieve the receptive state, estrogen and progesterone regulate the pre-implantation events by regulating the communication between stromal and epithelial compartments and many factors function in uterine receptivity. The secreted protein indian hedgehog *(IHH)* was identified as a gene differentially regulated by P4 in the luminal and glandular epithelium of the uterus during the peri-implantation period. IHH acts by binding the transmembrane receptor Patched1 (PTCH1) and by blocking its inhibitory action on another receptor, Smoothened (SMO) (Fig. 7.1); (Table 7.8).[108] The loss of inhibition of SMO activates the transcription of the glioma-associated oncogene homolog[48] (Gli) family of transcription factors and the orphan nuclear receptor Chicken Ovalbumin Upstream Transcription Factor II (COUP-TFII or nuclear receptor subfamily 2, group F, member 2, NR2F2). Because *Ihh* is expressed in the uterine epithelium and COUP-TFII is localized in the stromal compartment, this signaling pathway plays a central role in the regulation of the communication between epithelium and stroma of the uterus. In particular, IHH is required for the attachment of the embryo to the uterine epithelium, and mice lacking IHH in the uterus are sterile.[109] The ablation of *COUP-TFII* in the uterus phenocopies the defects observed in *Ihh* cKO mice and causes an increase in ER activity in the uterine epithelium. Thus, IHH and COUP-TFII play a central role as regulators of uterine receptivity by mediating the P4-dependent suppression of ER activity.

COUP-TFII also promotes the expression of another important factor involved in the regulation of the early pregnancy phases, BMP2.[110] *In situ* hybridization studies

Table 7.8 Genes Involved in the Regulation of Embryo Implantation

Gene	Phenotype
Ihh	Null mice die before or immediately after birth. Females with a conditional deletion of Ihh in the uterus show failure of blastocyst attachment. Decidualization is defective
NR2F2	Null mice show embryonic lethality. Females with a conditional deletion of COUP-TFII in the uterus are sterile; both embryo attachment and uterine decidualization are impaired
Bmp2	Null mice show embryonic lethality; females with a conditional deletion of Bmp2 in the uterus are sterile due to failure of uterine stromal cell decidualization
Wnt4	Null mice show neonatal lethality; female mice with a conditional deletion of Wnt4 in the uterus are severely subfertile due to impaired implantation

The full version of this table, including references, can be found on the companion Expert Consult Web site at www.expertconsultbook.com.

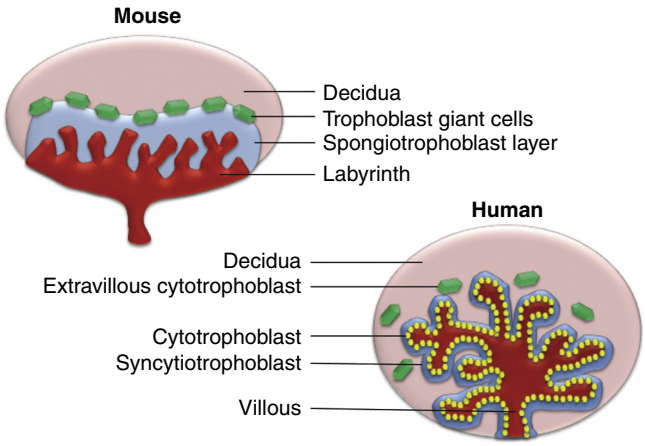


FIGURE 7.8 Schematic of the fetal-maternal interface in mouse and human. The placenta is composed of two main parts: the maternal decidua and the trophoblast of embryonic origin. During implantation, the extravillous cytotrophoblast in humans and the trophoblast giant cells in mouse, invade the uterine tissue and modify the maternal vasculature to obtain nutrients for the growing fetus as pregnancy progresses.

show that *Bmp2* is expressed in the uterine stroma at the moment of embryo implantation, and mice lacking BMP2 in the uterus are sterile.[111]

In wild-type mice, the attachment of the embryo to the uterine epithelium triggers a wave of intense proliferation and differentiation of the stromal compartment; this process is called decidualization and requires BMP2.[111] The role of BMP2 in the regulation of the decidualization has been investigated also in cells obtained from biopsies of human endometrium. Similar to the mouse, BMP2 is required in human endometrial stromal cells to induce their differentiation into decidual cells.[112] In agreement with a model in which IHH acts upstream to regulate the BMP pathway, mice lacking IHH or COUP-TFII, besides having an attachment defect, are also unable to decidualize. During decidualization, BMP2 activates several downstream targets. In particular, FK-506-binding protein 3 (FKBP3), FKBP4, and FKBP5 have been proposed to be induced by BMP2 and be required to modulate the activity of PR by acting as cochaperones of steroid hormone receptors. BMP2 is also necessary for inducing the expression of *Wnt4* and *Wnt6* during decidualization, and *Wnt4* cKO in the uterus are unable to support the invasion of the embryo through the endometrial wall and decidualize. When *Wnt4* is knocked down, differentiation of human endometrial cells is strongly reduced, demonstrating that the roles of WNT4 in decidualization is conserved in mice and humans. Moreover, besides its role in uterine development during embryonic life, WNT4 continues to be required for postnatal development of the uterus: *Wnt4* cKO mice show an abnormal stratification of the luminal epithelium and a reduced number of uterine glands.

Regulation of Trophoblast Proliferation, Invasion, and Migration

The trophectoderm is the outer layer of the blastocyst. The primary role of the trophoblastic cells is to differentiate to form the fetal part of the placenta, the organ that allows the fetus to connect to the maternal uterus, receive nutrients and hormones, eliminate waste, and exchange gas. Distinct epithelial cell types arise from trophoblast progenitor cells; in humans, 6 days after fertilization, two populations of trophoblast cells can be distinguished. The inner layer is composed of proliferating mononucleated cells called cytotrophoblasts. These cells can further differentiate by fusing together to form an outer, nonproliferating syncytiotrophoblast layer. The syncytiotrophoblast covers the villi (labyrinth in mice), providing a large surface for exchanging gas and nutrients. Additionally, this population of cells exhibits endocrine activity. The extravillous cytotrophoblasts (EVT, trophoblast giant cells in mice) arise from the villi and are responsible for actively invading the maternal decidua and rupturing the uterine capillaries to establish an interface between the maternal circulation and embryonic extracellular fluids (Fig. 7.8).[113-116]

Decidual cells, uterine natural killer cells,[117] and macrophages secrete a number of factors that regulate proliferation and invasion of the trophoblast at the fetal-maternal interface. The activity of the trophoblast is finely regulated by a balance of substances with distinct effects (Table 7.9). The factors that play a role in the regulation of the trophoblast activity can be divided in three main groups: (1) factors that promote proliferation of the EVT; (2) factors promoting the EVT migration/invasion; and (3) factors that inhibit proliferation, invasion, and migration of the trophoblast. The trophoblast is also an active source of factors; in particular, it secretes matrix metalloproteinases (MMPs), which destroy the extracellular matrix and facilitate the penetration into the endometrial environment. The invasion of the trophoblast relies on the precise control of the balance between MMPs and their inhibitors (TIMPs) on one hand and between urokinase-type plasminogen activator (uPA) and its inhibitors (PAI-I and PAI-II). uPA is a key protease for invasion, both independently and for its ability to activate other proteases. The interaction between uPA and its receptor (uPAR) at the cell surface has been shown to regulate local proteolysis and facilitate invasion and migration of the trophoblast. Moreover, human EVT shows a polarized

Table 7.9 Factors Produced at the Maternal-Fetal Interface; Their Effects on the Invasion, Proliferation, and Migration of the Trophoblast; and the Intracellular Molecular Pathways That They Regulate

Factor	Expression	Invasion	Proliferation	Migration	Downstream Pathway(s)
EGF	Placenta, deciduas	↑	↑	↑	MAPK, PI3K
CSF1	Trophoblast, placenta, deciduas	↔	↑	n.d.	MAPK, JAK/STAT
IGFII	Trophoblast	↑	↔	↑	$G_{i,}$ MAPK
IGFBPI	Decidua	↑	↔	↑	FAK, MAPK
HGF	Placenta	↑	n.d.	↑	PI3K, MAPK, iNOS
IL11	Decidua, placenta, trophoblast	↑	↔	↑	JAK/STAT
LIF	Decidua, placenta	↑	↑	n.d.	JAK/STAT
TGFβ	Decidua, trophoblast	↓	↓	↓	SMAD2/3
NODAL	Placenta, trophoblast	↓	↓	↓	SMAD2/3
DECORIN	Decidua	↓	↓	↓	-

n.d., not determined.
The full version of this table, including references, can be found on the companion Expert Consult Web site at www.expertconsultbook.com.

expression of uPAR at the invasion front. The uPA-uPAR system in rodents is different because uPA knockout mice do not show fertility or placentation defects.[118]

Immunohistochemical and *in situ* analyses have shown that several growth factors and cytokines are expressed at the fetal-maternal interface. EGF receptor *(Egfr)* is expressed by human trophoblast cells, and its ligands (EGF, AREG, and transforming growth factor α, *TGFA*) are broadly expressed in both placental and uterine cells. EGF promotes the proliferation, invasion, and migration of EVT. Confirming the role of the EGF pathway during placentation, *Egfr* KO mice are embryonic lethal due to impaired placental growth (although this phenotype is observed only in some strains of mice). Using an invasive extravillous trophoblast cell line (HTR8/Svneo), it has been shown that EGF promotes the transcription of *Mmp9* and *Timp1* through the activation of phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. Colony-stimulating factor 1 (CSF1) has an effect on trophoblast cell proliferation similar to EGF.

Trophoblast-derived insulin-like growth factor 2, IGF2, and decidua-derived insulin-like growth factor binding protein 1, IGFBP1, bind their distinct receptors on EVT and independently stimulate the migration and invasiveness of the trophoblast in an autocrine and paracrine way, respectively. The two factors have been shown to act through independent pathways: whereas IGF2 binds IGFR2 and causes the activation of MAPK and the inhibition of G proteins in EVT cells, IGFBP2 acts by binding the α5β1 integrin with its RGD domain and triggering the activation of FAK and stimulation of MAPK pathway. Another key signaling pathway regulating the invasion of the trophoblast is hepatocyte growth factor (HGF); HGF has been shown to promote the motility in tumors by activating signal transducer and activator of transcription 3 (STAT3). The effects of both HGF and STAT3 on trophoblast invasion have been described, although no evidence of a HGF-induced activation of STAT3 has been reported in trophoblast cells. HGF is secreted by the placenta and acts in a paracrine way by binding its receptor on trophoblast cells. HGF regulation of the invasion process has been shown both *in vitro*, where HGF stimulates the invasion of trophoblast cells in a dose-dependent way, and *in vivo*, since *Hgf* knockout mice have a lethal placental failure defect (reviewed in ref. 116 and 119). Interleukin 11 (IL11) is another decidual-expressed cytokine that activates STATs to mediate its signal. IL11 receptor α *(Il11ra1)* KO females are sterile due to defective decidualization, which eventually leads to uncontrollable trophoblast invasion. STAT3 is activated through its phosphorylation by LIF, causing an increased secretion of TIMP1 and TIMP2 and enhancing the invasiveness of human EVT cell culture.[120] STAT3 activity must be finely regulated to allow the trophoblast to establish a functional connection with the maternal blood vessels without proceeding too far and damaging the uterine environment. One mechanism that has been shown to be essential for controlling STAT3 activation is the negative regulation of LIF by suppression of cytokine signaling 3 (SOCS3). The importance of this signaling pathway in preventing the uncontrolled invasion of the trophoblast is demonstrated by the finding that *SOCS3* KO leads to embryonic lethality due to placental failure.

Several members of the TGFβ superfamily are produced by both the decidua and the trophoblast during pregnancy and have been shown to inhibit the invasion and the proliferation of EVT by acting in a paracrine and autocrine manner. TGFβ1, -2, and -3 cause a decrease in the secretion of MMP9 and uPA, and Nodal, acting through its type 1 receptor ALK7, has been shown to inhibit the activity of MMP2 and MMP9 and to promote the activity of TIMP1. Moreover, Nodal has been shown to inhibit proliferation and induce apoptosis in human trophoblast cells through a SMAD2/3-dependent mechanism.[121] Another factor that negatively regulates invasion, proliferation, and migration of EVT is the TGFβ-binding proteoglycan decorin (DCN). This factor colocalizes with TGFβ in decidual extracellular matrix, although its inhibitory action has been shown to be independent from TGFβ when the combined effects of the two factors were tested on propagated EVT cell lines.[122] DCN appears to act as a reservoir for inactive TGFβ, and the proteolytic action of EVT causes the release and activation of TGFβ to prevent the overinvasion of the decidua. The effects of TGFβ are also modulated by SMAD ubiquitination regulatory factor 2 (SMURF2), a homologous to the E6-AP carboxyl terminus (HECT) type E3 ubiquitin ligase that targets TGFβ receptors and SMADs for proteasome-mediated degradation; SMURF2 is highly expressed in human placental villi during the first trimester of pregnancy, and, when overexpressed in EVT cells in culture, it

enhances their migration and invasion by reducing the protein levels of TGFβ type 1 receptor.[123]

Growth Factor Pathways in Male Reproduction

In addition to the roles of growth factors in PGC formation and migration, development of the male reproductive tracts, and sex determination, growth factor pathways are also involved in the testis and epididymis. In particular, BMP7, BMP8a, and BMP8b are expressed in testicular germ cells, regulate Sertoli cell activity, function during spermatogenesis, and are important for male fertility. These BMPs, along with BMP4, also function in the epididymis and regulate sperm motility. Mouse mutations in the *Bmp7*, *Bmp8a*, and *Bmp8b* genes lead to defects in the head and tail of the epididymis, while heterozygous mutation in *Bmp4* leads specifically to epithelial degeneration in the body of the epididymis.

Activin and inhibin are not only involved in the regulation of ovarian granulosa cell tumor formation but also play key roles in testicular Sertoli cell tumor development. Absence of *Inha* leads to Sertoli cell tumor formation, while DKO of SMAD1 and SMAD5 or DKO of INHA and AMH (or AMHR2) result in Sertoli cell and Leydig cell tumor development. A key role of activins signaling through SMAD3 to regulate development of these testicular tumors is clear since *Inha Smad3* DKO males fail to develop these tumors (unlike in female granulosa cells where SMAD2 and SMAD3 play redundant functions).

Growth Factors in the Clinic

Whereas proteins (e.g., recombinant and purified FSH, LH, and hCG and GnRH analogs) and steroid hormones (e.g., estrogens, progestins, and androgens) have a major place in the clinical setting, there are fewer examples where growth factors have been used clinically. However, based on our discussions above, it is obvious that growth factors must play a key role in the assisted reproductive technology (ART) clinic. For example, when recombinant human colon stimulating factor 2 (GM-CSF; 2 ng/mL) was added to different culture media, it statistically increased from 30% to 76% the proportion of 2-4 cell human embryos that could develop to the blastocyst stage.[124] As ART clinics optimize conditions for *in vitro* mutation (IVM) of human oocytes, strategies that utilize recombinant growth factors that are normally synthesized in mural granulosa cells (e.g., EGF-like peptides) or in the oocyte (e.g., GDF9 and BMP15) will likely develop for these cultures.[125] Small molecules that regulate these processes may also come into play.

Since growth factors are also important for interaction of the hatched blastocyst and the uterine endometrium and establishment of pregnancy, growth factors may also play a role *in vivo* in this setting. In the mouse, recombinant human BMP2 could partially rescue the uterine decidual response in mice with a knockout of *Bmp2*, indicating that similar strategies could be developed for women who do not have appropriate uterine responses to the implanting embryo.[111] Immunotolerance of the maternal endometrium to the implanting conceptions is also necessary for a successful pregnancy and factors such as interleukin 1b and other pro-inflammatory cytokines could be used for modulating this process.[126] Diagnostically, measurement of growth factors in the follicular fluid (e.g., colony stimulating factor 3 [G-CSF]) may help to define oocyte competence before fertilization is attempted while endometrial mRNA analysis of IL15, IL18, and tumor necrosis factor superfamily member 12 (TWEAK) in the mid-luteal phase may help to define uterine receptivity for women with repeated ART failures.[127] These latter genes appear to correlate with the appropriate numbers of CD56-positive uterine natural killer (uNK) cells, which are necessary for proper spiral artery development post-implantation.

In a rare controlled multicenter study to test the efficacy of recombinant human leukemia inhibitory factor (LIF), women with two unexplained implantation failures in an ART setting were given subcutaneous LIF or placebo over 7 days.[128] However, pregnancy rates with LIF (17.6%) were statistically suppressed compared to placebo (34.0%), indicating that LIF in this setting is contraindicated.

Conclusions

In this chapter, we discuss how various growth factors act and interact to regulate multiple aspects of reproduction in mammals. The use of mouse models combined with high throughput methods of data production and analysis has led to the accumulation of a notable amount of useful information on how fertility is regulated; several growth factor signaling pathways, like TGFβ, WNTs, Hedgehog, EGF, IGF and Notch, have been shown to be of central importance in reproductive biology and their reciprocal regulation and cooperation is a complex problem under intense investigation. Considering the role that a number of these factors have been suggested to play in several reproductive diseases (i.e., polycystic ovarian syndrome [PCOS], premature ovarian failure [POF], ovarian cancer, preeclampsia, and endometriosis), the goal for the future is that the knowledge resulting from these efforts will provide the tools necessary to improve the diagnosis of infertility and to design new approaches for its treatment.

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The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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Table S7.1 Phenotypes for Mice with Mutations in PGC Pathway Components (Ordered Based on Temporal Function During Embryogenesis)

Gene Symbol	Phenotype; Findings	References
Bmp2	Embryonic lethal; reduced PGC	1,2
Bmp4	Embryonic lethal; no PGC	3,4
Bmp8b	Viable; male infertility; reduced PGC	5,6
Smad1	Embryonic lethal; reduced PGC	7-9
Smad5	Embryonic lethal; reduced PGC	10-12
Smad4	Embryonic lethal; absent PGC	13-15
Prdm1 (Blimp1)	Embryonic lethal; PGC specification defect	16
Prdm14	Infertility; PGC specification defect	17
Pou5f1 (Oct4)	Pluripotency marker	18,19
Nanog	Pluripotency marker	19-21
Sox2	Pluripotency marker	19,22
Kitl	Variable phenotypes depending on mutation; PGC migration defect	23
Kit	Variable phenotypes depending on mutation; PGC migration defect	23
Tgfbr1 (ALK5)	Embryonic lethal; enhanced PGC migration	24,25

Table S7.2 Mouse Models for Ligands in the TGFB Family

Mutant Gene	Reproductive Phenotype	Fertility Status	References
Amh	Increased primordial follicle activation	Normal	26
Bmp6	Reduced ovulation	Subfertile	27
Bmp7	Reduced primordial germ cells	N.A.	28
Bmp15	Decreased ovulation and fertilization rates	Normal to subfertile	29
Gdf9	Follicular arrest at the primary follicle stage	Infertile	30
Fst	Loss of oocytes by birth	N.A.	31
Fst (Conditional)	Reduced growing follicles, ovulation and fertilization defects	Subfertile to infertile	32
Inha (Conditional)	Increased polyovular follicles	Subfertile	33
Inhbb	Lactation defects	Infertile	34
Inhba Inhbb (Conditional)	Increased numbers of antral follicles and accumulation of corpora lutea	Infertile	33
Tgfb2	Increased oocytes at birth	N.A.*	35

*N.A., Not available due to embryonic or perinatal lethality.

Table S7.3 Phenotypes of Mice with Ovarian Deletion of the SMAD Transcription Factors

Gene	Cre	Cell-Specific Deletion	Reproductive Phenotype	Fertility Status	References
Smad1	Amhr2cre	Granulosa cell	None	Normal	36
Smad2	Amhr2cre	Granulosa cell	None	Normal	37
Smad3	Amhr2cre	Granulosa cell	None	Normal	37
Smad4	Amhr2cre	Granulosa cell	Reduced antral follicles and ovulation rates, cumulus cell defects, increased luteinization, increased progesterone	Subfertile, then 50% infertile by 6 months	38
Smad4	Gdf9-icre	Oocyte	Reduced litter sizes	Subfertile	39
Smad4	Zp3-cre	Oocyte		Normal	
Smad5	Amhr2cre	Granulosa cell	None	Normal	36
Smad8	Knockout	Global	None	Normal	36
Smad2 Smad3	Amhr2cre	Granulosa cell	Reduced antral follicles and ovulation rates, cumulus cell defects	Subfertile, then infertile at 5 months	37
Smad1 Smad8	Amhr2cre	Granulosa cell	None	Normal	Unpublished data, S. Pangas
Smad5 Smad	Amhr2cre	Granulosa cell	None	Normal	36
Smad1 Smad5	Amhr2cre	Granulosa cell	Granulosa cell tumors	Subfertile, then infertile at 4-6 months	36
Smad1 Smad5 Smad8	Amhr2cre	Granulosa cell	Granulosa cell tumors	Subfertile, then infertile at 4-6 months	36

Table S7.4 Mutations Affecting Ovulation Rate and Prolificacy Sheep BMP15 and GDF9

Gene	Mutation	Breeds	Protein Domain	Mutation Type	References
GDF9	$FecG^{H}$	Belclare, Cambridge	Mature	Substitution	40
	$FecG^{E}$	Santa Inês*	Mature	Substitution	41
BMP15	$FecX^{B}$	Belclare	Mature	Substitution	40
	$FecX^{I}$	Inverdale	Mature	Substitution	42
	$FecX^{H}$	Hanna	Mature	Premature stop	42
	$FecX^{G}$	Galway	Prepro	Substitution; premature stop	40
	$FecX^{L}$	Lacaune	Mature	Substitution	43
	$FecX^{R}$	Rasa Aragonesa		Premature stop	44
BMPR1B	FecB	Booroola†, Merlino, Hu, Han, Garole, Javenese Thin-tail	Kinase domain	Substitution	45

*Heterozygotes have normal ovulation rates, but rates increase in homozygous sheep.
†Heterozygotes and homozygotes have increased ovulation rates with the heterozygotes midway between homozygotes and non-carriers.

Table S7.5 Mouse Models with Defects in the Periovulatory Period

Mutant Gene	Reproductive Phenotype	Fertility Status	References
Luteinizing hormone b *(Lhb)*	Block in folliculogenesis; early antral stage	Infertile	46
Luteinizing hormone/choriogonadotropin receptor *(Lhcgr)*	Block in folliculogenesis; early antral stage	Infertile	47,48
Prostaglandin-endoperoxide synthase 2 *(Ptgs2)*	Defects in ovulation, cumulus expansion, and implantation	Mostly infertile	49,50
Prostaglandin E receptor 2, subtype EP2 *(Ptger2)*	Defects in cumulus expansion; decreased fertilization	Subfertile	51-53
Pentraxin 3 (Ptx3)	Defects in cumulus-oocyte complex integrity and ovulation	Subfertile	54,55
Tumor necrosis factor alpha induced protein 6 *(Tnfaip6)*	Impaired cumulus matrix formation resulting in failed cumulus-oocyte complex expansion	Infertile	56
CCAAT/enhancer-binding protein b *(Cebpb)*	Reduced ovulation; fail to form corpus luteum	Infertile	57
Nuclear receptor interacting protein (*Nrip1*; RIP40)	Ovulation defect; ovaries accumulate luteinized and unruptured follicles	Infertile	58
Progesterone receptor (*Pgr*; PR)	Anovulation; corpora lutea absent	Infertile	59
Alpha 1 microglobulin/bikunin *(Ambp)*	Defects in ovulation and cumulus-oocyte complex integrity	Subfertile	60,61
Amphiregulin *(Areg)*	Suppressed cumulus expansion, delayed meiotic reentry	Subfertile	62
Bone morphogenetic protein 15 *(Bmp15)*	Defects in cumulus expansion and ovulation	Subfertile	29
Bone morphogenetic protein receptor, type IB *(Bmpr1b)*	Defects in estrous cyclicity, cumulus expansion, and endometrial gland development	Subfertile	63
Epiregulin (*$Ereg^{wa2/wa2}$*; hypomorph)	Suppressed cumulus expansion, defective meiotic resumption, ovulation defect	Subfertile	62
MAD homolog 4 *(Smad4)* (cKO)	Premature luteinization; decreased ovulation with trapped oocytes	Subfertile	38

Table S7.6 Main Genes Involved in the Development of the Reproductive Tracts

Gene	Phenotype	References
Pax2	Null mice show neonatal lethality; males and females lack kidneys, ureters, and genital tracts	64
Emx2	Null mice show neonatal lethality; females and males lack kidneys, ureters, gonads, and genital tracts	65
Lim1	Null mice show embryonic lethality; females lack müllerian duct	66

Table S7.7 Genes Involved in Müllerian Duct Regression and Development

Gene	Phenotype	References
Amh	Null males have a complete male reproductive tract and functional sperm, but also uterus and oviducts; females are fertile with enlarged ovaries. Female mice overexpressing Amh show blind vagina and no uterus or oviducts	67,68
Amhr2	Null males have a normal male reproductive tract, but also uterus and oviducts	69,70
Alk2, Alk3	Alk2 and Alk3 null mice show embryonic lethality; conditional ablation of Alk2 and Alk3 in the müllerian duct mesenchyme (Alk2/Alk3 double conditional knockout mice) causes duct retention in males	71,72
Smad1, Smad5, Smad8	Smad1 and Smad5 null mice show embryonic lethality; Smad8 null mice are viable and fertile. Deletion of Smad5, Smad1/Smad5 or Smad5/Smad8 in the müllerian duct mesenchyme causes partial müllerian duct retention. Smad1/Smad5/Smad8 triple conditional knockout mice show complete müllerian duct retention	71
Wnt7a	Null mice show partial perinatal lethality; null males fail to undergo Mullerian duct regression. Females are infertile due to abnormal development of uterus and oviduct	73,74
Wnt4	Null mice show neonatal lethality; null females are masculinized: they have Wolffian duct and lack müllerian duct	75,76
Wnt5a	Null mice show perinatal lethality; females show caudal truncation of the reproductive tract and defective uterine glandular development	77,78
Ctnnb1	Null mice show embryonic lethality; male mice with a conditional deletion of Ctnnb1 in the müllerian duct mesenchyme show müllerian duct retention	79

Table S7.8 Genes Involved in the Regulation of Embryo Implantation

Gene	Phenotype	References
Ihh	Null mice die before or immediately after birth. Females with a conditional deletion of Ihh in the uterus show failure of blastocyst attachment. Decidualization is defective	80,81
NR2F2	Null mice show embryonic lethality. Females with a conditional deletion of COUP-TFII in the uterus are sterile; both embryo attachment and uterine decidualization are impaired	82
Bmp2	Null mice show embryonic lethality; females with a conditional deletion of Bmp2 in the uterus are sterile due to failure of uterine stromal cell decidualization	83
Wnt4	Null mice show neonatal lethality; female mice with a conditional deletion of Wnt4 in the uterus are severely subfertile due to impaired implantation	84

Table S7.9 Factors Produced at the Maternal-Fetal Interface; Their Effects on the Invasion, Proliferation, and Migration of the Trophoblast; and the Intracellular Molecular Pathways That They Regulate

Factor	Expression	Invasion	Proliferation	Migration	Downstream Pathway(S)	References
EGF	Placenta, decidua	↑	↑	↑	MAPK, PI3K	85-87
CSF1	Trophoblast, placenta, decidua	↔	↑	n.d.	MAPK, JAK/STAT	88-90
IGFII	Trophoblast	↑	↔	↑	G_i, MAPK	91-93
IGFBPI	Decidua	↑	↔	↑	FAK, MAPK	92-94
HGF	Placenta	↑	n.d.	↑	PI3K, MAPK, iNOS	95-97
IL11	Decidua, placenta, trophoblast	↑	↔	↑	JAK/STAT	98-100
LIF	Decidua, placenta	↑	↑	n.d.	JAK/STAT	101-103
TGFβ	Decidua, trophoblast	↓	↓	↓	SMAD2/3	93,104,105
NODAL	Placenta, trophoblast	↓	↓	↓	SMAD2/3	106,107
DECORIN	Decidua	↓	↓	↓	-	108

n.d., not determined.

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CHAPTER 8

Neuroendocrine Control of the Menstrual Cycle

Janet E. Hall

The Reproductive Axis

Normal reproductive function in women involves repetitive cycles of follicle development, ovulation and preparation of the endometrium for implantation should conception occur in that cycle. This pattern of regular ovulatory cycles is achieved through precise functional and temporal integration of stimulatory and inhibitory signals from the hypothalamus, the pituitary and the ovary (Fig. 8.1). The reproductive system functions in a classic endocrine mode initiated by pulsatile secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus into the pituitary portal venous system. GnRH regulates the synthesis and subsequent release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary into the circulation. FSH and LH stimulate ovarian follicular development, ovulation, and corpus luteum formation and the coordinated secretion of estradiol, progesterone, inhibin A, and inhibin B. A key component of this system is the modulatory effect of ovarian steroids and inhibins on gonadotropin secretion, acting either directly at the pituitary level or through alterations in the amplitude or frequency of GnRH secretion. Negative feedback restraint of FSH secretion is critical to the development of the single mature oocyte that characterizes human reproductive cycles. In addition to negative feedback controls, the menstrual cycle is unique among endocrine systems in its dependence on estrogen positive feedback to produce the preovulatory LH surge that is essential for ovulation.

Neuroendocrine Components of the Reproductive Axis

Gonadotropin Releasing Hormones

Luteinizing-releasing hormone (LHRH) was isolated, characterized, and synthesized in 1971.[1] The central role of this decapeptide in propagation of the species makes it fitting that Drs. Schally and Guillemin received the Nobel Prize in Physiology and Medicine in 1977 for its isolation.[1] It was expected that separate releasing hormones for LH and FSH would be discovered. However, subsequent studies provided evidence that both LH and FSH are secreted in response to LHRH, resulting in the common use of the term gonadotropin-releasing hormone (GnRH) for the decapeptide originally referred to as LHRH. It is now known that GnRH is located on chromosome 8p and works through its own receptor, which is encoded on chromosome 4.

GnRH neurons differentiate in the olfactory placode, cross the cribiform plate into the forebrain, and migrate to the medial basal hypothalamus where they establish connections with the pituitary portal system in the median eminence as part of the hypothalamic tuberinfundibular system.[2] The initial leg of this migratory journey occurs along the scaffold of olfactory, vomeronasal, and terminal nerves. Genetic studies in patients with abnormalities in GnRH secretion, with concomitant disruption of the olfactory system (Kallmann Syndrome; KS) or without anosmia (normosmic idiopathic hypogonadotropic hypogonadism; nIHH), in combination with functional studies in animal and cell systems have identified key components of the developmental regulation of GnRH neurons and provided critical insights into the growing complexity of this process.[2] Among the genes associated with KS or nIHH, *KAL1*, fibroblast growth factor 8 *(FGF8)* and its receptor fibroblast growth factor 1 *(FGFR1)*, prokineticin 2 *(PROK2)* and its receptor *(PROKR2)*, heparin sulfate 6-O-sulfotranferase 1 *(HS6ST1)*,[3] chromodomain helicase DNA binding protein 7 *(CHG7)*,[4] nasal embryonic LH-releasing hormone factor *(NELF)*, semaphorin-3A *(SEMA3A)*,[5] and *(WDR11)*[4] are all involved in different aspects of early migration and axonal guidance of GnRH neurons on the path to their eventual home in the hypothalamus (Table 8.1). In the human there are approximately 7000 GnRH expressing neurons in areas of the brain linked to gonadotropin regulation.[6] Unlike neurons secreting other hypothalamic releasing factors, GnRH neurons do not exist in a defined nucleus, but are scattered throughout the medial basal hypothalamus, with an additional population in the preoptic area.[7]

Mammals, including humans, simultaneously express more that one molecular form of GnRH (Table 8.2).[8] GnRH II is located on chromosome 20p13 and works through its own receptor which is encoded on chromosome 1. GnRH II is widely expressed both within and outside the brain. It likely plays a role in reproductive behavior in lower animal species. It is a potent stimulator of LH and FSH *in vitro* and *in vivo* in animal models, but its role in the human is unknown. GnRH III has been identified in human brain by immunohistochemistry and HPLC, has a similar hypothalamic distribution to that of GnRH, and may act through

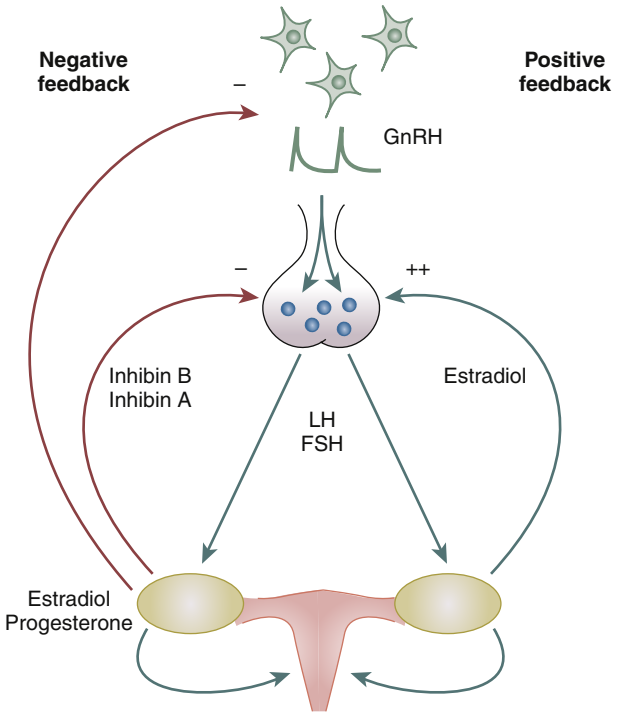


FIGURE 8.1 Neuroendocrine control of reproduction requires the pulsatile secretion of gonadotropin-releasing hormone (GnRH) released into the pituitary portal system to stimulate the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the gonadotropes. The gonadotropins, in turn, stimulate follicular development and secretion of gonadal steroids and peptides whose negative feedback effects on the hypothalamus and pituitary restrain gonadotropin secretion. At the midcycle, rising levels of estradiol are responsible for the positive feedback, which generates the preovulatory gonadotropin surge.

Table 8.1 KS and nIHH Patients with Gene Mutations*

	GnRH		
	Neuronal Migration	Secretion	Action
KAL1	X		
FGF8/FGFR1	X		
PROK2/PROKR2	X		
CHG7	X		
HS6ST1	X		
NELF	X		
SEMA3A	X		
WDR11	X		
KISS/KISSR1		X	
TAC3/TACR3		X	
GnRH1		X	
GnRHR			X

*Categorized in relation to their roles in gonadotropin-releasing hormone (GnRH) neuronal migration, secretion, and action.

Table 8.2 Amino Acid Sequences of Mammalian GnRH, GnRH II, and GnRH III

GnRH I (Mammal)	pGLu- His- Trp- Ser- **Tyr- Gly- Leu- Arg**- Pro- Gly- NH2
GnRH II (Chicken I)	pGLu- His- Trp- Ser- **His- Gly- Trp- Tyr**- Pro- Gly- NH2
GnRH III (Lamprey III)	pGLu- His- Trp- Ser- **His- Asp- Trp- Lys**- Pro- Gly- NH2

GnRH, gonadotropin-releasing hormone.

the GnRH receptor. Although there is some evidence in lower animal species that GnRH III may have preferential FSH releasing properties,[9] a GnRH III consensus sequence has not been found in the human genome[8] and thus, it is unlikely that GnRH III plays a role in human reproduction.

Pulsatile Secretion of GnRH

A prominent feature of the reproductive system is the absolute requirement for pulsatile secretion of GnRH into the pituitary portal system for normal gonadotropin secretion. The now classic studies of Knobil and colleagues in hypothalamic-lesioned monkeys receiving GnRH first showed that intermittent stimulation of the pituitary results in secretion of LH and FSH while constant GnRH stimulation is associated with suppression of gonadotropin levels.[10] Isolated GnRH neurons exhibit an intrinsic pulsatility,[11] but there is also a significant body of research indicating that external influences modify and coordinate the secretion of GnRH, influencing both the amplitude and frequency of pulsatile GnRH secretion.

Neuromodulators of GnRH Secretion

While a number of neurotransmitters are involved in the control of GnRH secretion in animal species, only a few have been shown to have an effect in the human.[12] Although there is evidence for a stimulatory role of the α-adrenergic system in a number of animal models, it is much less likely that it plays a role in control of the human menstrual cycle. The role of the dopaminergic system remains controversial, but studies that have documented an increase in LH pulse frequency in response to a dopamine antagonist in women with hypothalamic amenorrhea suggest dopamine may inhibit GnRH secretion in women.[13,14]

Kisspeptin

Knock out models suggest that there is considerable redundancy in the systems that ultimately control GnRH secretion; however, an emerging body of evidence indicates that the kisspeptin pathway is a key upstream modifier of GnRH secretion. As with the genes that are now known to control the developmental migration of GnRH neurons, a role for kisspeptin in reproduction was initially discovered by the combination of genetic studies in patients with IHH which identified mutations in the gene encoding the kisspeptin receptor (*KISS1R*, formerly known as G-protein coupled receptor 54 [*GPR54*]) and knock-out mouse models.[15] Kisspeptin is an extremely powerful stimulator of LH, an action that is blocked by a GnRH antagonist indicating that the effect on LH is through GnRH. The kisspeptin system is thought to play a dominant role in the onset of puberty and has also been implicated in estrogen negative feedback in the arcuate. This system has also been implicated in estrogen positive feedback in the anteroventral periventricular nucleus (AVPV) in rodents.[16,17] Its role in the midcycle surge in women is less clear as studies in women demonstrate a paucity of kisspeptin neurons in an analogous hypothalamic region[18] and evidence discussed below questions

the existence of a GnRH surge associated with the midcycle LH surge in women.

Neurokinin B

Neurokinin B (NKB), which is encoded by the tachychinin 3 gene *(TAC3)* and its receptor encoded by *TACR3* have also been implicated in the normal control of GnRH secretion through genetic studies in patients with KS or IHH. NKB stimulates LH secretion, acting upstream of the GnRH neuron.[19] *TAC3* and *KISS* are colocalized and there is mounting evidence that the effect of NKB on GnRH is exerted through kisspeptin.[20,21]

Endogenous Opioids/Dynorphin

There is substantial evidence for the involvement of endorphins in transducing the negative feedback effects of progesterone on pulsatile GnRH secretion.[22,23] Recent studies have identified dynorphin which binds to the kappa-opioid receptor as the key mediator of progesterone negative feedback.[24] In a variety of animal species and the human it has now been shown that kisspeptin, NKB, and dynorphin are coexpressed in cells in the median eminence that are now referred to as KNDy neurons. These neurons express estrogen, progesterone, and androgen receptors and are thought to mediate gonadal steroid feedback on GnRH secretion with increasing evidence that they are also involved in the specific generation of pulses of GnRH.[24] There is also evidence that gamma-amino butyric acid (GABA) may well be involved in mediating estrogen negative feedback on GnRH secretion.[12]

Gonadotropin Inhibitory Hormone

Gonadotropin inhibitory hormone (GnIH) is a hypothalamic neuropeptide that inhibits GnRH secretion. First discovered in the quail, there is now evidence that it is present in other avian species as well as in mammals, including the human.[25,26] GnIH is secreted into the pituitary portal system[27] and its receptor, G-protein coupled receptor 147 (GPR147) is present on both GnRH and gonadotrope cells, raising the distinct possibility that it functions at both the hypothalamic and pituitary level to regulate secretion of LH and FSH. Importantly, there is also evidence that GnIH increases food intake in sheep without reducing energy expenditure,[28] raising the possibility that GnIH may be a factor that mediates the inverse relationship between LH and body mass index in women.

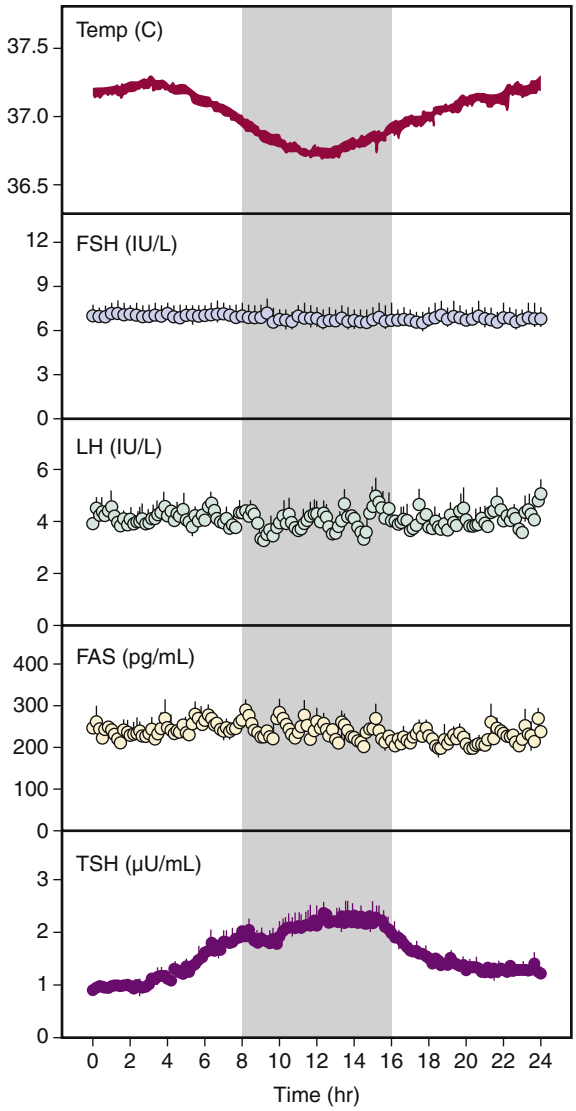


FIGURE 8.2 Mean + sem of temperature, FSH, LH, FAS, and TSH levels in early follicular phase women (n = 11) during a constant routine of light, position, wake, and nutritional intake over a 24 hour period indicate the constancy of gonadotropin levels across the day and night in the absence of sleep with maintenance of endogenous circadian rhythms of temperature and TSH. The shaded area represents the subjects' traditional sleep time during which they were kept awake. (*Adapted from Klingman KM, Marsh EE, Klerman EB, et al. Absence of circadian rhythms of gonadotropin secretion in women.* J Clin Endocrinol Metab *96:1456–1461, 2011.*)

Sleep and Circadian Effects on GnRH Secretion in Women

Endocrine systems are profoundly influenced by both sleep and endogenous circadian rhythms, which persist in the absence of sleep or other environmental cues. Diurnal (day and night) rhythms of LH and gonadal steroids have been well described in men and women. Studies in which sleep and other environmental cues were controlled have failed to demonstrate an endogenous circadian rhythm of LH or FSH in early follicular phase and postmenopausal women (Fig. 8.2), despite the presence of robust circadian rhythms of temperature, cortisol, and TSH.[29,30] In contrast, there is compelling evidence that sleep directly affects the pulsatile secretion of LH and presumably that of GnRH. Studies that have separated the effects of sleep from time of day demonstrate that during puberty in boys and girls, pulsatile LH secretion is increased during sleep.[31] Recent studies have further shown that in pubertal children, LH pulses are most commonly preceded by slow wave sleep (SWS).[32] These studies suggest that factors associated with SWS stimulate GnRH secretion or that there is an upstream regulator of both GnRH secretion and deep sleep in puberty. Paradoxically, with maturation of the reproductive system and the onset of ovulatory menstrual cycles, there is a notable slowing of pulsatile LH secretion at night in the early follicular phase of the cycle.[33,34] Sleep reversal studies in women[35] have demonstrated not only that the early follicular phase

night time slowing of LH pulses is due to sleep rather than time of day but also that, within sleep, brief periods of wakefulness are associated with the onset of LH pulses while SWS appears to be inhibitory to LH pulses (Fig. 8.3). It is tempting to speculate that prior progesterone exposure sensitizes the GnRH pulse generator to the inhibitory effects of sleep in the early follicular phase, possibly explaining the paradoxical effect of sleep on GnRH secretion in the early follicular phase compared with puberty.

Gonadotropin-Producing Cells of the Pituitary

LH and FSH are synthesized in gonadotropes, which comprise between 7% and 15% of the cells in the pituitary. Immunohistochemical studies in the rat indicate that approximately 70% of gonadotropes stain for both LH and FSH while the remainder stains for a LH or FSH with approximately equal numbers of cells staining for each. As the animals approach the day of the gonadotropin surge in proestrus, monohormonal cells begin to express both βLH and βFSH, while a population of cells that express growth hormone (GH) also express the gonaodtropin subunits.[36]

The process of biosynthesis and secretion of intact gonadotropins involves: (1) translation of βLH, βFSH, and the common gonadotropin α-subunit; (2) posttranslational modification and folding; (3) combination of the β− and α−subunits; and (4) hormone packaging and secretion. LH, FSH, and uncombined or free α-subunit (FAS) are all secreted in response to GnRH stimulation of the gonadotrope.

Multiple isoforms of LH and FSH, differing in their carbohydrate structure and charge, coexist in both pituitary and serum. More basic forms of both LH and FSH yield a greater *in vitro* potency, but a shorter half-life in the circulation while the opposite is true for less basic forms.[37] The greater number of sialic acid residues on FSH prolongs its half-life,[38] whereas the greater number of sulfonated N-acetyl-galactosamine (GalNAc) asparagine-linked oligosaccharides on LH is associated with more rapid clearance due to binding to a specific hepatic receptor.[39] Sulfonation and sialylation of LH and FSH vary across the menstrual cycle and in the absence of gonadal steroids; postmenopausal women have a greater preponderance of sialylated forms of both LH and FSH.[40] Consistent with these changes, the disappearance of LH following GnRH receptor blockade with a potent GnRH antagonist is significantly prolonged in postmenopausal women compared to women in the follicular phase and at the midcycle surge (MCS), while the disappearance of FAS is unaffected by the absence of gonadal function (Table 8.3).[41] The number of sulfonated and sialylated residues on LH and FSH is tightly linked to hormone clearance in women.[42]

There is also evidence that gonadotropin secretion is modulated by a factor or factors related to obesity. Serum levels of LH are inversely related to body mass index (BMI)

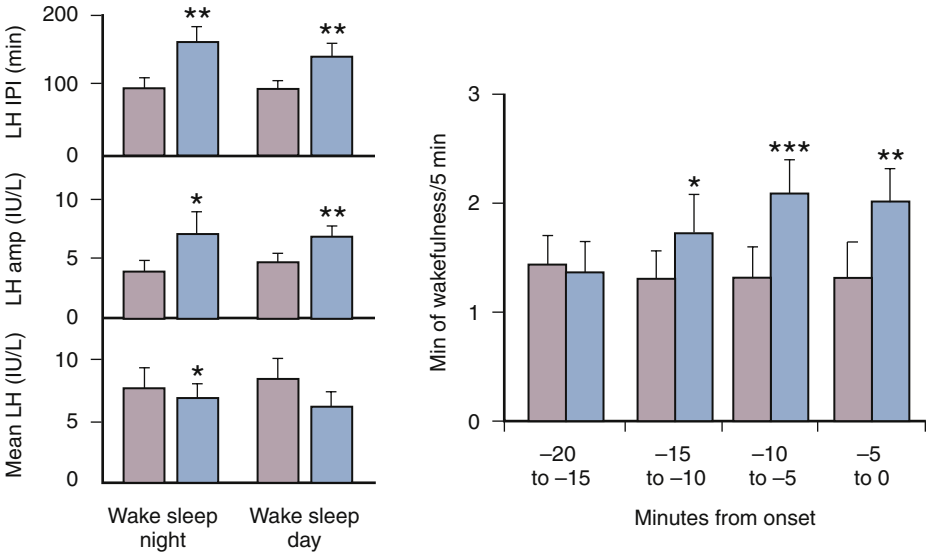


FIGURE 8.3 As indicated in the *left panel*, sleep is specifically associated with slowing of luteinizing hormone (LH) pulses. The LH interpulse interval (IPI) is longer and amplitude is higher in normal women during sleep *(blue bars)* compared with wake *(purple bars)*, whether sleep occurs at night or during the day. As indicated in the *right panel*, wakefulness is more likely in the 5 to 10 minutes before the onset of an LH pulse *(blue bars)* than in a similar time frame indexed to a random LH point that is not associated with an LH pulse *(purple bars)*. (*Adapted from Hall JE, Sullivan JP and Richardson GS. Brief wake episodes modulate sleep-inhibited luteinizing hormone secretion in the early follicular phase.* J Clin Endocrinol Metab *90:2050–2055, 2005.*)

Table 8.3 The Half-Life of LH, but not FAS, Is Influenced by the Gonadal Steroid Milieu

	LH		FSH	
	Baseline (IU/L) Mean ± SEM	T1/2 (min) Mean ± SEM	Baseline (pg/mL) Mean ± SEM	T1/2 (min) Mean ± SEM
Postmenopausal	62 ± 3	139 ± 35	774 ± 45	51 ± 26
EFP, LFP, ELP	10 ± 1	57 ± 28	266 ± 44	41 ± 12
MCS	56 ± 11	78 ± 20	627 ± 122	41 ± 19

LH, luteinizing hormone; FSH, follicle-stimulating hormone.
(From Sharpless JL, Supko JG, Martin KA, et al. Disappearance of endogenous luteinizing hormone is prolonged in postmenopausal women. *J Clin Endocrinol Metab* 84:688–694, 1999, with permission.)

in normal women and in women with polycystic ovary syndrome (PCOS).[43] Further studies have indicated that the inhibitory effect of obesity on LH secretion in PCOS is not mediated at the hypothalamus, but is associated with a decrease in both the pituitary response to GnRH and the half-life of endogenous, but not exogenous, LH.[44,45] The latter finding is consistent with the increase in sulfonated isoforms of LH and FSH as a function of increasing BMI in women with PCOS.[40]

Differential Control of LH and FSH Secretion

Although secreted from a common cell type within the gonadotrope, LH and FSH have markedly different functions in the control of ovarian physiology. These differences in function are reflected in their different patterns of secretion during normal reproductive cycles. The divergent control of LH and FSH is achieved through a combination of differential control of the synthesis and secretion of LH and FSH by the pattern of GnRH stimulation, the preferential control of FSH synthesis by the activin/follistatin system, and differential feedback by ovarian steroids and the inhibins on the two gonadotropins. Understanding the control of LH and FSH secretion is critical to our understanding of the dynamics of the menstrual cycle.

Table 8.4 Differential Effects of GnRH Pulse Frequency on LH and FSH

	LH	FSH
Increased Frequency		
'In Vitro'		
mRNA	↑	→
GnRH-deficient men/women		
Mean	↑	→↓
Amplitude	↓	
Decreased Frequency		
'In Vitro'		
mRNA	↓	↑
GnRH-deficient men/women		
Mean	↓	→↑
Amplitude	↑	

Gonadotropin Releasing Hormone

FSH is secreted along with LH in response to acute stimulation by GnRH, but the relative role of GnRH in the overall control of FSH synthesis is much less than for LH. Blockade of the GnRH receptor using a specific GnRH receptor antagonist results in 90% inhibition of LH secretion, but only 40% to 60% inhibition of FSH.[46]

Synthesis and secretion of LH and FSH are differentially controlled by the amplitude and frequency of GnRH stimulation.[47,48] LH secretion is highly responsive to increases in the dose of GnRH, while FSH is relatively insensitive to GnRH dose. A physiological frequency of GnRH results in synthesis and secretion of all three gonadotropin subunits. However, increases or decreases from the physiological frequency have differential effects on LH and FSH (Table 8.4). Slow frequencies of GnRH stimulation favor the synthesis and secretion of FSH *in vitro* and are associated with an increase in FSH *in vivo* in settings in which gonadal feedback is low.[49] In GnRH-deficient men and women, an increase in the frequency of GnRH stimulation results in an increase in mean levels of LH with no appreciable change in FSH (Fig. 8.4),[50,51] effects that have implications for the pathophysiology of PCOS in which the frequency of GnRH is consistently increased.[44] The direct effect of GnRH pulse frequency on GnRH receptor number[52] and the downstream effects of modulation of receptor number underlie the frequency modulation of LH and FSH secretion, at least in part.

While an increase in GnRH pulse frequency increases the synthesis and mean levels of LH, the LH pulse amplitude is inversely related to GnRH pulse frequency.[53,54] Thus, studies in GnRH-deficient men and women have shown that slower GnRH pulse frequencies are associated with higher LH amplitude in response to GnRH while faster frequencies are associated with a decrease in the amplitude

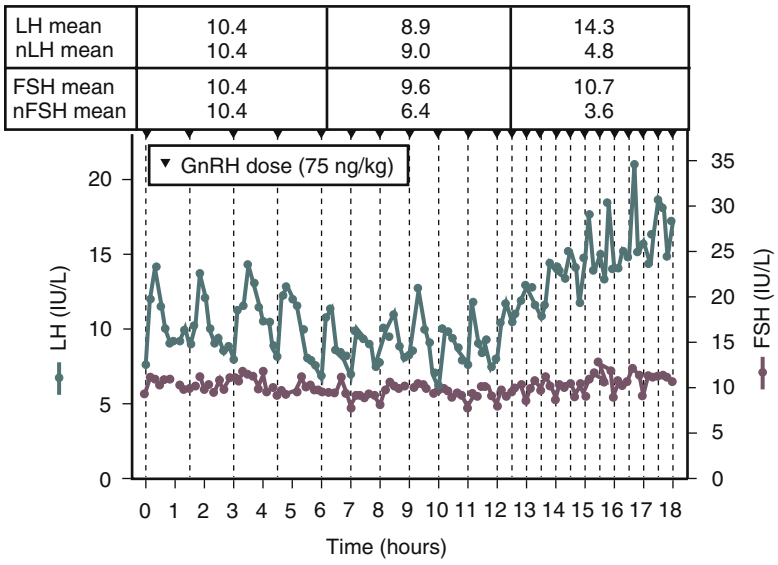


FIGURE 8.4 The differential effect of increasing gonadotropin-releasing hormone (GnRH) pulse frequency on luteinizing hormone (LH) and follicle-stimulating hormone (FSH) as demonstrated in a GnRH-deficient woman receiving IV pulsatile GnRH. After 7 days of administration of pulsatile GnRH at a dose of 75 ng/kg and a frequency of every 90 minutes, blood was sampled for 6 hours and the frequency was then increased to every 60 minutes for 6 hours and every 30 minutes for 6 hours with evidence of an increase in LH but not FSH. The mean LH and FSH are given for each frequency as indicated. The means of LH and FSH normalized for the overall amount of GnRH administered (nLH and nFSH), suggest that faster GnRH pulse frequencies are associated with early stages of pituitary desensitization. (*From Hall JE, Taylor AE, Hayes FJ, et al. Insights into hypothalamic-pituitary dysfunction in polycystic ovary syndrome.* J Endocrinol Invest *21:602–611, 1998.*)

response to GnRH. The decrease in LH amplitude seen with pulse frequencies that are faster than those encountered in physiological settings[50,51] may be the earliest sign of the pituitary desensitization that is well recognized in association with continuous infusions of GnRH or the use of a GnRH agonist.

Autocrine/Paracrine Regulation of Gonadotropins: Activin, Inhibin, and Follistatin

Activins, inhibins, and follistatins were first discovered as gonadal factors with preferential actions on FSH secretion from pituitary gonadotropes.[55] The inhibins, named for their inhibitory effect on FSH secretion in pituitary cell cultures, are heterodimers that are composed of one of two β-subunits, βA or βB, and a closely related α-subunit to form inhibin A or inhibin B. In contrast, the activins, which stimulate the synthesis and secretion of FSH, are composed of two β-subunits. Activin is secreted by gonadotropes and other pituitary cell populations and like other members of the TGF-β superfamily of growth and differentiation factors, activins exert most of their effects by autocrine or paracrine mechanisms. Activins sequentially interact with one of the two known type-II activin receptors, ActRII or ActRIIB and the type-1 receptor, activin receptor-like kinase 4 (ALK4). The expression of FSHβ is extremely sensitive to the stimulatory action of activins, which work in concert with and are permissive for the actions of GnRH through promotion of transcription of both FSHβ and the GnRH receptor.[56]

The inhibins act as specific antagonists of the FSH stimulatory actions of activin by binding to betaglyglycan and sequestering the type-II activin receptors. While inhibin is made in the pituitary, circulating inhibin derived from the ovary plays a far greater role in the negative control of FSH. In addition, bone morphogenic proteins, BMP-6 and BMP-7 are capable of modulating FSH synthesis in gonadotropes suggesting that other systems may also be involved in the control of FSH.[57]

Follistatin is a monomeric protein that is distinct from the activin and inhibin family and acts as a virtually irreversible binding protein by complexing with activin and masking the binding sites on activin for the type-I and type-II receptors.[55] The follistatin 288 isoform (FS288) has a high affinity for cell-surface proteoglycans and is presumed to act within the pituitary.[57] Follistatin is synthesized in many tissues in the body, including the pituitary where its synthesis in pituitary folliculostellate cells and gonadotropes is controlled by activin and GnRH and may be additionally modulated by gonadal steroids. Activin synthesis does not appear to vary during female reproductive cycles. However, specific changes in follistatin have been noted during the rat estrus cycle, suggesting that the effects of activin on FSH synthesis and secretion may be modulated through changes in follistatin.[57] Follistatin is increased by fast frequencies of GnRH stimulation and decreased with slower frequencies[58] compatible with the hypothesis that the activin and follistatin system mediates the effect of GnRH pulse frequency on FSH. Thus, while GnRH appears to be sufficient for control of LH synthesis and secretion, the activin-inhibin-follistatin system and perhaps BMPs play a significant role in conjunction with GnRH in regulating the synthesis and secretion of FSH.

Ovarian Feedback on the Hypothalamus and Pituitary

Negative Feedback

Estrogen

It is well known that low doses of estrogen inhibit gonadotropin secretion. Increased gonadotropin levels in patients with aromatase deficiency provide the most specific evidence of estrogen negative feedback on the control of both LH and FSH in men and women[59] although this is supported by the marked increase in LH and FSH levels in postmenopausal and ovariectomized women.[60] The loss of gonadal feedback in ovariectomized rats is associated with an increase in LH and FSH secretion; increased expression of LHβ, FSHβ, and α-mRNA; an increase in the number of cells expressing LHβ; an increase in cell size; and an increase in the amount of expression per cell. These changes are reversed by administration of low doses of estradiol,[61] which has also been shown to reverse the increases in gonadotropin secretion in the ovariectomized sheep and monkey.

There is considerable evidence that supports a primary hypothalamic site of estrogen negative feedback on pituitary gonadotropin secretion.[12,62] Studies, in which GnRH secretion has been measured, using a push-pull hypothalamic perifusion technique in the rat or cannulation of the pituitary portal circulation in the sheep and monkey, indicate that GnRH increases following ovariectomy and decreases with estrogen replacement. Estradiol administration is associated with a decrease in GnRH expression in rat hypothalamic tissue slices and in a GnRH neuronal cell line. Both indirect and direct mechanisms for estrogen regulation of hypothalamic GnRH neurons have been proposed. Studies in estrogen receptor (ER) knock-out animals support an important role of ERα in mediating estrogen negative feedback although both forms of the receptor are present in the hypothalamus.[62] Estrogen-receptive GABA neurons within the preoptic area may play a role in mediating the negative feedback effects of estrogen on GnRH secretion. There is also compelling evidence for a role of kisspeptin in estrogen negative feedback in rodents,[17,63] while β−endorphin is unlikely to be involved.[12]

In women, the mechanisms underlying estrogen negative feedback have generally been investigated in postmenopausal or ovariectomized women. The quantity of GnRH, estimated *in vivo* using submaximal GnRH antagonist administration, is higher in postmenopausal compared with premenopausal women,[64] consistent with the increase in GnRH mRNA that has been demonstrated in postmenopausal women studied at autopsy.[65] The quantity of GnRH returned to follicular phase levels with administration of low levels of estradiol, suggesting that the increase in GnRH in the absence of gonadal feedback can be attributed to loss of estrogen.[64] To further support a hypothalamic site of estrogen negative feedback in women, neuroimaging studies have now provided evidence of decreased metabolical activity in the medial basal hypothalamus in association with brief exposure to relatively low doses of estradiol.[66] Estradiol administration does not decrease GnRH pulse frequency in

the majority of studies in postmenopausal women and thus, the negative feedback effect of estradiol on the hypothalamus is likely to be mediated through changes in GnRH pulse amplitude.[60] These results in women are similar to those in which GnRH was measured directly in pituitary portal blood in ovariectomized sheep and monkeys and showed a decrease in GnRH pulse amplitude, but not frequency in response to estradiol administration.[67,68] Autopsy studies in women are consistent with a role for neurons expressing kisspeptin, NKB, substance P, dynorphin, and ERα in mediating the negative feedback of estrogen in the medial basal hypothalamus.[69,70]

While the studies detailed above demonstrate a significant hypothalamic effect of estrogen negative feedback, they do not exclude a pituitary site of action. In cultured pituitary cells, estradiol transiently reduces the LH response to GnRH[71] and administration of estradiol to hypothalamic-lesioned monkeys receiving pulsatile GnRH decreased LH secretion.[72] Both ERα and ERβ are present on gonadotropes[62] and characterization of the gonadotrope specific ERα (ESR1) knock-out mouse has now provided definitive evidence of a direct inhibitory effect of estradiol at the pituitary in the rodent.[73] Recent studies in postmenopausal women that isolated the pituitary from hypothalamic input have confirmed that physiological levels of estradiol have a direct pituitary effect on gonadotropin secretion that is greater for FSH than for LH.[74]

Progesterone

Progesterone has a profound effect on gonadotropin secretion that manifests at the hypothalamic level through slowing of pulsatile GnRH secretion.[75,76] This effect requires estrogen priming,[77] likely through upregulation of progesterone receptors in the hypothalamus.[78] In postmenopausal women receiving low doses of estradiol, addition of progesterone uniformly suppresses GnRH pulse frequency using either LH or FAS as markers of GnRH secretion[79-81] and administration of progesterone decreases the overall amount of GnRH secretion.[64] It is likely that progesterone exerts its effects on GnRH secretion through both direct and indirect mechanisms.[12,82] There is ample evidence that the β-endorphin system plays a critical role in mediating the effects of progesterone on GnRH pulse frequency and studies in sheep indicate that the inhibitory effect of progesterone on GnRH secretion is mediated by dynorphin.[24]

Inhibin/Activin/Follistatin

Evidence for a nonsteroidal gonadal factor with feedback effects on the pituitary dates to the early 1900s but it was not until the mid-1980s that inhibin was isolated and subsequently found to be part of a family of peptides that includes inhibin A, inhibin B, activin, and the functionally related protein, follistatin.[57] Inhibin B is present in the circulation in men while both inhibin A[83-85] and inhibin B[86-88] are detected in serum and follicular fluid in women during their reproductive years. Further studies have determined that inhibin B is produced by granulosa cells in the ovary[89] while inhibin A is produced by the luteinized granulosa cells of the corpus luteum.[89,90] Inhibin subunits are expressed in a variety of tissues including the adrenal. However, the only significant source of circulating dimeric inhibins is the gonads and there is compelling evidence that the principle mechanism of action of inhibin in suppressing pituitary FSH secretion is an endocrine action.

Activin acts as a local growth and differentiation factor in a variety of tissues, including the pituitary and ovary.[57] While activin has been measured in serum, circulating activin is irreversibly bound by the circulating isoform of follistatin, FS315.[91] As yet, no mechanisms have been identified within tissues that would alter neutralization by follistatin and therefore it is almost certain that within the pituitary activin acts in an autocrine and paracrine rather than endocrine fashion.

Gonadotropin Surge Attenuating Factor

Gonadotropin Surge Attenuating Factor (GnSAF), also known as gonadotropin surge inhibiting factor (GnSIF), is an ovarian factor that reduces GnRH-induced LH secretion.[92,93] Despite many years of investigation, the molecular structure of GnSAF[92,94,95] has not been completely characterized, hampering efforts to fully understand its regulation and physiological roles. Its name derives from the initially hypothesized role of this compound in preventing an early LH surge and thus, premature luteinization of the preovulatory follicle. However, there is now evidence in animal models and women that there is an inverse relationship between GnSAF bioactivity and follicle size, with highest concentrations in small growing follicles,[92] suggesting that it may well act earlier in the cycle than previously thought.

Positive Feedback

Estrogen

In addition to inhibition of gonadotropin secretion, estrogen exerts a stimulatory effect to generate the preovulatory LH surge. This positive feedback effect is seen in multiple animal species[62] and in women. There are two critical questions that form the basis of a mechanistic understanding of gonadotropin surge generation: the first is how estrogen can exert both inhibitory and stimulatory effects on LH secretion, and the second, is whether the site of estrogen positive feedback is at the pituitary, the hypothalamus, or both. The direction of estrogen feedback is dependent on both the degree of estrogen exposure and its duration with low levels of estradiol administration resulting in decreases in LH secretion within 12 to 24 hours, while positive feedback requires exposure to higher concentrations over a more prolonged duration.[96-98] There is ample evidence that high levels of estrogen augment the pituitary response to GnRH across species. Studies in animal models demonstrate an increase in the number of cells expressing the gonadotropin subunits, an increase in GnRH receptor number, an impact on the function of ion channels in the plasma membrane and regulation of both gene expression and second messenger systems within gonadotropes.[52,99,100] Studies in LβT2 pituitary cells indicate that activin may act in concert with estrogen to increase GnRH receptors on pituitary gonadotropes[56] while NPY secretion from the median eminence may contribute to pituitary sensitization through changes in the affinity of the GnRH receptor for its ligand.[101]

In the rat and sheep there is evidence that in addition to pituitary augmentation of the GnRH signal, estrogen positive feedback on gonadotropin secretion requires an increase in GnRH secretion[102] and in rodents, this increase

in GnRH secretion requires specific circadian signals.[103] In rodents, estrogen negative and positive feedback occur in distinct areas of the hypothalamus: the arcuate nucleus of the medial basal hypothalamus and the anteroventral periventricular nucleus (AVPV), respectively. While kisspeptin appears to be involved in both negative and positive estrogen feedback in the rodent, kisspeptin neurons in the arcuate nucleus coexpress NKB and dynorphin, whereas this is not the case in the AVPV.[24,63]

Generation of the preovulatory surge in nonhuman primates appears to be fundamentally different from that in the rodent—while there may be some alteration in GnRH input, this is not controlled in the preoptic area (the site of the AVPV), it is not tied to circadian signals, and the gonadotropin surge does not require an increase in GnRH.[104] As in nonhuman primates, an increase in GnRH secretion is not required for generation of a normal gonaodtropin surge in women.[105] In contrast to nonhuman primates, there is no evidence for augmentation of GnRH secretion or even an altered pattern of GnRH stimulation associated with generation of the surge in women.[106-108] Taken together, these data suggest that the gonadotropin surge in normal women requires ongoing pulsatile GnRH stimulation, but is otherwise mediated through the marked increase in pituitary sensitivity to GnRH (discussed later in the chapter in Midcycle Surge). Thus, while an increase in GnRH is required for generation of the gonadotropin surge in rodents and sheep, GnRH appears to play a permissive role in generation of the preovulatory LH surge in normal women as it does in the monkey.[102,104]

The Normal Menstrual Cycle

Clinical Characteristics

By convention, the first day of menses is designated Day 1 and marks the onset of the follicular phase of the menstrual cycle. The follicular phase encompasses the period of recruitment of multiple follicles and emergence and growth of the dominant follicle (Fig. 8.5). During the follicular phase, rising levels of estradiol are associated with endometrial proliferation. The luteal phase, which begins on the day after the LH surge, is characterized by formation of the corpus luteum, secretion of progesterone and a coordinated series of changes in the endometrium as it first prepares for implantation and then, with decline of the corpus luteum in the absence of pregnancy, loses its blood supply and sloughs.

The classic studies of Treloar and colleagues[109] indicate that median menstrual cycle length is 28 days, with a normal range between 25 and 35 days (Fig. 8.6). For the majority of reproductive life, there is little cycle-to-cycle variability although the intermenstrual interval declines between the ages of 36 and 40 years old. Both immediately following menarche and preceding menopause there is a significant degree of variability in the intermenstrual interval between and within individuals. Fluctuations in the length of the follicular phase are primarily responsible for the variations in cycle length both between women and as a function of normal aging in an individual woman. Luteal phase duration is more constant, lasting between 10 and 16 days in 95% of cycles. During the follicular phase, the progressive increase in diameter of the largest follicle as assessed by ultrasound is

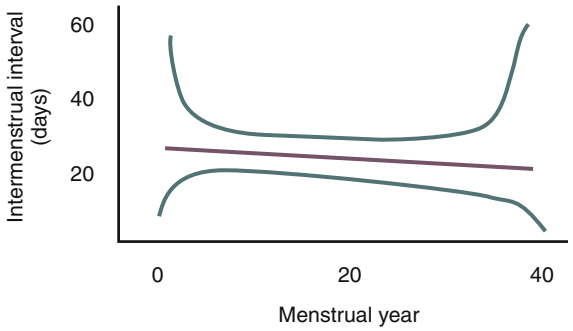


FIGURE 8.6 Median menstrual cycle lengths *(purple line)* from menarche (menstrual year 0) until menopause. The *green upper* and *lower* lines indicate the 95% confidence limits. (*From Treloar AE, Boynton RE, Behn BG, et al. Variation of the human menstrual cycle through reproductive life.* Int J Fertil *12:77–126, 1967.*)

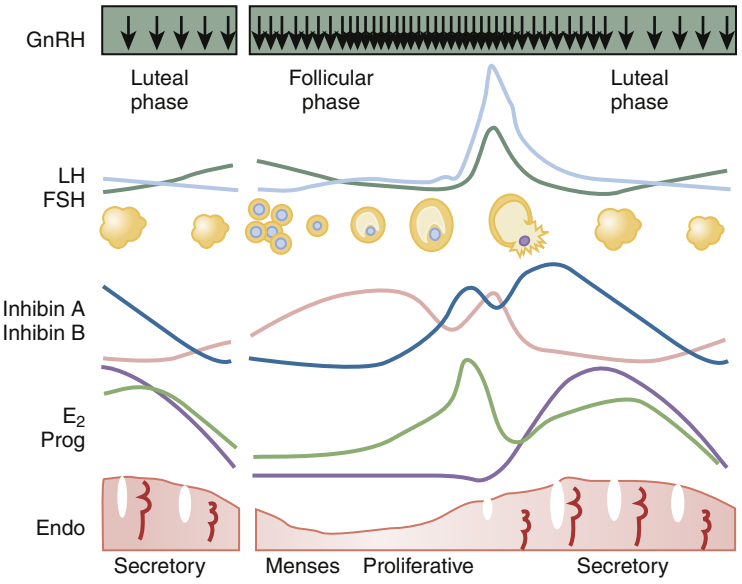


FIGURE 8.5 The hormonal, follicular and endometrial dynamics of the normal menstrual cycle from the late luteal phase, through menses and the beginning of a new cycle of follicle development, ovulation and corpus luteum function, as indicated. With the support of the changing frequency of pulsatile gonadotropin-releasing hormone (GnRH) secretion, the integrated actions of follicle-stimulating hormone (FSH; *green*) and luteinizing hormone (LH; *light blue*) are responsible for: (1) follicular development with secretion of estradiol (E_2; *light green*), inhibin B *(pink)* and inhibin A *(blue);* (2) the preovulatory surge and ovulation; and (3) secretion of progesterone (Prog; *purple*), estradiol and inhibin A from the corpus luteum. Secretion of estradiol and progesterone result in proliferative and secretory changes in the endometrium (Endo), preparing it for implantation should conception occur. In the absence of conception, endometrial shedding follows the decline in hormone secretion secondary to demise of the corpus luteum.

highly predictable at approximately 2 mm per day until ovulation (Fig. 8.7). The accompanying increase in estradiol is associated with a progressive increase in the thickness of the endometrium while the luteal phase effects on the endometrium result in increased echogenicity (Fig. 8.7).

GnRH Dynamics and Pituitary Responsiveness

GnRH secretion can be measured directly in large animal species, and studies have indicated that under physiological circumstances, peripheral LH secretion occurs concomitantly with secretion of GnRH measured in pituitary portal blood.[110-112] LH has therefore been used as a marker of GnRH pulse frequency in the human, based on these studies and two additional lines of evidence. The first is that pulsatile secretion of LH is absent in patients with congenital isolated GnRH deficiency and can be restored with pulsatile administration of GnRH.[113] The second is that pulsatile secretion of LH in normal subjects is reversibly abolished by administration of a specific GnRH antagonist.[46] Thus, the occurrence of LH pulses can be taken as evidence for the occurrence of a preceding stimulatory GnRH pulse and LH pulse frequency can be used as a peripheral monitor of the frequency of pulsatile GnRH secretion. Although the glycoprotein free alpha subunit (FAS) is secreted from both the gonadotrope and thyrotrope under the control of GnRH and TRH, the pulsatile component of FAS secretion is entirely under the control of GnRH in euthyroid women.[106] Thus, FAS can also be used as a surrogate marker of GnRH pulse frequency. As its clearance is faster than that of intact LH, it is a preferable marker when GnRH pulse frequency is rapid or when the clearance of LH is prolonged. The amplitude of the LH or FAS response to GnRH depends on both the amplitude of the GnRH signal and on pituitary responsiveness to GnRH. Other techniques must therefore be used to assess the amplitude of GnRH secretion.

Results of frequent sampling studies (every 5 or 10 minutes for up to 48 hours) have demonstrated marked variations in the frequency and amplitude of LH pulses across the normal menstrual cycle (Fig. 8.8) and their precise regulation in relation to the preovulatory LH surge.[106,113-115]

Follicular Phase

In the early follicular phase (days 14 to 9 from the LH surge), the mean interpulse interval of GnRH is approximately 90 to 100 minutes.[33,115] The early follicular phase of established reproductive cycles is characterized by a marked slowing of GnRH pulse frequency during sleep (Figs. 8.3 & 8.8),[35] which may serve the function of maintaining FSH synthesis during this critical period of follicle recruitment.

In the midfollicular phase, GnRH pulse frequency increases and the interpulse interval shortens to approximately 60 minutes. LH pulse amplitude is markedly attenuated in the midfollicular phase, reflecting the negative feedback of estradiol secreted from developing follicles on the amplitude of GnRH pulses and possibly the increased frequency of GnRH stimulation and its effect on gonadotrope responsiveness.[50,51]

The circhoral frequency of GnRH secretion is maintained through the late follicular phase as LH pulse amplitude begins to increase due to the stimulatory effects of rising levels of estradiol on gonadotrope responsiveness to GnRH.

Midcycle Surge

In response to the exponential increase in estradiol secretion in the late follicular phase, LH levels increase tenfold over

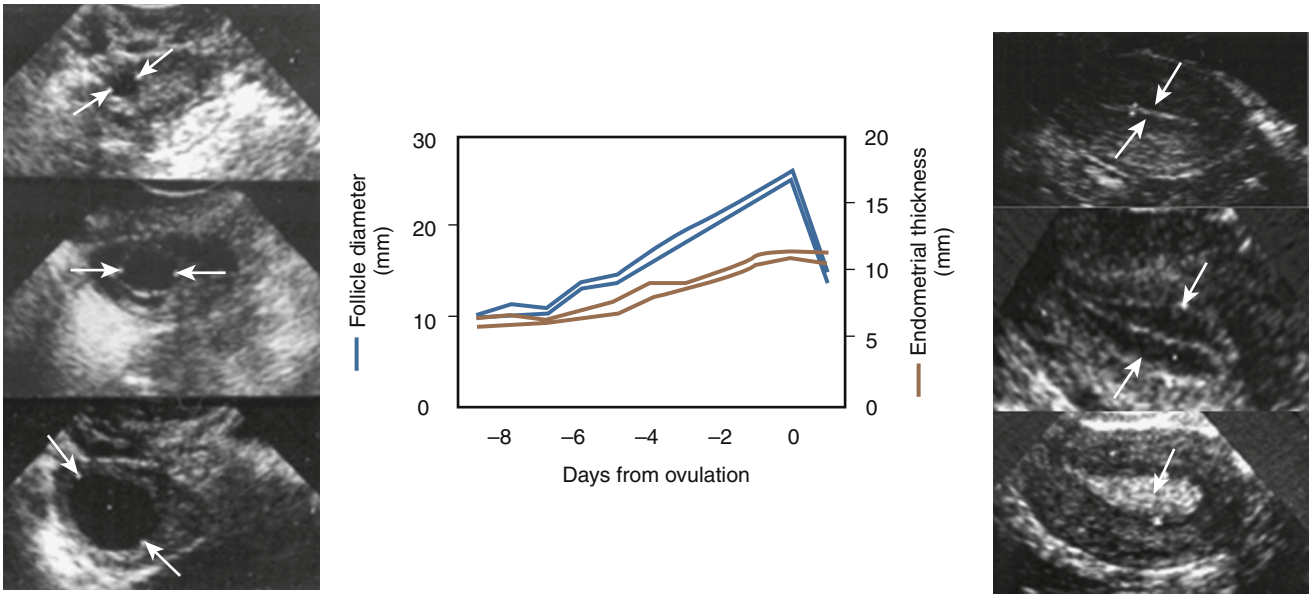


FIGURE 8.7 Follicular development is associated with a progressive increase in the diameter of the dominant follicle *(arrows)*, as seen on ultrasound *(left panels, top to bottom)* and an increase in endometrial thickness during the follicular phase *(right top and middle panels)*. Follicle diameter *(blue lines)* and endometrial thickness *(brown lines)* derived from studies in 42 women with normal menstrual cycles are presented in relation to the day of ovulation, which is indicated as 0. The appearance of the endometrium *(single arrow)* is markedly different in the luteal phase, showing increased echogenicity *(right bottom panel)*. (*From Adams JM, Hall JE. Increase in the size of the dominant follicle and endometrial thickness as measured by ultrasound during the follicular phase in 42 normal women. Personal Communication, 2003.*)

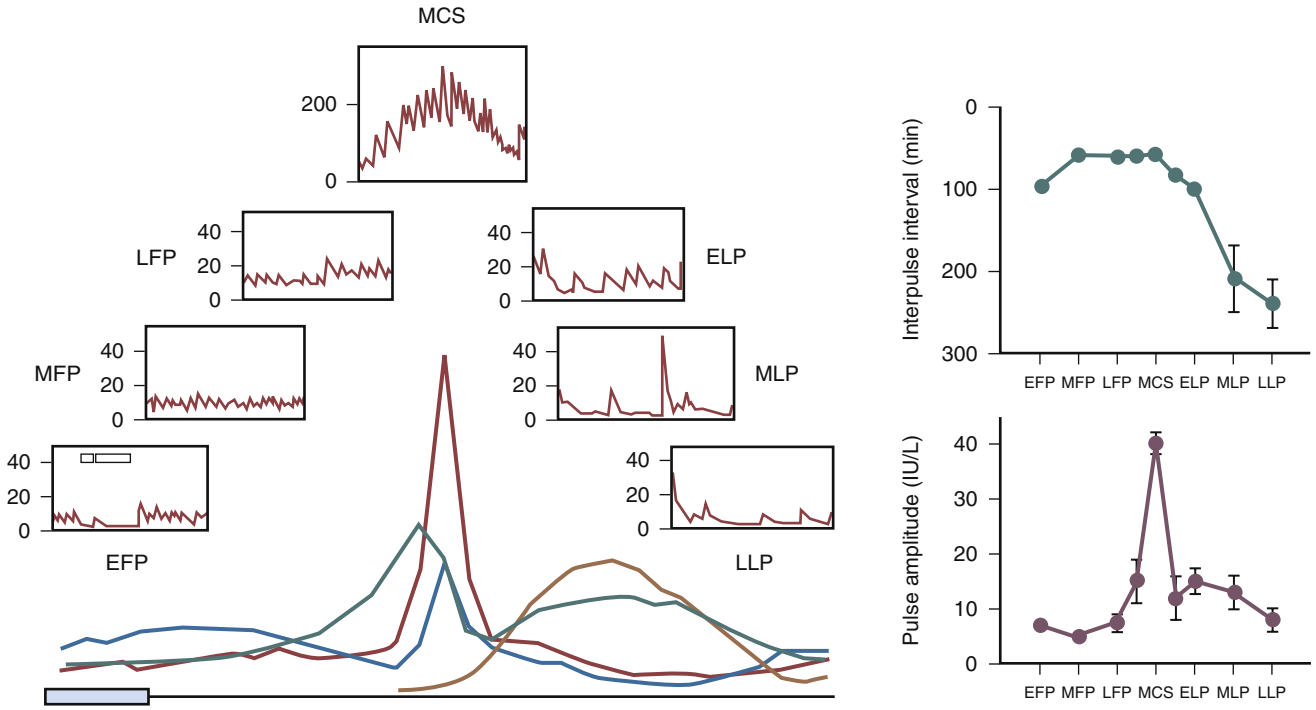


FIGURE 8.8 Dynamics of pulsatile luteinizing hormone (LH) secretion in relation to LH *(red)*, FSH *(teal)*, estradiol *(green)*, and progesterone *(brown)* in the early follicular phase (EFP), midfollicular phase (MFP), and late follicular phase (LFP), during the midcycle surge (MCS) and in the early luteal phase (ELP), midluteal phase (MLP), and late luteal phase (LLP) in normal women *(left panel)*. Menses is indicated by the *red rectangle*. The dynamic changes in the interpulse interval and amplitude of pulsatile LH secretion in relation to the phases of the menstrual cycle are indicated in the *right panel*. (*Adapted from Hall JE, Martin KA, Taylor AE. Body weight and gonadotropin secretion in normal women and women with reproductive abnormalities. In Hansel W, Bray, GA, Ryan DH.* Nutrition and Reproduction *[8]:378–393. 1998, Baton Rouge, Louisiana State University Press, Pennington Center Nutrition Series and Hall JE. Neuroendocrine physiology of the early and late menopause.* Endocrinol Metab Clin North Am *33:637–659, 2004.*)

a period of 2 to 3 days while FSH levels increase fourfold (Fig. 8.8). This midcycle surge of LH is absolutely required for final maturation of the oocyte and initiation of follicular rupture, which generally occurs 36 hours after the surge. The gonadotropin surge is therefore a critical component of normal reproductive cycles. However, the mechanisms responsible for this dramatic increase in gonadotropin secretion are incompletely understood and appear to be species specific. The classic studies of Yen and colleagues demonstrated that the responses of LH and FSH to exogenous GnRH administration are markedly influenced by the stage of the menstrual cycle with an exaggerated increase in secretion of both gonadotropins at the time of the midcycle surge.[116]

The pattern of estrogen exposure is critical to positive feedback. Exogenous administration of estradiol to normal women in the early follicular phase[96,97] or to postmenopausal women[74] induces an increase in both basal and GnRH-stimulated LH secretion that is dependent on both the dose and duration of estrogen exposure. There is further evidence that the surge occurs in response to the increase in estradiol rather than the drop in estradiol that frequently accompanies the onset of the surge.[97] In other words, the surge results from estrogen positive feedback rather than removal of estrogen negative feedback.

Other evidence indicates that a small increase in progesterone augments this surge. Secretion of progesterone is typically associated with the luteal phase, however, the earliest increase in progesterone is evident in normal women prior to the LH surge. Blockade of progesterone receptors by RU486 delays the surge by up to 3 days despite continued growth of the dominant follicle and rising levels of estradiol.[117] In studies in normal women in whom a graduated estrogen infusion was initiated in the early follicular phase, it has now been demonstrated that progesterone does not influence the height of the surge, but decreases the variability in its timing relative to the onset of the infusion.[97] Although an LH surge can be generated in response to recreation of normal preovulatory estradiol and progesterone levels, the amplitude of the surge is less that in normal women. This suggests that there may also be other ovarian factors required for generation of a surge of normal amplitude. One possibility is inhibin A which increases dramatically before the LH surge in normal cycles[85,118] and has been shown to increase GnRH receptor number and LH secretion in an ovine pituitary cell culture system.[119]

A key question is whether estrogen positive feedback in women is mediated at the hypothalamus, the pituitary or both. There is increasing evidence that the mechanisms underlying this critical process may be species specific. As reviewed above, there is compelling evidence from a variety of animal and *in vitro* studies that estradiol acts directly at the pituitary to increase gonadotrope sensitivity to GnRH.[52,99,100] In lower animal species, there is also evidence that hypothalamic mechanisms play a critical role in generating the preovulatory surge although studies in sheep indicate that the amplitude of the GnRH surge exceeds

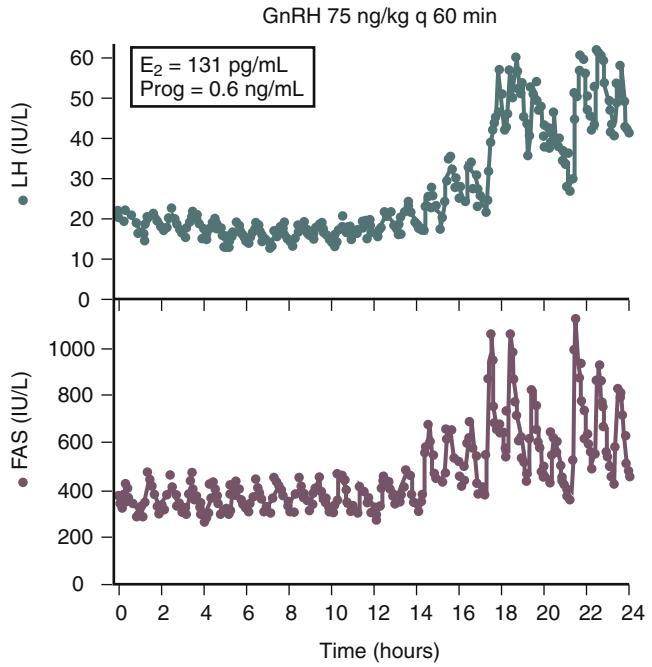


FIGURE 8.9 Luteinizing hormone (LH) and free α-subunit (FAS) sampled every 5 minutes in a gonadotropin-releasing hormone (GnRH)-deficient woman receiving pulsatile GnRH intravenously, demonstrating the abrupt increase in LH and FAS pulse amplitude and mean levels associated with estrogen positive feedback in the absence of any change in the dose or frequency of GnRH. E_2, estradiol; Prog, progesterone.

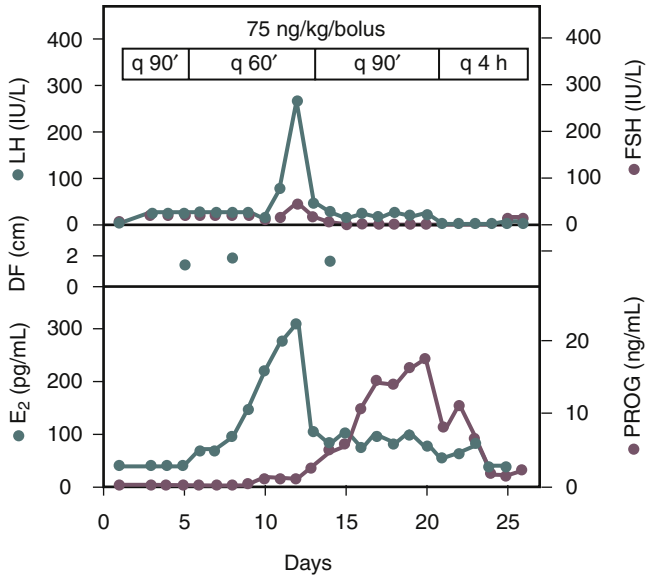


FIGURE 8.10 Administration of intravenous pulsatile gonadotropin-releasing hormone (GnRH) to a GnRH-deficient woman at a physiological frequency with follicular development, ovulation, and normal luteal phase function. Note that the luteinizing hormone (LH) surge is generated in association with an increase in follicle size and estradiol (E_2), but in the absence of an increase in the dose or frequency of pulsatile GnRH administration. FSH, follicle-stimulating hormone; DF, dominant follicle. (*Adapted from Hall JE, Martin KA, Whitney HA, et al. Potential for fertility with replacement of hypothalamic gonadotropin-releasing hormone in long term female survivors of cranial tumors.* J Clin Endocrinol Metab *79:1166–1172, 1994.*)

that needed to generate the LH surge.[102] Studies in GnRH-deficient women receiving exogenous GnRH replacement provide the most compelling evidence for the importance of pituitary sensitization to GnRH in generation of the midcycle surge in women. When GnRH is administered at a dose and frequency that mimic the GnRH pulse frequency in the normal menstrual cycle with development of a single dominant follicle, an abrupt increase in LH and FAS pulse amplitude is observed in the absence of any change in the dose or frequency of GnRH (Fig. 8.9) and a normal LH surge is achieved (Fig. 8.10).[120] These studies indicate that positive feedback can be achieved in women through pituitary mechanisms alone in the absence of any alteration in hypothalamic input.

Further studies indicate that gonadotropin surges are achieved with no increase in GnRH secretion in normal women. At the onset of both spontaneous and steroid-induced LH surges in normal women, complete GnRH receptor blockade results in termination of the surge,[107,121,122] indicating that ongoing GnRH secretion is essential for generation of the gonadotropin surge. However, neither the frequency nor the overall amount of GnRH is increased in association with the onset of the gonadotropin surge. Studies in normal women in which blood samples were drawn every 5 minutes for up to 36 hours at midcycle indicate a striking increase in LH and FAS pulse amplitude from the late follicular phase to the early portions and midportions of the surge with no change in pulse frequency during this same time period (Fig. 8.8).[106] To address the question of whether generation of the surge in women is associated with an increase in the amplitude of GnRH secreted with each bolus, submaximal GnRH receptor blockade was used to provide a semiquantitative estimate of the overall amount of endogenous GnRH secreted.[107] Results of these studies provided no evidence for an increase in the overall amount of GnRH secreted and, in fact, suggested that the amount of GnRH at the surge is less than in the early and late follicular phase. Consistent with this finding, further studies have shown that in GnRH-deficient women, the replacement dose of GnRH can be reduced by two-thirds in the late follicular phase from that required for development of a single dominant follicle without compromising the timing or height of the midcycle surge or the subsequent luteal phase (Fig. 8.11).[108]

Taken together, these studies indicate that GnRH is absolutely required for generation of the midcycle surge in normal women; however, there is no evidence for an increase in either the amplitude or frequency of GnRH secretion. Consistent with this conclusion, neuroimaging studies in postmenopausal women, in whom a gonadotropin surge was induced by administration of a graded estradiol infusion, show a marked increase in metabolic activity at the pituitary, but not the hypothalamus, in association with estrogen positive feedback on LH.[66]

The termination of the LH surge is associated with a dramatic decrease in pulse amplitude accompanied by a decrease in pulse frequency to approximately every 70 minutes (Fig. 8.8).[106] The decline in pulse frequency that accompanies the termination of the surge is due, at least in part, to the hypothalamic effects of progesterone on the GnRH pulse generator.

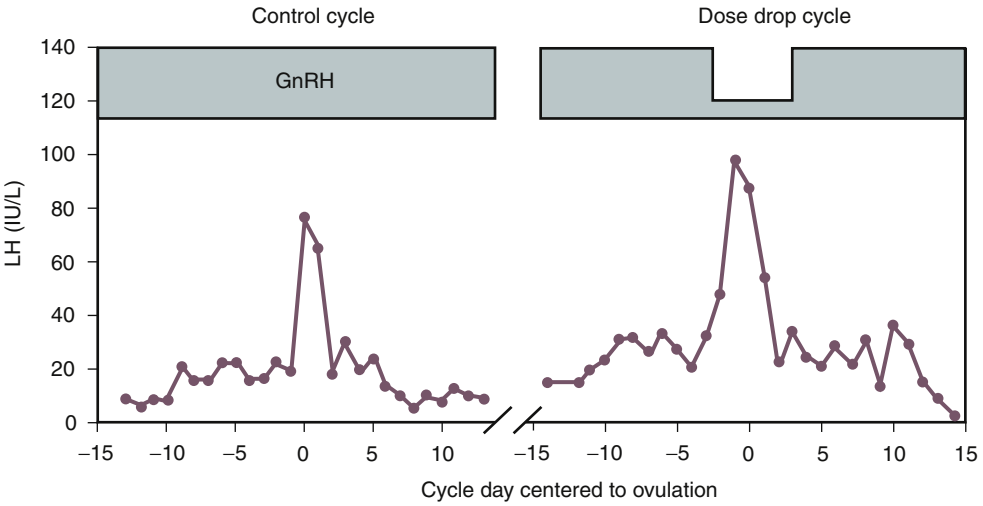


FIGURE 8.11 A normal surge of luteinizing hormone (LH) can be recreated in gonadotropin-releasing hormone (GnRH)-deficient women when the dose of exogenous GnRH *(green bars)* is reduced from 75 ng/kg to 25 ng/kg before the onset of the LH surge. (*From Martin KA, Welt CK, Taylor AE, et al. Is GnRH reduced at the midcycle surge in the human? Evidence from a GnRH-deficient model.* Neuroendocrinology *67:363–369, 1998.*)

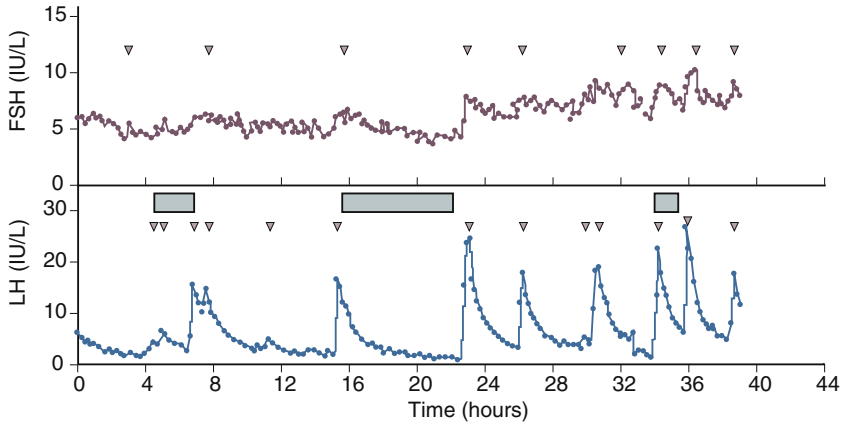


FIGURE 8.12 Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in a subject who underwent blood sampling every 10 min over a 2 day period during the luteal-follicular transition. This study was conducted beginning 12 days after the subject's preovulatory LH surge and 3 days before menses. Note the rise in FSH that is clearly evident before menses and is associated with an increase in the frequency of pulsatile LH secretion. The horizontal bars indicate sleep and the inverted triangles indicate statistically identified pulses. (*Adapted from Hall JE, Schoenfeld DA, Martin KA, et al. Hypothalamic gonadotropin-releasing hormone secretion and follicle-stimulating hormone dynamics during the luteal-follicular transition.* J Clin Endocrinol Metab *74:600–607, 1992.*)

Luteal Phase

Slowing of pulsatile GnRH secretion begins during the termination of the midcycle surge and continues through the early luteal, midluteal, and late luteal phases (Fig. 8.8). In the late luteal phase, interpulse intervals as long as 4 to 8 hours are observed. This slowing of the GnRH pulse generator is due to the effect of progesterone,[123] but is not expressed without the additional presence of estradiol.[77]

In the luteal phase, LH pulse amplitudes are significantly higher than in the follicular phase. The known inverse relationship between LH responsiveness to GnRH and GnRH pulse frequency[53,54] suggests that this increase in LH pulse amplitude is secondary to progesterone-induced slowing of GnRH pulse frequency. However, there is additional evidence from studies in GnRH-deficient women, in whom it is possible to control the dose of GnRH administered, that progesterone may act directly at the pituitary to increase LH responsiveness to GnRH.[124]

Luteal-Follicular Transition

The luteal follicular transition is characterized by declining function of the corpus luteum and declining levels of progesterone, estradiol, and inhibin A. Release from negative feedback permits FSH to rise, an increase that begins before menses and is critical for recruitment of a new cohort of follicles into the developing pool (Fig. 8.5). Maintenance of midluteal phase levels of estradiol prevents this increase in FSH.[125,126] Thus, it has been proposed that release from estrogen negative feedback is the key factor in the luteal-follicular rise in FSH and that other factors such as the decline in inhibin A secretion from the corpus luteum may not play a role. However, studies using tamoxifen to block the estrogen receptor in normal cycles suggest that inhibin A also plays a role in restraining FSH secretion during the normal luteal phase.[127]

LH pulse frequency increases before the onset of menses (Fig. 8.12). LH pulse frequency is inversely related to progesterone levels[114] and administration of midluteal phase levels of progesterone in conjunction with estradiol prevents the normal luteal-follicular increase in GnRH pulse frequency in normal women.[77] There is convincing evidence that the increase in GnRH pulse frequency that occurs between the luteal and follicular phases facilitates the luteal-follicular increase in FSH secretion. In normal women, the increase in FSH is significantly correlated with LH pulse frequency, while the inverse relationship between FSH and estradiol was not significant.[114] In GnRH-deficient women receiving pulsatile GnRH at a physiological frequency to recreate normal cycle dynamics, there is a partial increase in FSH associated with release from the negative feedback effects of estradiol and

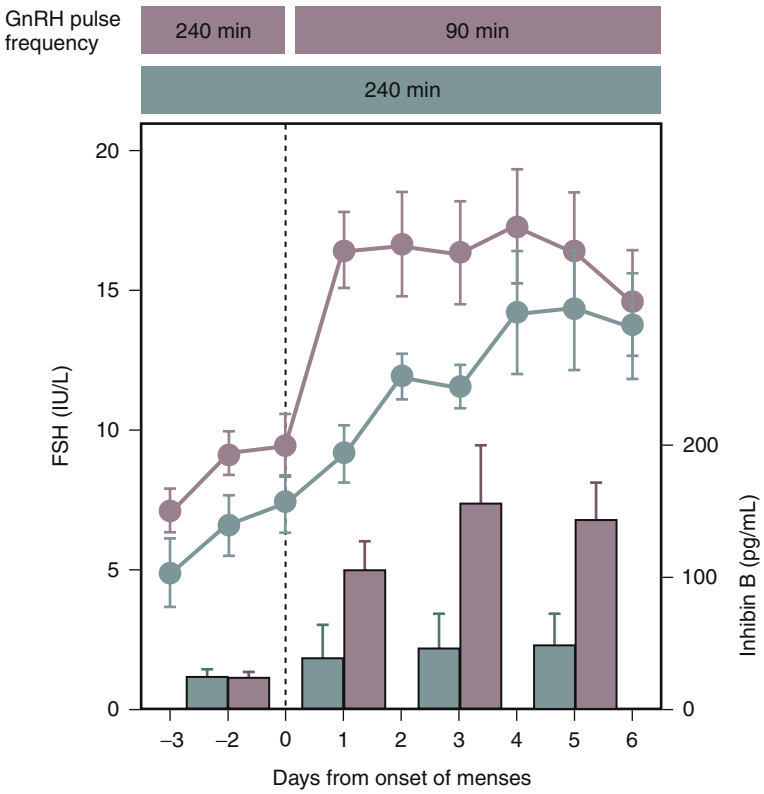


FIGURE 8.13 The increased frequency of pulsatile gonadotropin-releasing hormone GnRH during the luteal follicular transition facilitates the secretion of follicle-stimulating (FSH) as indicated in this study in GnRH-deficient women. The normal rise in FSH in relation to menses is attenuated when the frequency of intravenous GnRH (75 ng/kg) remains at the luteal phase frequency of every 240 minutes *(green circles)* compared to the usual increase to every 90 minutes at the time of menses *(purple circles)*. The bars indicate that the follicular phase increase in inhibin B is also attenuated in association with a persistently slow frequency of GnRH replacement *(green bars)* compared to the usual increase in frequency to every 90 minutes *(purple bars)* secondary to the inadequate rise in FSH. (*From Welt CK, Martin KA, Taylor AE, et al. Frequency modulation of follicle-stimulating hormone [FSH] during the luteal-follicular transition: evidence for FSH control of inhibin B in normal women.* J Clin Endocrinol Metab *82:2645–2652, 1997.*)

possibly inhibin A.[128] However, an increase in GnRH pulse frequency from the luteal phase frequency of every 4 hours to the follicular phase frequency of every 90 minutes is essential to recreate the normal intercycle rise in FSH (Fig. 8.13).

Thus, the slow frequency of GnRH secretion in the luteal phase is hypothesized to increase FSH synthesis either directly or through a decrease in follistatin and a concomitant increase in activin signaling. However, FSH secretion is inhibited by estradiol and possibly inhibin A. With the demise of the corpus luteum, estradiol and inhibin A levels fall as do those of progesterone. FSH is allowed to increase with release from negative feedback and is further stimulated by the increase in GnRH pulse frequency to its early follicular phase frequency.

There is now evidence that the progressive increase in GnRH pulse frequency from the early to the midfollicular phase represents a gradual loss of the restraining effects of low levels of progesterone on the GnRH pulse generator.[129] As the early follicular phase is characterized by sleep-related inhibition of pulsatile LH secretion, it is intriguing to speculate that a similar prolonged effect of progesterone is also involved in sensitizing the hypothalamus to the inhibitory effects of sleep during this cycle phase. The possibility that sleep-related slowing of GnRH pulse frequency in the early follicular phase may play an important role in maintaining synthesis of FSH at this critical time is suggested by the association of menstrual cycle abnormalities with altered sleep wake cycles in women exposed to transmeridian travel or rotating shifts.[130,131]

Secretion of Inhibin A and Inhibin B

The pattern of inhibin A secretion is characterized by a periovulatory increase and maximal levels in the luteal phase (Fig. 8.5).[83,84,118] In contrast, inhibin B is at its nadir in the luteal phase, begins to rise during the luteal-follicular transition to attain its highest levels in the early follicular and midfollicular phases, and declines in the late follicular phase followed by a brief periovulatory increase (Fig. 8.5).[85,87] The differential patterns of inhibin B and inhibin A in serum across the menstrual cycle suggest different sources and differential control of these two inhibin forms in normal reproductive cycles. Interestingly, their relationship to the secretion of the gonadotropins and estradiol does not easily confirm the anticipated endocrine role for either inhibin A or inhibin B in the control FSH. Inhibin B and FSH levels are positively rather than negatively related during the luteal-follicular transition, while the potential negative feedback effects of inhibin A on FSH might just as easily be accounted for by estradiol whose levels change in parallel to those of inhibin A.

Sources of Inhibin A and Inhibin B

The peak in inhibin A in the luteal phase and its decline with luteolysis are consistent with production by the corpus luteum, as expected from the high levels of βA inhibin subunit expression in the corpus luteum.[89] During folliculogenesis, peak levels of inhibin A are attained in the preovulatory period and inhibin A is correlated with the size of the dominant follicle in normal cycles[84,85] as is estradiol. Inhibin A is primarily the product of granulosa cells; however, there is some evidence for theca cell production in the mature follicle.[89] Studies of βA inhibin subunit expression[89] and measurement of dimeric inhibin A in follicular fluid,[132] are consistent in indicating maximal follicular phase levels in the preovulatory follicle. However, it is now appreciated that inhibin A is synthesized and secreted at earlier stages of follicular development than previously suggested by serum levels.[133]

In contrast to inhibin A, the pattern of inhibin B in serum suggests that it is primarily secreted from small antral follicles. A correlation of inhibin B levels with the size of the dominant follicle is remarkably absent in women undergoing spontaneous ovulatory cycles,[85] suggesting that inhibin B is not regulated in relation to dominant follicle growth. These data are consistent with studies showing that inhibin B levels in follicular fluid do not change as a function of follicle size or maturity.[132] They are also consistent with earlier studies demonstrating that expression of βB inhibin subunit mRNA is highest in early antral follicles, with lower levels in the dominant follicle and no evidence of βB-subunit expression in the corpus luteum.[89] The latter studies have also shown that inhibin B synthesis is confined to the granulosa cells and is absent in theca cells.

Regulation of Inhibin A and Inhibin B by Gonadotropins

A significant body of data has now demonstrated an increase in inhibin B secretion in conjunction with early follicular development stimulated by physiological levels of FSH. This was initially suggested by the relationship of the rise in FSH during the luteal-follicular transition to the concomitant increase in inhibin B (Fig. 8.5). In GnRH-deficient women, replacement of pulsatile GnRH at the slower luteal phase frequency of every 4 hours compared with the early follicular phase frequency of every 90 minutes was associated not only with failure of FSH to rise normally during the luteal-follicular transition, but also with the absence of both follicular growth and any increase in secretion of inhibin B (Fig. 8.13).[128] This study demonstrated the remarkable sensitivity of serum inhibin B levels to changes in FSH stimulation within the physiological range during the earliest stages of follicular development.

In studies designed to examine the time course of the response to specific and physiological gonadotropin stimulation, recombinant human LH (rhLH) or FSH (rhFSH) was administered to women following downregulation of endogenous GnRH secretion using a potent GnRH agonist.[133] Consistent with failure to observe LH receptors in small antral follicles, rhLH alone had no effect on follicle growth or hormone secretion when administered daily for up to 7 days. However, daily subcutaneous administration of rhFSH resulted in normal early follicular phase levels of FSH and an increase in inhibin B secretion that preceded that of estradiol and inhibin A (Fig. 8.14). One interpretation of these studies is that FSH directly stimulates inhibin B secretion from granulosa cells. However, administration of FSH at this stage of development also results in the recruitment of a cohort of follicles into the growing pool and a dramatic stimulation of granulosa cell number.

To determine whether the increase in inhibin B observed *in vivo* in the early stages of folliculogenesis results from direct stimulation of granulosa cell secretion or from an increase in granulosa cell number, studies were conducted using pre-antral and small antral follicles obtained from

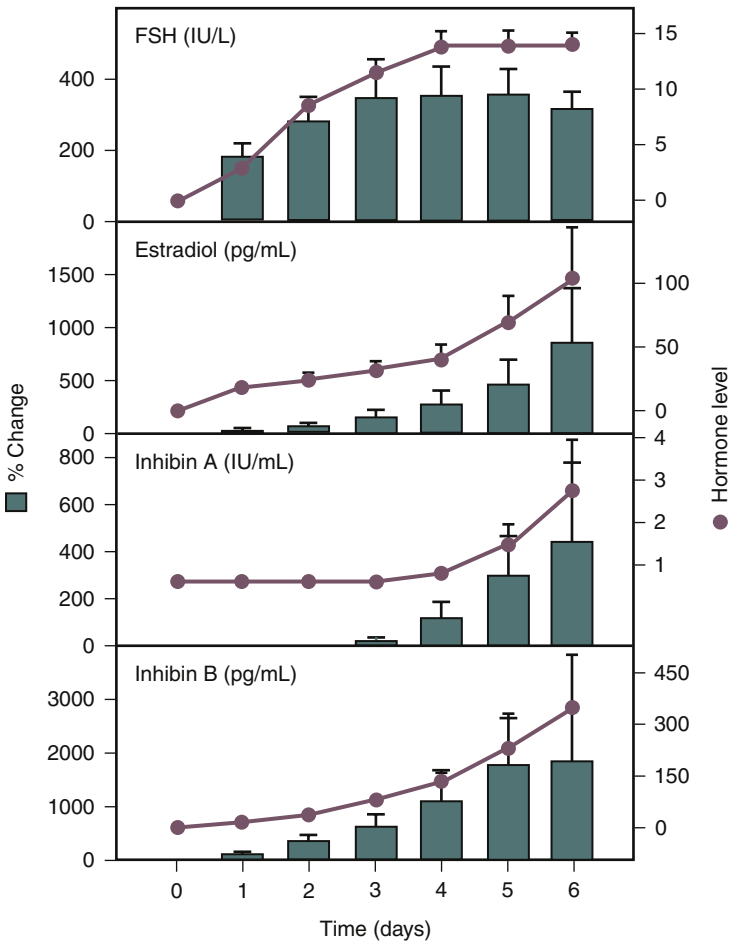


FIGURE 8.14 Hormone response to recombinant human follicle-stimulating hormone (FSH; 150 IU daily) for 6 days in normal women after GnRH agonist downregulation of endogenous gonadotropin secretion. (*From Welt CK, Smith ZA, Pauler DK, et al. Differential regulation of inhibin A and inhibin B by luteinizing hormone, follicle-stimulating hormone, and stage of follicle development.* J Clin Endocrinol Metab *86:2531–2537, 2001.*)

ovaries at the time of oophorectomy for nonovarian indications.[134] Results of these studies indicated that inhibin B is constitutively secreted from pre-antral follicles and that FSH stimulates secretion of inhibin A, but not inhibin B. Taken together, these studies demonstrate that inhibin B is constitutively secreted from granulosa cells and that the increase in inhibin B observed *in vivo* in response to FSH results from an increased number of granulosa cells rather than direct stimulation of secretion by FSH. At later stages of follicular development, both LH and FSH stimulate secretion of inhibin A and estradiol from the dominant follicle, but have no effect on inhibin B.[133]

Evidence for an Endocrine Role for Inhibin A or Inhibin B

Although an endocrine role of either inhibin A or inhibin B cannot be implied from their relationship to FSH during the normal menstrual cycle, several lines of evidence support such a role in the negative feedback regulation of FSH. Inhibin was initially discovered based on its ability to inhibit FSH secretion in pituitary cell cultures.[135] In addition, FSH levels decrease in response to administration of pharmacological doses of inhibin A administered in the follicular and luteal phases in the rhesus monkey.[136,137]

The most compelling line of evidence that inhibin regulates FSH secretion under normal physiological circumstances in women is the failure of physiological levels of gonadal steroids to restore FSH levels to normal in postmenopausal women. The model of reproductive aging has been used by a number of investigators to refine this evidence. FSH levels increase with age, before increases in LH or decreases in estradiol[138-140] and a number of studies have demonstrated an inverse relationships between increasing FSH and decreasing inhibin B[140-143] in association with reproductive aging.

Reproductive aging is associated with a decline in fertility that begins in the third decade, but accelerates rapidly after age 35. A gradual decrease in the pool of ovarian follicles which first accelerates around the same age would appear to underlie this decline in fertility.[144] It is also at age 35 that an increase in follicular-phase FSH is first seen. In women 35 or older with regular ovulatory cycles and follicular phase FSH levels within the normal range, there is a small, but significant increase in FSH in the early follicular phase only, decreased inhibin B levels across the entire follicular phase and estradiol levels that do not differ in the early follicular phase, but increase in the midfollicular and late follicular phases.[85] In the luteal phase, inhibin B, inhibin A, and progesterone are lower in the older cycling women while estradiol levels are preserved. Thus, the very earliest decrease in inhibin B occurs at a time that is marked by the beginning of the more rapid follicular depletion that heralds the menopause, suggesting that lower inhibin B levels reflect a decrease in available follicle number with aging. This change in inhibin B is associated with higher levels of FSH in the absence of alterations in estradiol, providing evidence for an endocrine role of inhibin B in the control of FSH secretion in reproductive-aged women. Studies conducted at later stages in the process of reproductive aging have uniformly confirmed increased FSH in conjunction with normal to higher levels of estradiol and lower inhibin B and further support the negative feedback role of inhibin B in control of FSH. While a decrease in inhibin A may occur slightly later in reproductive aging, it precedes the decline in estradiol levels.[145]

While studies of reproductive aging provide evidence of a negative feedback role of inhibin B on FSH secretion in normal women, these studies have not clarified the role of inhibin A or inhibin B relative to that of estradiol in the dynamic control of FSH during normal reproductive cycles. A key question is whether inhibin A and inhibin B contribute to the midfollicular decrease in FSH that is critical to the monofollicular development that characterizes reproductive cycles in normal women. The tools to determine the role of inhibin directly by either administration or blockade are not available. However, it is possible to investigate the estrogen component of FSH negative feedback and thereby infer the physiological role of inhibin. Studies in which estradiol levels were maintained by estradiol administration during the luteal-follicular transition have been interpreted to suggest that inhibin A is not involved in the negative feedback control of FSH in the luteal phase.[125,126] An alternative approach is to block estrogen negative feedback. The use of tamoxifen to block the estrogen receptor permitted evaluation of the relative role of estradiol and the inhibins in negative feedback on FSH.[127] Results suggest that the low levels of estradiol in the early follicular phase do contribute to negative feedback on FSH, as attested to by a prompt increase in early follicular phase FSH levels. An increase in FSH in response to estrogen receptor blockade was not seen in GnRH-deficient women receiving pulsatile GnRH replacement,[127] suggesting that the negative feedback of estradiol on FSH secretion in normal women is mediated entirely at the hypothalamic level in the early follicular phase.

Taken together, these studies suggest that estradiol plays a critical role in FSH negative feedback in the luteal phase and during the luteal-follicular transition. However, the failure of luteal phase FSH to increase to menopausal levels in the presence of estrogen receptor blockade suggests additional FSH restraint by inhibin A.

The control of FSH is dependent not only on inhibin and estradiol, but also on the activin/follistatin system. During the menstrual cycle, total activin A levels are highest at the midcycle and during the luteal follicular transition of the normal menstrual cycle.[146] However, there is no change in activin A in follicular fluid as a function of follicular development,[132] no change in free activin across the menstrual cycle,[147] and no difference in activin B between the follicular and luteal phases.[148] Taken together, these data suggest that activin does not play an endocrine role in the control of FSH secretion.

Racial Differences in Menstrual Cycle Dynamics

Racial disparities between African-American women and Caucasian women in the incidence of breast cancer, leiomyomas and osteoporosis raise the possibility of a greater lifetime exposure to estrogen in African-American women. Recent studies have now indicated that estradiol levels are significantly higher in weight-matched regularly cycling, ovulatory African-American women compared to their Caucasian counterparts with estradiol levels that are 18% higher in the late follicular phase, and 40% higher in the midluteal and late luteal phases.[149] In these studies, higher estradiol in the face of similar androstenedione levels suggests that

ovarian aromatase activity is higher in African-American women. Importantly, this apparent increase in aromatase activity in African-American women is not accounted for by differences in FSH levels.

Recent evidence also suggests that fertility and the results of fertility treatment are also highly influenced by race.[150,151] While the etiology of these differences is undoubtedly multifactorial, racial differences in reproductive hormones suggest that differences in fertility and fertility outcome should not be attributed to psychosocial influences without further investigation.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

Suggested Readings

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CHAPTER 9

The Ovarian Life Cycle

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The ovary is a dynamic organ that undergoes some of the most dramatic changes in structure and function of any adult human tissue. The follicles are the major endocrine and reproductive compartments of the ovary, and their health and numbers determine both reproductive potential and reproductive life span (Fig. 9.1). The multiple cellular components of follicles interact in a highly integrated manner to secrete sex steroids and protein hormones, and control oocyte development. The follicle-derived hormones program secretion of gonadotropins that promote follicular development and entrain an ovulatory surge of luteinizing hormone (LH) when follicular maturation is completed, as well as prepare and sustain the reproductive tract for conception, implantation, and early pregnancy. The oocyte is a large and rare cell that is both totipotent and highly specialized in that it is capable of undergoing meiosis and fertilization, with the resulting formation of a new human being. Remarkably, some of these attributes can be suspended for years, only becoming operative when a lengthy process of maturation has been completed. Although many follicles initiate development, only a few (some 400 to 500 follicles or less than 1% of the endowment) make the complete journey to ovulation. The factors that govern the seemingly profligate expenditure of follicles during ovarian development and reproductive life remain largely unknown.

Primordial Germ Cells

The primordial germ cell lineage is established early in development, originating in the proximal region of the epiblast, close to the extraembryonic endoderm. A small number of cells emerge under the influence of inductive signals delivered by members of the transforming growth factor-β (TGF-β) superfamily, including bone morphogenetic protein (BMP)-2, BMP-4, and BMP-8B (see Chapter 7).[1-5] Studies conducted using genetically manipulated mice demonstrated that loss of any of these signals prevents the appearance of all or most of the primordial germ cells.

Primordial germ cell precursors must express SMAD1, SMAD5, and SMAD8, which are phosphoprotein downstream mediators of BMP signaling. Absence of these SMAD proteins results in a marked reduction in the founder cells of the germ cell lineage.[6,7] The *Bmp–Smad* gene dosage is critical, and distortion of the ratios impairs germ cell development. Mice homozygous for null mutations of the *Bmp2*, *Bmp4*, *Prdm1* (previously known as *Blimp1*), and *Prdm14* genes do not generate primordial germ cells.[1,8-10]

Primordial germ cells are identifiable as early as the end of the third week of human gestation by their large size and clear cytoplasm, which contains fewer organelles than the endoderm cells.[11,12] In mice, the expression of the interferon-induced transmembrane proteins *Ifitm1*, *Ifitm3* (previously known as *Fragilis*), and *Prdm1* signals their emergence, with the expression of *Prdm14* and *Dppa3* (previously known as *Stella*), marking the founder primordial germ cells.[8,9,13,14] PRDM1 coordinates the repression of genes normally expressed in somatic cells, and both PRDM1 and PRDM14 have roles in maintaining primordial germ cell pluripotency.[10,15] In addition to these protein regulators, microRNAs, which are short RNA sequences that regulate gene expression by transcriptional and post-transcriptional gene silencing, also control primordial germ cell specification. LIN28, a protein that represses the *Let7* microRNA processing pathway, is expressed in founder primordial germ cells, and prevents *Let7*-mediated destruction of *Prdm1*.[16] Other markers of primordial germ cells include tissue-nonspecific alkaline phosphatase[17] and POU5F1 (previously known as OCT4), a transcription factor present in embryonic stem cells and primordial germ cells.[18,19]

Once specified, primordial germ cells enter a period of migration and proliferation (Fig. 9.2). In the human, they migrate from the yolk sac epithelium to the hindgut by approximately 4 weeks post-fertilization, and then migrate through the dorsal mesentery, finally reaching the genital ridge by approximately 6 weeks post-fertilization. The cells change during this migration from a "resting" morphology, taking on an irregular shape, with protrusions and pseudopodia required for active amoeboid movement.[20,21]

Primordial germ cell proliferation is characterized by incomplete cytokinesis, resulting in clusters of cells known as "oocyte nests" that form a network as a result of cytoplasmic continuity via intracellular bridges.[22-25] Primordial germ cells that migrate to aberrant locations undergo apoptosis, so migration along the proper route is essential for successful proliferation.

Genes involved in primordial germ cell proliferation in mice include *Tiar1*, which encodes the RNA-binding protein TIAR23, and *Fancl* (Fanconi anemia, complementation group L), the gene responsible for the mouse "germ cell–deficient" mutation.[26,27] *Fancl* encodes a subunit of the Fanconi anemia nuclear protein complex that functions in DNA damage repair.[28] In addition, leukemia inhibitory

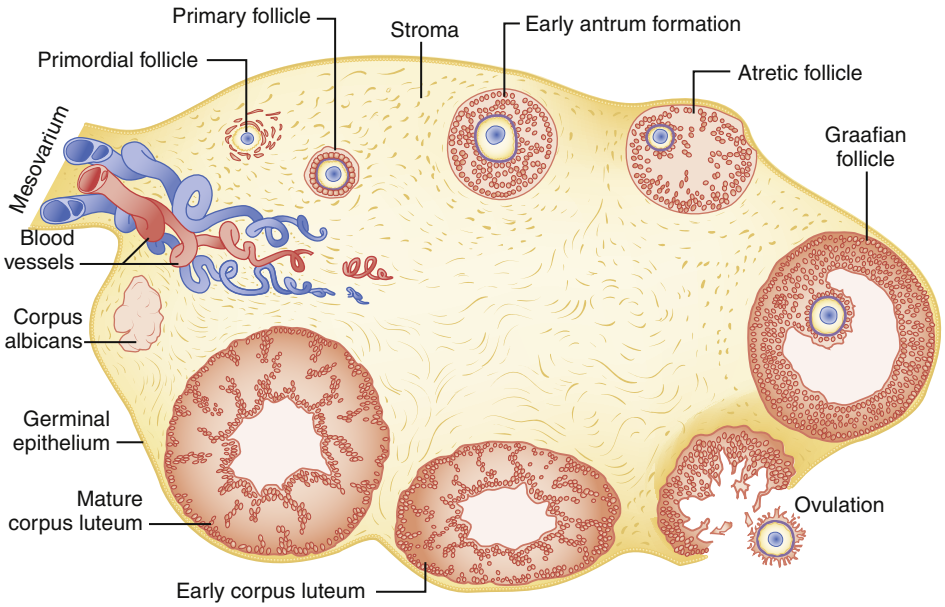


FIGURE 9.1 The follicular cycle of the human ovary. *(Modified from Ham AW, Leeson TS.* Histology, *4th ed. Philadelphia,* JB Lippincott, *1961.)*

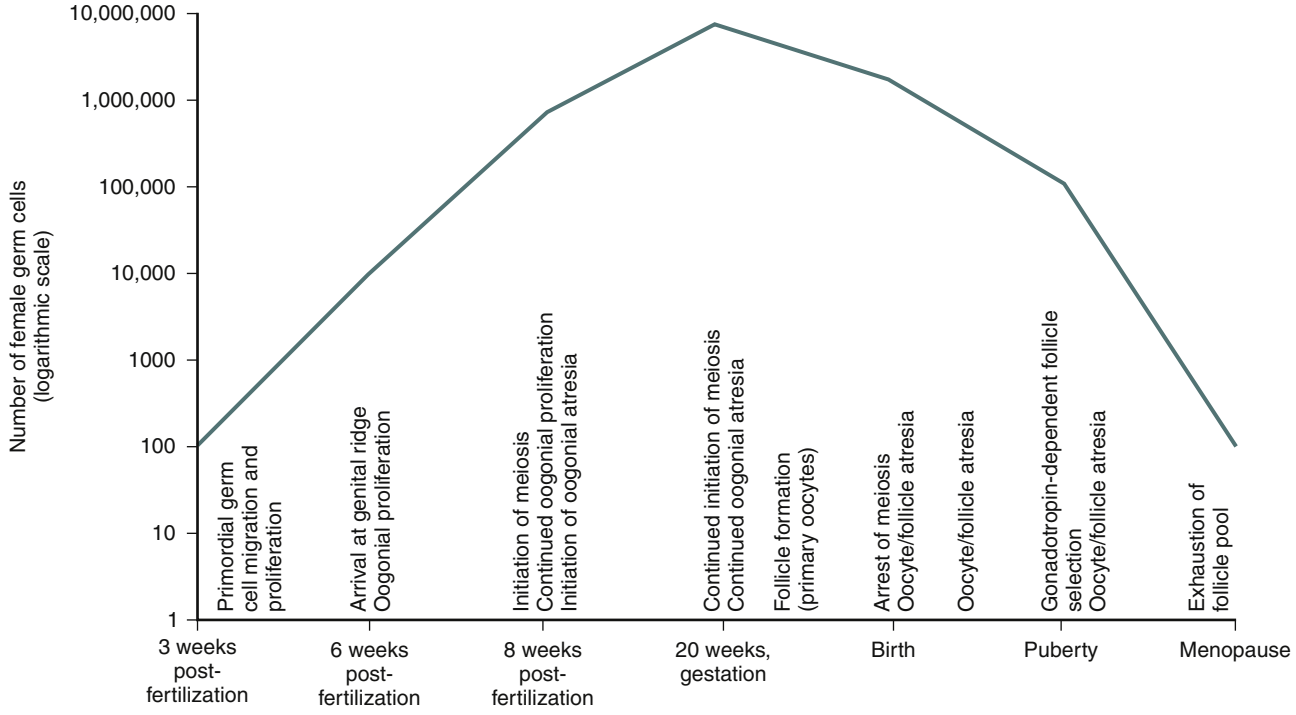


FIGURE 9.2 Changes in oocyte numbers during fetal and postnatal life. The total number of oocytes is a reflection of the balance between active proliferation and oocyte/follicle atresia.

factor (LIF) and related cytokines appear to be responsible, in part, for primordial germ cell proliferation.[29]

The transcription factor LHX1 maintains proper positioning of primordial germ cells in the hindgut.[30] The two interferon-induced transmembrane proteins expressed in primordial germ cells cause repulsive (IFITM1) and homing (IFITM3) activities involved in the migration process.[14,31] The cell adhesion protein integrin β1 must be expressed on the primordial germ cell surface for successful migration to the genital ridge.[32]

Two ligand/receptor signaling pathways, Kit ligand/Kit receptor tyrosine kinase and Cxcl12/Cxcr4, are required for primordial germ cell migration.[33-36] *Wnt5a*, which encodes a secreted morphogen, also provides a migration cue for primordial germ cells.[37] The genital ridge is absent or does not develop normally in mice homozygous for null mutations in the transcription factors *Wt1*, *Nr5a1*, *Lhx1*, *Lhx9*, and *Emx2* (Table 9.1).[38] In addition, nullizygous mice have other abnormalities because some of these genes regulate renal or adrenal development.

Table 9.1 Genes Involved in the Development of the Ovary

Gene Name	Other Aliases	Protein Type	Role
Wt1		Transcription factor	Genital ridge formation
Nr5a1	Sf1	Transcription factor	Genital ridge formation
Lhx1	Lim1	Transcription factor	Genital ridge formation
Lhx9		Transcription factor	Genital ridge formation
Emx2		Transcription factor	Genital ridge formation
Bmp2		Extracellular growth factor	Primordial germ cell specification
Bmp4		Extracellular growth factor	Primordial germ cell specification
Bmp8B		Extracellular growth factor	Primordial germ cell specification
Smad1		Transcription regulator	Primordial germ cell specification
Smad5		Transcription regulator	Primordial germ cell specification
Prdm1	Blimp1	Transcription repressor	Primordial germ cell development
Prdm14		Transcription regulator, epigenetic reprogramming	Primordial germ cell development
Cxcl12		Chemokine ligand	Primordial germ cell migration
Cxcr4		Chemokine receptor	Primordial germ cell migration
Ifitm1	Fragilis2	Transmembrane protein	Primordial germ cell migration
Ifitm3	Fragilis	Transmembrane protein	Primordial germ cell migration
Dazl	Dazla	Translation regulator	Primordial germ cell development
Tial1	Tiar	RNA-binding protein	Primordial germ cell development
Nanog		Transcription factor	Primordial germ cell proliferation, survival
Dnd1	Ter	RNA-binding protein	Primordial germ cell proliferation, survival, affect miRNA pathway
Apobec3		RNA-binding protein	Primordial germ cell proliferation, survival, affect miRNA pathway
Fancl	Pog	Ubiquitin ligase involved in DNA damage repair	Primordial germ cell proliferation
Itgb1		Cell adhesion	Primordial germ cell migration
Kitl	Steel, SCF	Transmembrane growth factor	Primordial germ cell migration, follicle formation and growth
Kit	W, c-kit	Tyrosine kinase receptor	Primordial germ cell migration, follicle formation and growth
Figla		Transcription factor	Primordial follicle formation, follicle growth
Foxo3		Transcription factor	Follicle growth
Nobox		Transcription factor	Follicle growth
Gdf9		Growth factor	Follicle growth
Bmp15		Growth factor	Follicle growth
Zfx		Transcription factor	Oocyte proliferation/survival
Atm		Cell cycle kinase	Meiosis
Wnt4		Secreted ligand	Oocyte-somatic cell contacts, meiosis entry, steroidogenic enzyme expression

The microRNA cluster, *Mir290-295*, is highly expressed in primordial germ cells, and female mice null for *Mir290-295* have premature ovarian failure due to primordial germ cell migration defects.[39,40] The downstream targets of these microRNAs have not been identified, and could include some of the protein regulators mentioned above.

Ovarian Morphogenesis

Germ cells play an indispensable role in the induction of gonadal development. In the absence of two functional X chromosomes, as in Turner syndrome (45, X, or other X chromosome structural abnormalities), primordial germ cells can proliferate and migrate to reach the developing gonad, but the germ cells fail to survive because pregranulosa cells are not maintained. As a result, streak gonads containing only stromal cells are present by the time of birth in classical cases, although some germ cells remain in individuals who are mosaics.[41]

Germ cells that arrive at the genital ridge are referred to as *oogonia*. By 6 to 7 weeks of intrauterine life, the oogonia population has expanded by mitosis to reach some 10,000 cells, and it reaches approximately 600,000 cells by 8 weeks of intrauterine life (Fig. 9.2). However, from this juncture, the oogonial endowment is influenced by three concurrent processes: mitosis, meiosis, and oogonial atresia. As a result of the combined impact of these processes, the number of germ cells peaks at 6 to 7 million by 20 weeks of gestation, and then declines.

Between weeks 8 and 13 of fetal life, some of the oogonia enter prophase of the first meiotic division. Entry into meiosis is triggered at least in part by retinoic acid that is produced locally in the fetal ovary and also by the mesonephros.[42] If correctly executed, meiosis appears to provide temporary protection from oogonial atresia. Oogonia that persist beyond the seventh month of gestation without entering meiosis undergo programmed cell death.[43]

Primordial follicle formation begins at around 20 weeks of gestation, and is accompanied by oocyte nest breakdown as the intercellular bridges are lost and pre-granulosa cells surround the primary oocytes. At midgestation, when the ovarian germ cell endowment is at its apex, two thirds of the total germ cells are intrameiotic primary oocytes; the remaining one third are oogonia. One reason for the subsequent decline in germ cell numbers is the declining rate of oogonial mitosis, a process that ends by approximately 7 months of intrauterine life. The reduction in germ cells is also the result of an increasing rate of oogonial atresia, which peaks at about month 5 of gestation, followed by follicular atresia, which begins around the sixth month of gestation.[44] Apoptosis is believed to be triggered by either a deficiency in survival factors, such as Kit ligand, LIF, or basic fibroblast

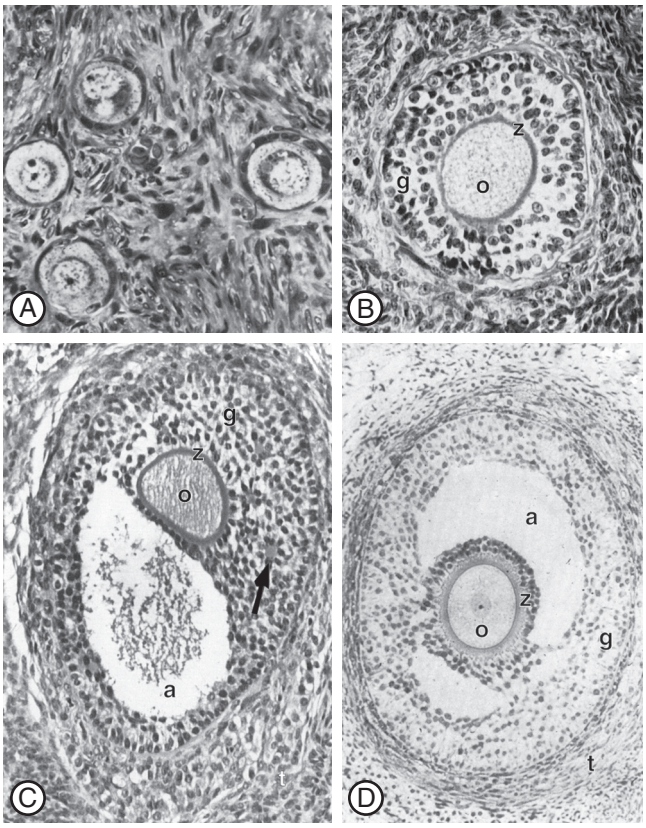


FIGURE 9.3 Ovarian follicles. **A,** Primordial follicles. Primary oocytes are surrounded by a single layer of flattened granulosa cells. **B,** Secondary follicle. The oocyte is surrounded by the early zona pellucida and several layers of cuboidal granulosa cells. **C,** Antral follicle. The oocyte is surrounded by a fully formed zona pellucida and many layers of granulosa cells. A Call-Exner body *(arrow)* is seen in the granulosa cell layer. Follicular fluid has accumulated, forming an antrum, and the theca interna layer is visible. **D,** Graafian follicle. The oocyte is surrounded by a fully formed zona pellucida and several layers of cumulus cells. Follicular fluid has accumulated, forming a large antrum. The mural granulosa cells and the theca interna layer are well defined. a, antrum; c, cumulus cells; g, granulosa cells; o, oocyte; t, theca interna; z, zona pellucida. ***(A** originally published in Baca M, Zamboni L. The fine structure of the human follicular oocyte.* J Ultrastruct Res *19:354, 1967; **B** to **D** adapted from Kurman RJ.* Blaustein's Pathology of the Female Genital Tract, *3rd ed. New York,* Springer-Verlag,*1989.)*

growth factor, or by death-inducing factors, such as Fas ligand, TGF-β, and activin.

Primordial follicles in the human ovary (30 to 60 μm in diameter) are composed of a late diplotene primary oocyte (9 to 25 μm in diameter) surrounded by a single layer of flattened granulosa cells (Fig. 9.3).[45] Follicles at this stage of development are not believed to be influenced by gonadotropins. Primary follicles (>60 μm in diameter) are characterized by a primary oocyte surrounded by a single layer of cuboidal granulosa cells. Secondary follicles (<120 μm) consist of a primary oocyte surrounded by several layers of cuboidal granulosa cells (<600 cells), as shown in Figure 9.3.

Inactivating mutations in the oocyte-specific transcription factor, folliculogenesis specific basic helix-loop-helix *(Figla)*, prevent the formation of primordial follicles. Mice homozygous for a null mutation in the X-linked zinc finger gene, *Zfx*, have a reduced number of oocytes, resulting in a diminished reproductive life span. Mutations in the *Atm* gene, the murine homologue of the human ataxia-telangiectasia gene, disrupt gametogenesis at the leptotene stage of meiosis, and consequently result in sterility. Deletion of the *Wnt4* gene alters the expression of steroidogenic enzymes in the ovary, resulting in increased testosterone synthesis and wolffian duct masculinization, and also affects germ cell entry into meiosis.[46,47] *WNT4* mutations are also associated with müllerian defects (Mayer-Rokitansky-Kuster-Hauser Syndrome) and hyperandrogenism in women. A more extensive list of mouse genes involved in ovary development and follicle formation is provided in Table 9.1.

Relentless and irreversible attrition from midgestation onward progressively diminishes the gonadal germ cell complement, leaving approximately 700,000 primordial follicles in the ovaries at birth.[48] This number decreases further to approximately 300,000 by the onset of puberty; of these follicles, only 400 to 500 will ovulate in the course of a reproductive life span (Fig. 9.2). Although there is good evidence that embryonic stem cells can give rise to oocyte-like cells and follicle-like structures in culture, the notion that germ cells and follicles can arise *in vivo* in the mammalian ovary during postnatal life from an oogonial stem cell population and contribute to the ovarian reserve remains controversial.[49,50]

Compared with the fetal testis, the human fetal ovary is generally believed to be steroidogenically quiescent, although cholesterol side-chain cleavage activity and 17α-hydroxylase/17, 20-desmolase activities are detectable. Even though follicles are present in the fetal and infant ovary, their steroidogenic capacity only becomes evident at puberty.

Epigenetic Programming and Germ Cell Development

Dramatic alterations in epigenetic programming of primordial germ cells are initiated soon after their specification, and continue after entry into the developing gonad. Nearly complete DNA demethylation occurs across the entire genome, including the inactive X-chromosome, which allows transcriptional activity to resume from the X-chromosome.[51] This global demethylation process is in part passive, i.e., simply a consequence of DNA replication in the absence of DNA methyltransferase activity, so maintenance methylation cannot be accomplished. An enzyme-mediated active demethylation process also occurs in combination with activation of the DNA base excision repair pathway, which is required to resolve the proper DNA sequences.[52] Post-translational modifications to DNA-associated histone proteins are either transiently or permanently lost, due to a combination of histone replacement by nonmodified histone proteins and repression of histone modifying enzymes such as histone methyltransferases.[53] These modifications are temporally associated with pronounced alterations in nuclear architecture, including chromatin decondensation and an increase in nuclear size.[54] Together, these epigenetic changes serve to almost fully erase the inherited somatic differentiation program, resetting the cells to a "naive" epigenetic state.

A critical step in mammalian germ cell development, the modification of genomic imprinting, occurs with these

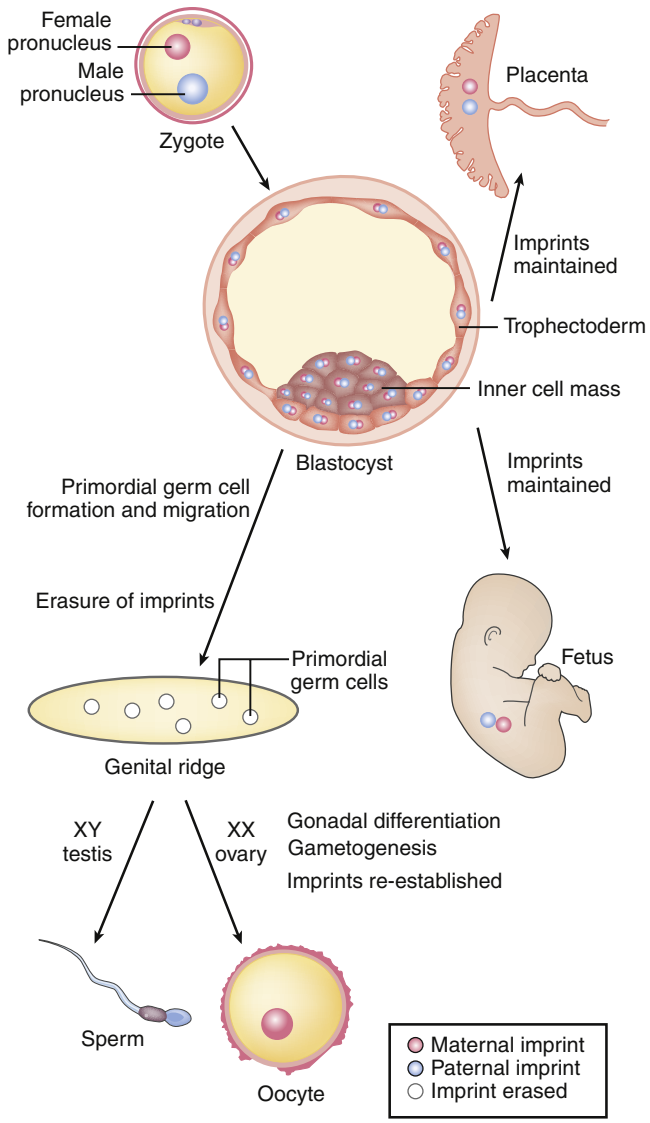


FIGURE 9.4 Erasure and reestablishment of genetic imprints during development. *(Adapted from Surani MA. Reprogramming of genome function through epigenetic inheritance.* Nature *414:122–128, 2001.)*

epigenetic reprogramming events. In mammals, there is an absolute requirement for the inheritance of a chromosomal complement from both the mother and the father.[55-57] The basis for this requirement is that specific genes must be expressed from *one* allele, not both, for successful development.[58-60] This task is accomplished by a tightly orchestrated set of modifications of the maternal and paternal chromatin that include methylation of CpG sequences of specific "imprinted" genes, depending on the parent of origin; the result is differential transcriptional regulation.[60-67] Complete erasure of these imprints occurs during the primordial germ cell epigenetic reprogramming process and is required in preparation for switching the inherited maternal and paternal imprinted patterns to reflect the sex of the progeny.[64] In the case of oogonia, both maternal and paternal imprints are erased, and maternal imprints are later established during oogenesis in the fetal ovary and during oocyte growth (Fig. 9.4).[63,66,67]

The Follicle and Its Surroundings

The Oocyte

Oocyte-Specific Genes

The growing oocyte expresses a number of genes that are essential for successful follicular development, fertilization, and preimplantation development. Mouse knockout studies have identified several oocyte–specific transcription factors essential for folliculogenesis. *Figla*, *Sohlh1* (spermatogenesis- and oogenesis-specific basic helix-loop-helix 1), and *Lxh8* (LIM homeobox 8) appear to be required for the formation of primordial follicles from naked primordial oocytes, whereas *Nobox* (newborn ovary homeobox) is involved in the transition of primordial to primary follicles. *Dazl* (deleted in azoospermia-like), *Cpeb1* (cytoplasmic polyadenylation element binding protein 1), and *Ybx2* (previously known as *Msy2*) are DNA or RNA-binding proteins involved in regulating mRNA translation within the oocyte.[68-70]

Oocyte growth is accompanied by formation of the *zona pellucida*, an extracellular matrix surrounding the oocyte.[68-72] The zona pellucida protects the developing germ cell in the follicle, the ovulated egg in the oviduct, and the cleavage-stage embryo. It also serves as the initial site of contact with sperm, and after fertilization, it becomes a barrier that discourages polyspermy. Three genes have been characterized that encode ZP1 (ZPA), ZP2 (ZPB), and ZP3 (ZPC), which are the main sulphated glycoproteins of the zona. A polymer of ZP2 and ZP3 proteins forms filaments that are interconnected by ZP1, a minor component of the zona pellucida. FIGLA regulates the coordinated expression of these genes during the oocyte growth phase. Humans and rats have a fourth ZP-1 like subunit (ZP4), which is not expressed in mice.[73]

In the mouse, ZP3 was believed for many years to be the primary sperm receptor, with ZP2 being a secondary receptor. However, recent compelling evidence indicates that this "ligand-receptor" model of sperm–ZP3 interaction may not be correct, and suggests that, instead, sperm specifically recognize and bind, not to ZP3 alone, but to the three-dimensional matrix formed by all three zona proteins.[74,75] Indeed, in the human, it is evident that components beyond ZP3 are required for sperm binding to the zona. After fertilization, a cortical granule metalloprotease named ovastacin is released from the egg and cleaves ZP2, which prevents additional sperm from binding the ZP and results in the ZP to block polyspermy.[76]

Mice lacking ZP1 form structurally abnormal zonae and have reduced fecundity. In mice lacking ZP2, a thin zona forms that is not retained in preovulatory follicles. The antral-stage follicle number is substantially reduced, few eggs are ovulated, and no two-cell–stage embryos can be found in mated animals. Moreover, blastocysts derived from *in vitro* fertilized oocytes from females lacking ZP2 do not undergo normal development. Mice lacking ZP3 form no zona pellucida despite the expression of the other zona proteins. Few eggs are ovulated, and the females are sterile. As in the ZP2 mutant, *in vitro* fertilized eggs from ZP3-deficient mice do not develop beyond the blastocyst stage.

Several oocyte-specific "maternal effect" genes have been identified in mouse models that are expressed in

the oocyte, but are only required during preimplantation embryo development. Four maternal effect genes encode proteins that form a "subcortical maternal complex" in oocytes, including *Nlrp5* (NACHT, leucine-rich repeat and PYD-containing 5; also known as *MATER*), *Filia*, *Ooep* (oocyte-expressed protein; also known as *Floped*), and *Tle6* (transducin-like enhancer of split 6).[77,78] Although *Nlrp5* and *Filia* transcripts are degraded during meiotic maturation, the proteins remain until the blastocyst stage. NLRP5 protein is required for development of embryos beyond the two-cell stage, possibly because it regulates mitochondrial function.[79,80] FILIA is required in oocytes and early embryos to regulate spindle function; embryos of *Filia*-null female mice develop poorly because of a high incidence of aneuploidy.[81] *Ooep*-null females have oocytes that lack cytoplasmic lattices, structures that store maternally contributed components required for embryo development. [82] *Nlrp2*, a gene related to *Nlrp5*, is expressed in both oocytes and granulosa cells during folliculogenesis; the maternal protein is required in early embryos for successful development.[83]

Zar1 (zygote arrest 1) encodes a cytoplasmic protein required for the transition from fertilized egg to cleaving embryo by an unknown mechanism.[84] *Gclm* (glutamate-cysteine ligase, modifier subunit) encodes a protein that regulates synthesis of glutathione, which is critical for controlling cellular redox status. Mouse embryos deficient in maternal GCLM have deficits in their ability to develop to the blastocyst stage.[85] *Npm2* (nucleoplasmin 2) encodes a nuclear protein generated before oocyte maturation that affects heterochromatin organization and histone deacetylation. In the absence of *NPM2*, ovulation and fertilization occur normally, but there is a complete failure of preimplantation embryo development. *Dppa3*, in addition to its role in primordial germ cell specification, is a maternal effect gene required for normal preimplantation embryo development.

Oocyte-Derived Factors and the Control of Follicular Growth and Maturation

The concept that the oocyte plays an important role in follicular function and is far more than a passive inhabitant of the follicle emerged from two observations: (1) that follicular survival depends on the presence of viable germ cells and (2) that removal of the oocyte from an antral follicle is followed by luteinization, indicating that the oocyte produces factors that restrain the terminal differentiation of granulosa cells.[86,87] Additional support for this concept was derived from experiments in which midsized oocytes from mouse secondary follicles were transferred by a grafting procedure into primordial follicles. This transfer resulted in a doubling of the rate of development of the primordial follicular cells in the presence of the secondary follicle oocyte.[88]

The effects of the oocyte on follicular growth are mediated in part by closely related members of the TGF-β superfamily that are selectively or specifically produced by the oocyte. These factors, growth differentiation factor (GDF)-9 and BMP-15, influence granulosa and theca cell function (Fig. 9.5) (see Chapter 7). The importance of GDF-9 and BMP-15 has been established through genetic manipulation of mice and the discovery of spontaneous

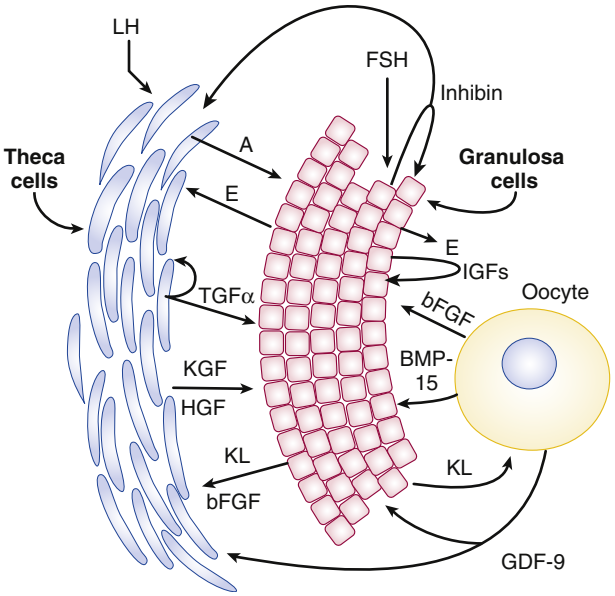


FIGURE 9.5 Paracrine and autocrine interactions between theca cells, granulosa cells, and the oocyte. A, androstenedione; BMP, bone morphogenetic protein; E, estradiol; FGF, fibroblast growth factor; FSH, follicle-stimulating hormone; GDF, growth differentiation factor; HGF, hepatocyte growth factor; KGF, keratinocyte-derived growth factor; KL, kit ligand; LH, luteinizing hormone; TGF, transforming growth factor; IGF, insulin-like growth factors. *(Modified from Nillson E, Skinner MK. Cellular interactions that control primordial follicle development and folliculogenesis.* J Soc Gynecol Investig *8[Suppl 1]:S17–S20, 2001.)*

mutations in the genes encoding GDF-9 and BMP-15, which affects follicular dynamics in sheep.[70,89-91]

Growth Differentiation Factor-9

Growth differentiation factor-9, encoded by a gene on chromosome 5q31.1, is highly expressed by oocytes and, to a lesser extent, by primate granulosa cells. The follicles of GDF-9–deficient mice arrest in growth at the primary stage, yet the oocytes continue to grow at a faster rate than wild-type oocytes, progressing to advanced stages of differentiation seen in the antral follicles of normal mice. However, there are ultrastructural abnormalities in the interconnections between granulosa cells and oocytes; the oocytes ultimately die, leaving a ribbon of zona pellucida behind. The theca also does not form around the follicles, implicating GDF-9 in the organization or proliferation of this follicular component. Studies in the rat also indicate that GDF-9 stimulates the growth of primary follicles, consistent with the block to progression at the primary stage in GDF-9–deficient mice.[70,92]

GDF-9 has a variety of actions on granulosa cells and theca cells that are species-specific, acting at least in part through interaction with the ALK5 (TGF-βRI) and BMP receptor type 2 (BMPRII) receptor complex.[93] In rodents, GDF-9 stimulates granulosa cell differentiation, including induction of LH receptors and steroidogenesis. In cumulus cells, GDF-9 promotes expression of hyaluronan synthase 2, pentraxin 3 and tumor necrosis factor inducible gene 6 (TSG-6), the latter being proteins that are incorporated into the proteoglycan extracellular matrix of the cumulus

oophorus complex. It also suppresses urokinase expression while stimulating COX-2 and prostaglandin synthesis and progesterone formation.[70,94,95] LH receptor expression is suppressed by GDF-9, which would discourage luteinization of the cumulus cells. These actions of GDF-9 promote unique phenotypes in the granulosa cells surrounding the oocyte, which are exposed to the highest GDF-9 concentrations. GDF-9 inhibits human theca cell steroidogenesis *in vitro*.[96] It also stimulates theca cell proliferation, a finding consonant with the apparent role of GDF-9 in the murine ovary in controlling thecal development.

Bone Morphogenetic Protein-15

Bone morphogenetic protein-15, also known as GDF-9b, encoded by a gene on the X chromosome, is another member of the TGF-β superfamily produced by oocytes.[97] It is related structurally to GDF-9 and shares a similar pattern of expression. Targeted deletion of the murine *Bmp15* gene causes a modest ovarian phenotype in nullizygous animals of subfertility with diminished ovulation and fertilization rates. However, mice nullizygous for *Bmp15* and heterozygous for a *Gdf9* mutation have severely impaired fertility, with abnormalities in folliculogenesis and cumulus cell function. Spontaneous point mutations in the ovine *Bmp15* gene (e.g., Inverdale and Hanna sheep) result in phenotypes that differ from those in *Bmp15* knockout mice.[70] In the heterozygous state, the number of follicles ovulating is increased, and thus there is an increase in fecundity. However, primary ovarian failure, with a phenotype resembling the murine *Gdf9* knockout, is observed in ewes homozygous for the mutations. *In vitro*, BMP-15 stimulates granulosa cell mitosis. Thus, its absence *in vivo* would be predicted to impair follicular growth, which is consistent with the ovarian abnormalities in homozygous mutant sheep.

BMP-15 binds to a receptor complex consisting of BMPR1B (activin receptor-like kinase 6, or ALK6) and BMPRII. A point mutation in BMPR1B in Booroola sheep is associated with an additive increase in ovulation rate, based on the copy number of mutant alleles. Targeted deletion of the *Bmpr2* gene in mice does not affect follicular development, but it does yield an infertility phenotype as a result of defects in cumulus cell expansion that prevent *in vivo* fertilization.

Both BMP-15 and kit ligand participate in a negative feedback loop: BMP-15 stimulates kit ligand expression by granulosa cells, whereas kit ligand inhibits BMP-15 expression in oocytes. In the presence of an oocyte, both BMP-15 and kit ligand stimulate granulosa cell mitosis. The observations that only the oocyte expresses kit, the kit ligand receptor, and that kit ligand suppresses expression of BMP-15, a granulosa cell mitogen, suggest that the oocyte must be involved in producing another granulosa cell mitogen.

GDF-9 and BMP-15 are both synthesized as proproteins that form dimers and are then proteolytically processed to yield the bioactive molecules. There is evidence that GDF-9 and BMP-15 can form heterodimers and that processing of the heterodimers is impaired.[98] Notably, the Inverdale mutation that inactivates BMP-15 dramatically impairs the proteolytic processing of both the mutant BMP-15 and wild-type GDF-9 in coexpressing cells. This observation suggests that the phenotype of the Inverdale sheep may be the result, at least in part, of GDF-9 deficiency due to interference by mutant BMP-15 with wild-type GDF-9 processing. Similarly, cells coexpressing mutant human forms of BMP-15 and GDF-9 associated with premature ovarian failure have decreased production of the mature proteins, likely because of impaired post-translational processing.[99]

Granulosa Cells

The granulosa cells are believed to originate from ovarian surface epithelium.[100] The cohort of granulosa cells that surrounds each oocyte has an oligoclonal origin, with three to five parent cells giving rise to the full complement of granulosa cells in a mature follicle.[101] Granulosa cells receive no direct blood supply, and because a basal lamina separates them from the vascularized theca interna, there is a relative blood–follicle barrier that restricts the entry of leukocytes and high–molecular-weight substances (such as low-density lipoproteins). The absence of a blood supply also necessitates intimate intercellular contact between neighboring granulosa cells and the oocyte.

Intercellular Communication Between Granulosa Cells and the Oocyte

Granulosa cells are interconnected by an extensive network of gap junctions, which are important for metabolic exchange and transport of small molecules between neighboring cells. The number of gap junctions per granulosa cell increases as follicles develop, coupling them into a functional syncytium. Moreover, granulosa cells extend cytoplasmic processes through the zona pellucida to form gap junctions with the plasma membrane of the oocyte. Cyclic GMP (cGMP), produced by granulosa cells, is one of the factors passed into the oocyte via gap junctions to maintain oocytes in a state of maturational arrest.

Gap junctions are composed of hexameric arrays of proteins called *connexins*. Connexin-37 and connexin-43 are two of the important follicular connexins, and they form gap junctions with different permeability properties. Connexin-37 is reported to be the predominant connexin in the oocyte, whereas connexin-43 predominates in the granulosa cells. However, at least the first layer of granulosa cells surrounding the oocyte also expresses connexin-37. Communication between granulosa cells and the oocyte occurs through homotypic connexin-37 gap junctions, whereas gap junctional communication between granulosa cells is via homotypic connexin-43 complexes.[102] Follicle stimulating hormone (FSH) induces connexin-43 expression in granulosa cells, whereas the ovulatory surge of LH suppresses connexin-43 mRNA expression and causes mitogen-activated kinase-mediated phosphorylation of connexin-43 protein that results in gap junction closure.

The importance of connexins to follicular function was shown in the ovarian phenotype of connexin-37 and connexin-43 knockout mice.[103,104] In connexin-37–deficient mice, created by targeting the *Gja4* gene, follicle growth is arrested at the preantral stage; oocyte growth, although it commences, is also subsequently arrested before meiotic competence is achieved, resulting in loss of oocytes and formation of luteinized structures. Connexin-43–deficient

mice, created by targeting the *Gja1* gene, have an ovarian phenotype characterized by diminished germ cell number and impaired growth of follicles beyond the primary stage.

Granulosa Cells and the Convergence of Multiple Signaling Pathways

Granulosa cells express receptors for and respond to a large number of factors that are either generated locally within the follicle or that enter the follicular compartment from the blood. These include oocyte-derived factors, autocrine factors produced by the granulosa cells themselves, products of theca cells, and circulating factors. The repertoire of signaling molecules that have been shown to affect primate as well as other animal granulosa cells *in vitro* and *in vivo* beyond FSH and LH is extensive, encompassing hypothalamic factors (e.g., gonadotropin releasing hormone [GnRH]), other pituitary hormones (e.g., growth hormone and prolactin), a large array of growth factors (e.g., epidermal growth factor [EGF] family members, TGFβ family members, and insulin-like growth factors), hormones controlling metabolism (e.g., insulin) and vascular tone (e.g., endothelins), cytokines (e.g., TNF-α) and adipokines (e.g., adiponectin). Some of these factors have a critical role as documented from the phenotypes associated with mutations in humans or spontaneous or induced gene mutations in animals as previously described for GDF-9 and BMP-15. However, functional genomics has not revealed a critical physiological or pathophysiological role for many of these factors in granulosa cells and follicles.

Granulosa Cell Endocrine Activity

The primary steroid hormone produced by the preovulatory granulosa cells is estradiol. The synthesis of this hormone requires a collaborative relationship with adjacent theca cells, which produce the immediate precursors for the aromatization reaction. The control of this process is under the direction of LH, acting on thecal elements, and FSH, acting on the granulosa compartment through multiple signaling pathways including protein kinase A and Akt (Fig. 9.6). The two cell–two gonadotropin model is a formidable example of the integrated function of the different cellular components of the follicle.

Studies of isolated granulosa cells have shown that FSH, but not LH, stimulates estrogen production when the cells are provided with an aromatizable substrate.[105] In contrast, isolated human theca cells do not produce substantial amounts of estrogens, but instead secrete dehydroepiandrosterone, androstenedione, and smaller amounts of testosterone when adenylate cyclase activity is stimulated. The aromatase activity of granulosa cells is estimated to be at least 700 times greater in the granulosa cells of large preovulatory follicles than in theca cells, arguing strongly for the cellular compartmentalization of estrogen synthesis outlined in the two-cell–two-gonadotropin model.

Inhibin, a member of the TGF-β protein superfamily, is produced by granulosa cells. It is a heterodimeric 32-kDa glycoprotein composed of two subunits, α (18 kDa) and β (12 kDa), linked by disulfide bonds. There is a single common α subunit, but there are different β subunits, denoted β_A and β_B. The $\alpha\beta_A$ and $\alpha\beta_B$ heterodimers are named inhibin A and B, respectively. In the ovary, the primary source of inhibin is granulosa cells. The main endocrine role for

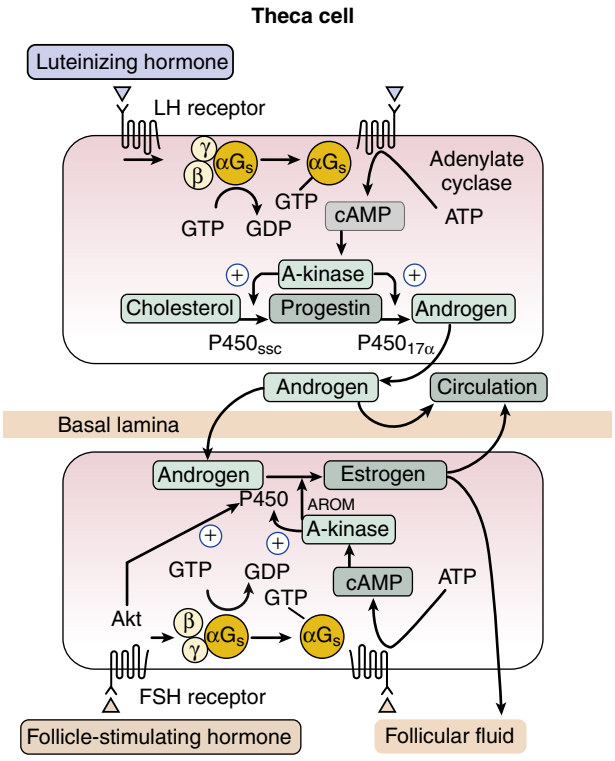


FIGURE 9.6 The two-cell–two-gonadotropin system for estradiol synthesis in the follicle. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are shown to stimulate adenylate cyclase via G-protein–coupled receptors. The cyclic AMP (cAMP) generated from ATP activates protein kinase A to stimulate expression of the respective steroidogenic enzymes in theca and granulosa cells. In addition, in granulosa cells, FSH binding to the FSH receptor leads to activation of AKT, also known as protein kinase B, probably via a phosphatidyl inositol second message, which augments aromatase expression. GDP, guanosine diphosphate; GTP, guanosine triphosphate. *(Modified from Erickson CF, Shimasaki S. The physiology of folliculogenesis: the role of novel growth factors.* Fertil Steril *76:943–949, 2001, with permission.)*

inhibin, for which it was discovered and named, is to suppress pituitary FSH production.[106,107] *In vitro*, inhibin augments LH- and IGF-stimulated androgen production by theca cells, and elevated inhibin production by granulosa cells in polycystic ovary syndrome may contribute to the hyperandrogenemia that is associated with this condition.

Although both isoforms of inhibin seem to have similar biological properties, their synthesis is regulated differently during the follicular and luteal phases. Inhibin B is secreted mainly during the early follicular phase, with levels decreasing in the midfollicular phase and becoming undetectable after the LH surge. Concentrations of inhibin A are low during the first half of the follicular phase, but increase during the midfollicular phase and peak during the luteal phase.

The secretion of inhibin A is regulated by gonadotropins, but production of inhibin B evidently is not. This differential regulation of inhibin A and B production was exemplified in measurements made on follicles of different sizes that showed that inhibin A was present in follicles down to a size of 6 mm, with levels increasing with increased follicular

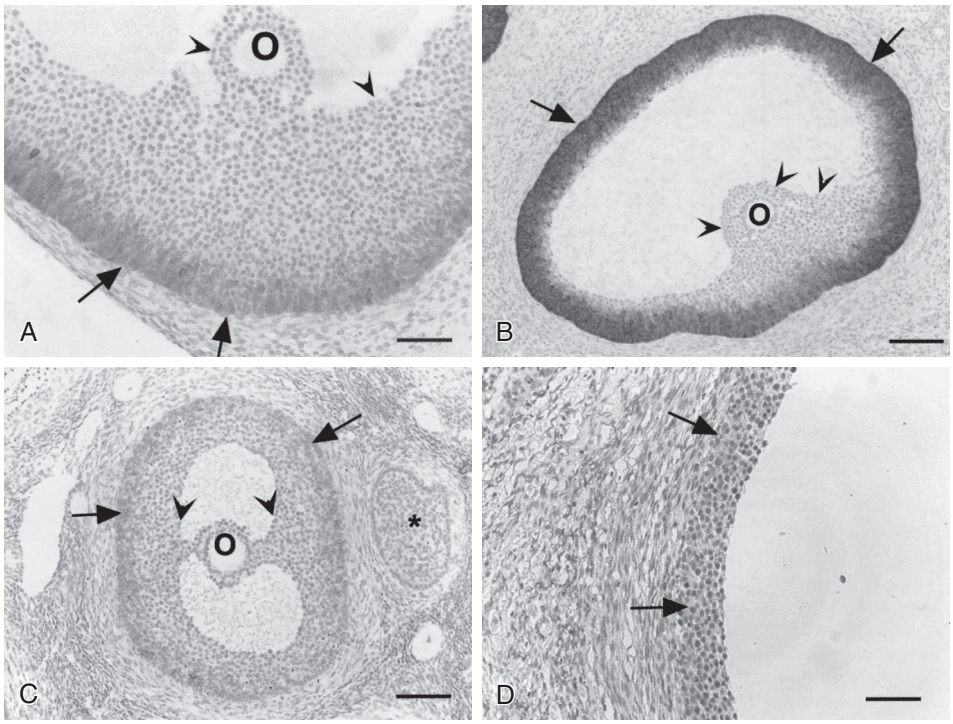


FIGURE 9.7 Heterogeneity of granulosa cell function in the developing follicle. Differential expression of aromatase is shown in a rat ovary (**A** and **B**) and a marmoset ovary (**C** and **D**). *Arrows* point to immunostained granulosa cells; *arrowheads* indicate absent staining in cumulus cells. *, preantral follicle; o, oocyte. Bar = 50 μm in **A, C,** and **D,** and 100 μm in **B**. *(From Turner KJ, Macpherson S, Millar MR, et al. Development and validation of a new monoclonal antibody.* J Endocrinol *172:21–30, 2002, with permission.)*

size. In contrast, inhibin B levels showed no relationship to follicle size or maturation state.

Granulosa Cell Phenotypic Heterogeneity

Granulosa cells display different phenotypes within the follicle, depending on their location.[108-111] The mural granulosa cells, antral granulosa cells, and cumulus granulosa cells each have distinguishing features that are likely determined by their proximity to the oocyte and theca cells and by the paracrine substances that they produce. The mural granulosa cells in the antral follicle express the greatest steroidogenic activity (Fig. 9.7). In addition, mural granulosa cells in the preovulatory follicle have the highest level of LH receptors. The granulosa cells closest to the antral cavity have a lower expression of steroidogenic enzymes, whereas those in the middle region have greater mitotic activity than the antral and mural granulosa cells.

The cumulus cells, which are released with the oocyte at ovulation, do not express aromatase, and their LH receptor content and level of LH responsiveness are substantially lower than those of their mural counterparts. In mice, they have distinguishing gene expression patterns that include expression of *Slc38a3*, which encodes a sodium coupled neutral amino acid transporter, and higher expression of the anti-müllerian hormone gene, *Amh*. These cells proliferate after the LH surge and are active in producing an extracellular matrix consisting of hyaluronan, proteoglycans, and proteoglycan-binding proteins when stimulated by the prostaglandins generated in response to the ovulatory stimulus. The elaboration of this matrix leads to the preovulatory expansion of the cumulus–oocyte complex, which appears to be essential for ovulation (Fig. 9.8). Differential patterns of expression of prostaglandin E (EP) receptors allow granulosa cell subpopulations to respond uniquely to PGE2 during ovulation. Cummulus cells appear to respond via EP2

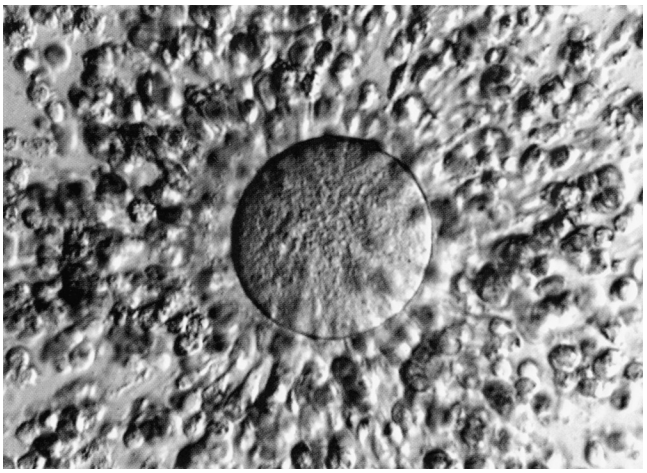


FIGURE 9.8 Appearance of a mature ovulated human oocyte. This metaphase II–arrested oocyte is surrounded by a highly expanded cumulus cell layer. The zona pellucida is visible only as a blurred region immediately around the oocyte (100×). *(Adapted from Veeck LL. An Atlas of Human Gametes and Conceptuses. New York, Parthenon Publishing, 1999.)*

and EP3, whereas rupture of a specific region of the follicle is through EP1 in granulosa cells near the apex. A growing body of literature suggests that cumulus cell viability (e.g., apoptosis) and expression of certain proteins and mRNAs correlates with oocyte competence and success in assisted reproduction.[112]

Luteinized granulosa cells of the ovulated follicle undergo terminal differentiation to give rise to the large luteal cell population of the corpus luteum. These cells have an increased capacity to synthesize progesterone, resulting from up-regulation of the machinery required to acquire cholesterol from the circulating lipoproteins, which now

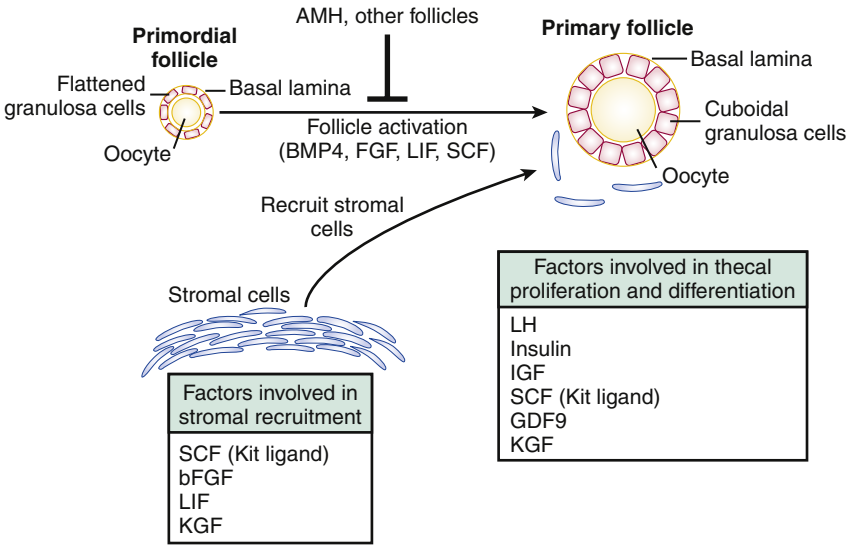


FIGURE 9.9 Recruitment to and differentiation of theca cells in the developing follicle. AMH, anti-müllerian hormone; bFGF, basic fibroblast growth factor; BMP, bone morphogenetic protein; GDF9, growth differentiation factor 9; IGF, insulin-like growth factor; KGF, keratinocyte growth factor; LIF, leukemia inhibitory factor; SCF, stem cell factor also known as Kit ligand. *(Modified from Young and McNeilly Reproduction 140: 489–504, 2010.)*

have access to the granulosa-lutein cells as a result of neovascularization of the developing corpus luteum. The granulosa-lutein cells also retain the capacity to synthesize estrogens from androgen precursors produced by theca lutein cells.

Theca Cells

The theca and interstitial cells are believed to arise from fibroblast-like mesenchymal cells in the stromal compartment. They first appear around follicles that have two or more layers of granulosa cells (Fig. 9.9).[113]

The Kit and Kit ligand system is thought to play a prominent role in early development of the androgen producing cells of the ovary and testis.[114] Production of GDF-9 by the oocyte is required for development of the theca layer. In the absence of GDF-9, theca cells are recruited to surround the follicle but do not undergo proper functional differentiation. Theca cells also express Kit, the receptor for Kit ligand, and researchers have postulated that Kit ligand produced by granulosa cells is important in organizing the theca layer around the developing follicle.

Theca cells engage in a bidirectional dialogue with granulosa cells through the production of keratinocyte-derived growth factor (KGF) and hepatocyte growth factor (HGF). KGF and HGF, like FSH, stimulate granulosa cells to produce Kit ligand, whereas Kit ligand acts on the theca cells to promote expression of KGF and HGF in a positive feedback loop (Fig. 9.5). Kit ligand, KGF, and HGF achieve their highest concentrations in large antral follicles. As the oocyte expresses Kit receptor, this feed-forward loop also affects oocyte function. Moreover, theca-derived insulin-like factor-3 (INSL3) acts on the oocyte to promote maturation.

Theca cells of the ovulated follicle are incorporated into the corpus luteum, where they represent the population of small luteal cells (theca lutein cells), expressing the steroidogenic machinery to produce androgen precursors that are transformed by the larger luteal cells derived from the granulosa cells of the luteinizing follicle, which express aromatase activity.

Hypertrophied theca interna cell remnants of atretic follicles are found in the ovary. These cells are thought to be targets of noradrenergic innervation that control steroidogenic activity. Hilar interstitial cells are large lutein-like cells with structural and functional characteristics indistinguishable from those of differentiated Leydig cells. Like Leydig cells, they contain the hexagonal Reinke crystals. Hilar cells are intimately associated with nonmyelinated sympathetic nerve fibers. The endocrine activity of these cells is assumed to be correlated with their prominence at the time of puberty, during pregnancy, and around the menopause.

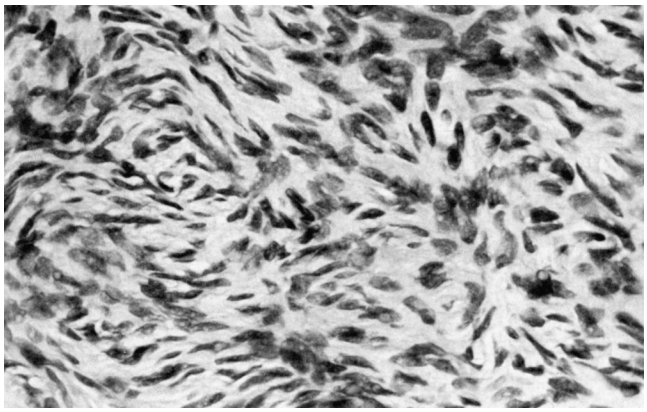


FIGURE 9.10 Ovarian stroma consisting of whorls of fibroblastic cells. *(Reproduced from Kurman RJ. Blaustein's Pathology of the Female Genital Tract, 3rd ed. New York, Springer-Verlag, 1989.)*

Ovarian Stroma

The ovarian stroma contains fibroblastic cells that express some steroidogenic enzymes. The stroma may also harbor precursors of androgen-producing cells (Fig. 9.10).[115] Ovarian stroma cells express androgen receptors, and may proliferate under the influence of androgens, contributing to the increased stromal density characteristic of hyperandrogenemia of ovarian origin (e.g., polycystic ovary syndrome and androgen-producing ovarian tumors).

The stromal cells serve as insulators in the ovary, separating follicles and corpora lutea from adjacent structures

physically, as well as biochemically. Stromal cells produce growth factors and growth factor–binding proteins, the latter perhaps being key to the proposed insulator role. Ovarian stromal cells express substantial levels of gremlin, a protein that binds and inactivates BMPs; follistatin, which binds and inactivates activin; insulin-like growth factor (IGF)-binding proteins, and secreted frizzled-related proteins, which bind members of the WNT signaling family.

Ovarian Surface Epithelium

The ovarian surface epithelium, a flat to cuboidal epithelial layer of mesodermally derived cells, is also known as the *ovarian mesothelium* and by the misnomer of "germinal epithelium" because of the erroneous belief that it gave rise to germ cells.[116,117] During development, the ovarian surface epithelium may be one source of granulosa cells in addition to the rete ovarii, which has a common origin with the surface epithelium. In the adult ovary, the surface epithelium is characterized by expression of the mucin gene, *MUC1*, and the presence of cilia and apical microvilli. The cells sit on a basement membrane that covers a dense connective tissue layer.

The ovarian surface epithelium plays a role in the transport of material to and from the peritoneal cavity and in the repair of surface defects resulting from ovulation. During the ovulatory process, epithelial cells overlying the follicle undergo apoptotic cell death, followed by activation of a subsequent repair process. This process includes cell proliferation, which begins right after ovulation, and the resynthesis of extracellular matrix components. Proinflammatory cytokines may initiate these events.

Invaginations of surface epithelium into the ovary result in the formation of inclusion cysts. The ovarian surface epithelium in these inclusion cysts may undergo metaplasia and neoplasia. Although some researchers have suggested that these inclusion cysts form from invaginations around the site of ovulation, they have been reported to be prominent in polycystic ovary syndrome (PCOS), a condition characterized by oligo-ovulation or anovulation. Others have proposed that cysts containing müllerian ductal epithelium that are not present in normal ovaries are the cause of epithelial cancers of the ovary, and represent a common link to fallopian tube and peritoneal cancers.[118]

Ovarian Leukocytes and Macrophages

Macrophages, lymphocytes, and polymorphonuclear granulocytes are present in the ovary at various stages of its life cycle.[119] These cells have roles in normal ovarian function as well as in ovarian pathology, where for example, lymphocyte infiltration, particularly in the theca interna, is a characteristic feature of autoimmune ovarian failure.

Macrophages are a major cellular component of the interstitium, usually found near the perifollicular capillaries. Few other white blood cells are observed in the ovary in the early phases of follicular development, but a substantial infiltration of white blood cells occurs in the periovulatory period and in association with follicular atresia.[120] Mast cells increase progressively in number during the latter portion of the follicular phase, and their release of histamine may contribute to ovarian hyperemia at ovulation.[121,122]

After ovulation, eosinophils and T lymphocytes migrate into the corpus luteum, recruited by the expression of chemoattractants.[123,124] The infiltration and subsequent activation of these cells occurs before there is evidence of either functional or structural luteal regression. Activated T cells produce lymphokines that attract and activate macrophages. The dark stellate K cells that are scattered among the luteal cells are thought to be macrophages. They influence luteal cell function through discrete cell–cell contacts and the production of growth factors and cytokines.[125-127] Regulatory T cells, a population of lymphocytes which maintain antigen-specific T cell tolerance, may be of particular importance.[127] An abundance of these cells in the midcycle corpus luteum have been hypothesized to promote an anti-inflammatory or "tolerant" state. Luteolysis is associated with a loss of regulatory T cells, which would engender a pro-inflammatory state. The importance of invasion of T lymphocyte subtypes and macrophages into the corpus luteum in the process of luteolysis is highlighted by the fact that pregnancy delays this invasion.

Ovarian Innervation

The ovary has both extrinsic and intrinsic innervation. The extrinsic nerves, which are mainly sympathetic and sensory fibers with a small parasympathetic component, enter the ovary via the hilar perivascular plexus.[128-130] The main function of this extrinsic innervation is to regulate ovarian blood flow. However, it may also affect pain in pathological states like ovarian endometriomas and the endocrine function of ovarian cells. Excessive activation of thecal adrenergic receptors possibly plays a role in the pathogenesis of ovarian hyperandrogenism. Consistent with this notion, histochemical evaluation of ovarian tissue obtained from patients with PCOS suggested enhanced innervation of the theca–interstitial cell compartment.[131]

The human ovary is also endowed with intrinsic innervation. Some of the neurons are catecholaminergic, as identified by the expression of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis. Most of these neurons express neurotrophin receptors.

The neurotrophins are a family of nerve survival and differentiation factors. These molecules act on the TRK proto-oncogene family of high-affinity receptors and the low-affinity p75 nerve growth factor (NGF) receptor.[132] NGF supports early follicular development. NGF-deficient mice, and mice lacking the NGF receptor, NTRK1, exhibit a substantial reduction in primordial follicle formation, and the ovaries have an increased number of oocytes that are not incorporated into a follicular structure.[133] The absence of NTRK2, the receptor for neurotrophin-4 (NTF4) and brain-derived neurotrophic factor (BDNF), is also associated with reduced primordial follicle formation. The studies of knockout mice indicate that NTRK1 and NTRK2 receptors are required for assembly of primordial follicles and early follicular development and that the action of NGF is in part through the induction of FSH receptors in developing follicles.

Mouse oocytes express the BDNF receptor TRK-B. BDNF, which is produced by granulosa cells in response to LH and NT-4/5, but not NT-3, stimulates mouse oocyte maturation, including extrusion of the first polar body,

and promotes early embryonic development *in vitro*.[134] Collectively, these findings suggest that the intraovarian neurotrophin system may be important for early follicular development and later oocyte maturation.

Neurotrophins and their receptors are also expressed in the human fetal ovary, with the TRK-B receptor being localized to germ cells and p75 NGF receptor in the stroma.[135] BDNF, NT-4/5, and NT-3 are detectable in human follicular fluid aspirated from women undergoing controlled ovarian hyperstimulation.

Ovarian Stem Cells

Embryonic stem cells are pluripotent and have been shown to give rise to oocyte-like structures *in vitro*, raising the possibility of an ovarian stem cell population that could generate germ cells in adult life.[49,50] Although this is a controversial notion, rare oogonial stem cells have been isolated from mouse and human ovaries through immunotagging with antibody to the N-terminus of DDX4, which is evidently expressed on the cell surface, and fluorescence-activated cell sorting.[136] The isolated cells divide in culture and form oocyte-like structures based on their morphology and expression of oocyte-specific markers, including DDX4 and LHX8. These oocyte-like cells from mouse ovary develop *in vitro* and *in vivo* into oocytes that are capable of fertilization and embryonic development. Oogonial stem cells isolated from human ovarian cortex show similar characteristics. Oogonial stem cells transfected with a green fluorescent protein (GFP)-tagged lineage marker and injected into the ovaries of adult mice, form GFP-expressing oocytes 5 to 6 months after injection. Some recovered GFP-tagged oocytes were successfully fertilized and subsequently developed in some instances to blastocyst stage. Oogonial stem cells isolated from adult human ovarian cortical strips and labeled with GFP were injected into human cortical strips that were transplanted into immunodeficient mice or re-aggregated with dissociated cells from cortical strips. Oocyte-like cells with meiotic markers and surrounded by somatic cells were found within grafts at 7 to 14 days after transplantation. Although the characterization of these oocyte-like cells was incomplete, these studies do provide evidence of a rare pluripotent stem cell population in the ovary. However, these cells do not appear to support follicular generation in numbers to prevent natural ovarian aging. Whether these stem cells make a quantitatively significant contribution to the ovarian follicular complement in normal women or women with ovarian dysfunction who have different follicular pool sizes (primary ovarian insufficiency or polycystic ovary syndrome) remains to be determined.[50]

The Follicular Life Cycle

Gougeon proposed that the various follicular classes, defined largely by size and the number of granulosa cells, represent sequential stages of development on the way to maturity (Fig. 9.11).[137] Based on this schema, approximately 1 year may elapse in the maturation of a primordial follicle to a dominant follicle. During much of this remarkably long period (approximately 300 days), follicles are believed to grow in a gonadotropin-independent manner. Gonadotropins influence the last 50 days of the maturation process.

Initiation of Follicular Growth

Follicular growth, which begins when primordial follicles emerge from their quiescent state, occurs continuously from the fifth to sixth month of intrauterine life until menopause. Although some researchers have suggested that the first formed follicles are the first ovulated, transition into the growth phase is more likely to be a random event

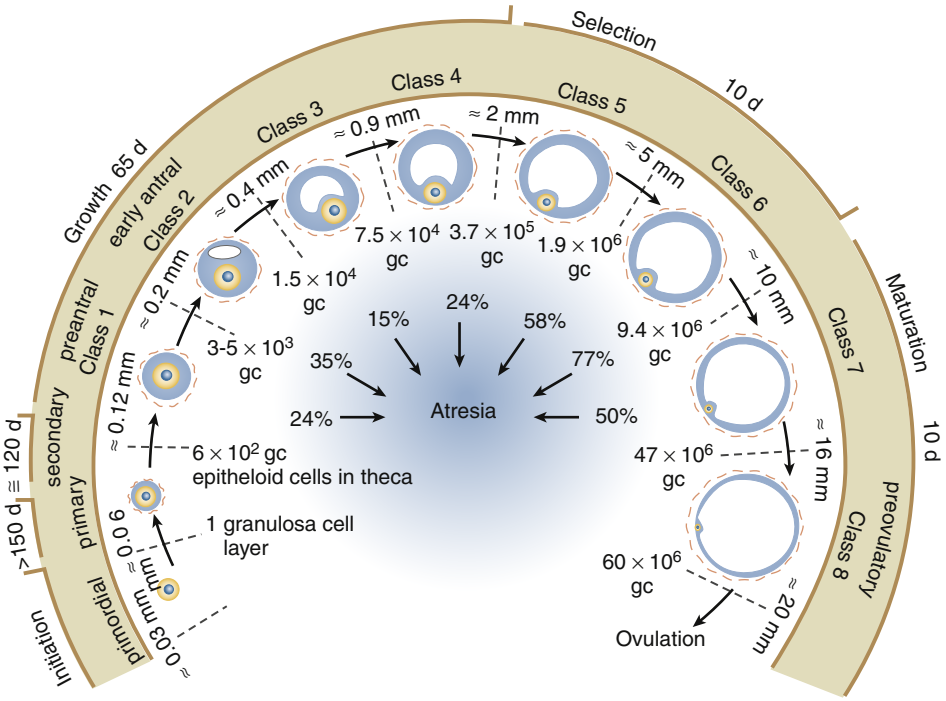


FIGURE 9.11 Stages of follicular development in the adult human ovary and extent of atresia in the eight classes of growing follicles. The classes are defined by the granulosa cell (gc) numbers and the corresponding estimated follicular diameter (mm). d, days. *(From Gougeon A. Dynamics of follicular growth in the human: a model from preliminary results.* Hum Reprod *1:81–87, 1986.)*

independent of the sequence in which follicles formed during development.

The initiation of follicular growth is characterized by morphological changes, including a change in granulosa cell shape from flattened to cuboidal, proliferation of granulosa cells, enlargement of the oocyte, and formation of the zona pellucida (Fig. 9.12). The transformation of flattened granulosa cells to a cuboidal shape has functional correlates, including the expression of certain mRNAs (e.g., follistatin mRNA). Granulosa cell proliferation and the change to a cuboidal shape precede increases in oocyte diameter. In the human, the first substantial increase in oocyte diameter occurs when there are 15 granulosa cells in the largest follicle cross-section. Oocyte growth is accompanied by elaboration of the zona pellucida, first evident as islands of periodic acid-Schiff–positive material. Subsequent increases in oocyte and follicular diameters are positively correlated until well into the secondary follicle stage, when oocytes reach a mean diameter of 80 μm. This stage corresponds to a follicular diameter of 110 to 120 μm and a granulosa cell endowment of approximately 600 cells. The germinal vesicle attains a mean maximal diameter of 26 to 27 μm at this follicular size.

The early theca interna is acquired at the end of the primary follicle stage. The theca externa forms as the follicle expands and compresses the surrounding stroma (Fig. 9.3).

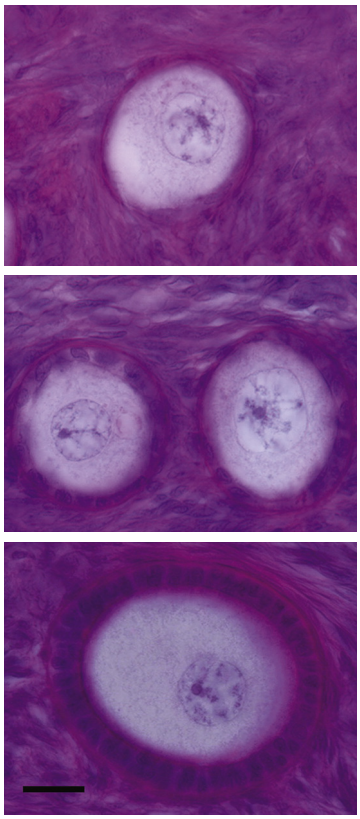


FIGURE 9.12 Changes in the structure of primordial follicles with initiation of growth. The change in morphology of the cumulus cells from flat to cuboidal represents the earliest morphological feature of this process. *(From Westergaard CG, Byskov AG, Andersen CY. Morphometric characteristics of the primordial to primary follicle transition in the human ovary in relation to age.* Hum Reprod *22[8]:2225–2231, 2007.)*

The migration of pretheca cells to the outer surface of the follicle is triggered by secreted signals from granulosa cells including Kit ligand and insulin-like growth factor-I.[138] Signals secreted from the oocyte including fibroblast growth factor (FGF2), platelet derived growth factor (PDGF), GDF-9, and BMP-15 also promote theca cell migration and proliferation (Fig. 9.5).[139]

As a secondary follicle is being formed, the granulosa cells develop FSH, estrogen, and androgen receptors and become coupled by gap junctions. The formation of the thecal layer is associated with the development of a follicular blood supply from arterioles that terminate in a wreath-like network of capillaries adjacent to the basement membrane. Concomitantly, the theca cells acquire LH receptors and the capacity to synthesize steroid hormones. The secondary follicles constitute the pool of preantral follicles from which FSH-dependent recruitment of follicles takes place.

Oocyte Growth

A significant component of the secondary follicle growth phase is dedicated to oocyte differentiation and growth. The growing oocyte is a metabolically active cell, synthesizing mRNAs and proteins that support growth and development through the early preimplantation embryo stages. The oocyte is supported in its growth by bidirectional transport of nutrients, growth factors, and other molecules across the gap junctions that directly connect the transzonal projections of the surrounding granulosa cells to the oocyte.[140]

Morphological alterations in the oocyte that occur during the growth phase include complete elaboration of the zona pellucida by the active production, assembly, and secretion of its component proteins. The quantity of several cytoplasmic organelles increases, particularly the mitochondria, which are estimated to number approximately 500,000 in a fully grown human oocyte.[141] In contrast, the centrioles that were present in oogonia are lost during the oocyte growth phase.[142] The distribution of the organelles changes, with clustering of the mitochondria, endoplasmic reticulum, and Golgi complex in the region immediately surrounding the germinal vesicle.[143]

During the growth phase, oocytes become competent to undergo meiotic maturation, or acquire "meiotic competence." The molecular basis for the acquisition of meiotic competence is not completely understood. However, it occurs only after the oocyte has reached a critical size. Meiotically competent oocytes do have certain attributes, including increased levels of the cell cycle proteins CDK1, cyclin B, and CDC25.[144,145] A threshold amount of these proteins is most likely a prerequisite for resumption of the cell cycle. Importantly, proper meiotic chromosome segregation depends on successful folliculogenesis and oocyte growth, processes that have substantial energy requirements. These requirements are met by transport of ATP and energy substrates from granulosa cells via gap junctions and also by oxidative phosphorylation of pyruvate within the oocyte proper. Mouse oocytes lacking PDH1A, an enzymatic subunit of pyruvate dehydrogenase, have decreased levels of ATP and nicotinamide adenine dinucleotide phosphate (NADPH) because they cannot perform oxidative phosphorylation of pyruvate. These oocytes grow and are ovulated, but do not successfully complete meiotic

maturation.[146] Similar processes may be compromised in the ovaries of older women, resulting in nondisjunction errors and aneuploidy in the embryo.[147,148]

Although it is not completed until late in preimplantation embryo development, reestablishment of genomic imprints begins to occur during oocyte growth, at least partially due to the activity of DNA methyltransferases.[149-152] Failure of the imprinting process in either oocytes or male germ cells results in aberrant expression of genes from the maternal or paternal alleles. These alterations in gene expression are associated with several inherited human diseases, including Beckwith-Wiedemann, Prader-Willi, and Angelman syndromes.[153,154] A global loss of maternal imprinting in the oocyte results in the formation of a complete hydatidiform mole.[155]

In addition to developing meiotic competence, oocytes in the growth phase begin to acquire the ability to support preimplantation embryo development and development to term, known as "developmental competence." Functional aspects of developmental competence are poorly defined, but include the ability of the oocyte cytoplasm to remodel sperm DNA and an enhanced ability to generate calcium oscillations.[156,157] Generation of a pool of maternal mRNAs that are highly stable but translationally repressed until maturation or fertilization is an important aspect of developmental competence.

At the completion of oocyte growth, transcription is actively silenced and protein translation slows substantially. Transcriptional silencing requires patent gap junctional communication with cumulus granulosa cells and is accompanied by changes in large-scale chromatin structure that are essential to confer growing oocytes with meiotic and developmental competence.[158,159] Maintenance of these chromatin changes requires the activity of histone deacetylases.[160] After transcriptional silencing, preovulatory oocytes rely on maternally derived proteins and mRNA stores to support the resumption of meiosis and the first cleavage divisions after fertilization.

Factors Initiating Follicular Growth

Intraovarian factors are believed to play key roles in regulating the early phases of follicular growth either as activators or inhibitors. Activators of follicular growth and development include LIF, basic fibroblast growth factor (FGF), and Kit ligand. Kit ligand, which is produced by granulosa cells and acts on Kit, a receptor on the oocyte and theca cells, is required for initiation of follicular growth and growth of the oocyte. An antibody to Kit that blocks interaction with Kit ligand interferes with the primordial stage of follicle development when injected into newborn mice. Conversely, recombinant Kit ligand accelerates the transition from primordial to primary follicles in neonatal rat ovaries. Anti-müllerian hormone (AMH), activin A, and the chemokine SDF-1/CXCL12, acting through its receptor, CXCR4, inhibit follicular growth.

The oocyte-derived proteins GDF-9 and BMP-15 are important for granulosa cell proliferation in a species-specific manner, as shown by the ovarian phenotypes of the *Gdf9* knockout mouse and sheep with homozygous mutations in the *Gdf9* and *Bmp15* genes. With GDF-9 deficiency, granulosa cells cease proliferating after approximately two doublings. The oocytes continue to grow, however, resulting in large oocytes that ultimately degenerate and are surrounded by a single layer of granulosa cells.[70,161]

Two signal transduction cascades have been identified to play critical roles in the activation of primordial follicles: the phosphatidylinositol-3-kinase (PI3 kinase)/Akt pathway and the mTOR (mammalian target of rapamycin) pathway. When disrupted, both pathways result in aberrant activation of follicular growth in mice and the ultimate depletion of primordial follicles.

PI3 kinase catalyzes the formation of the formation of phospholipid, 3,4,5 phosphoinositol phosphate (PIP3), which activates protein kinase PDK1, which in turn phosphorylates the kinase AKT, which phosphorylates the forkhead O3 transcription factor, FOXO3. Kit ligand, produced by granulosa cells, acts on Kit expressed on the oocyte and appears to activate this cascade, which ultimately leads to phosphorylation and inactivation (exclusion from the nucleus) of FOXO3, resulting in the inhibition of genes required for the initiation of follicular growth. Mice with null mutations for *Foxo3* do not restrain primordial follicle activation, which results in global follicle activation soon after birth and subsequent premature primordial follicle depletion and sterility.[162] The regulatory components involved in FOXO3 phosphorylation include PDK1 and PTEN (phosphatase and tensin homologue deleted on chromosome 10). Oocyte-specific deletion of *Pdk1* causes infertility secondary to depletion of primordial follicles. PTEN, is a PIP3 phosphatase and, therefore a negative regulator of phosphatidylinositol 3-kinase. Deletion of PTEN in the oocyte, which removes a restraint on activation of PDK1 and Akt results in activation of primordial follicular growth and early depletion of follicles because of FOXO3 phosphorylation.

mTOR is a member of the phosphatidylinositol-3-kinase family. mTOR complex 1 (mTORC1), one of two mTOR complexes with distinct functions, regulates transcription, translation and metabolism through downstream targets.[70] It is negatively regulated by a complex formed by two unrelated proteins, TSC1/TSC2, that work through the intermediacy of a small GTP hydrolase, RHEB, which activates mTORC1 that in turn activates translation by phosphorylating ribosomal S6 kinase and 4E-binding protein 1. When *Tsc1* and *Tsc2* function is ablated in mouse oocytes, the primordial follicle pool is established, but premature follicular activation depletes this pool resulting in infertility. The follicular activation in mice lacking TSC1 can be suppressed by the administration of the mTOR inhibitor, rapamycin. Although these observations provide strong evidence for the importance of the mTOR signaling pathway in follicular growth control, the upstream factor that initiates the signal transduction cascade has not been elucidated.

In addition to the PI3 kinase and mTOR pathways, several transcription factors are known to play roles in the transition from primordial to primary follicles based on functional genomic studies in mice, including NOBOX (newborn ovary homeobox), SOHLH1 (spermatogenesis and oogenesis helix-loop-helix 1), and SOHLH2. Mice bearing mutations in *Nobox*, *Sohlh1*, or *Sohlh2* have defects in the primordial to primary follicle transition and are infertile.[70]

Evidence suggesting that FSH is not required for initiation of follicular growth includes the fact that the process occurs in hypophysectomized animals. In humans and mice with inactivating mutations in the FSHβ subunit or FSH receptor gene, follicular development can occur to the secondary and early antral stages, but more slowly and with markedly reduced frequency than when normal levels of FSH activity are present. However, the importance of pituitary-derived factors (not necessarily FSH) in follicular growth and survival in the primate fetus was illustrated by the observation that hypophysectomy of the fetal Rhesus monkey results in oocyte depletion. Moreover, studies on rodent ovaries suggest that preantral follicles are gonadotropin-responsive. In human ovarian xenografts transplanted to immunodeficient and hypogonadal mice, FSH was shown to be required for growth of follicles beyond the two-granulosa–layer stage.[163] Thus, follicle growth before the antral stage, although possible in the absence of gonadotropins, may be facilitated by FSH.

Formation of Antral Follicles

There is little doubt that the transition from the secondary-follicle to the antral-follicle stage is promoted by FSH. Antral follicles are rarely observed in animals or humans with FSH deficiency (unless exogenous FSH is administered) or ovaries lacking FSH receptors. The antrum and its fluid facilitate the process of release of the cumulus–oocyte complex at ovulation and serve as a vehicle for nutrient exchange and waste removal in the avascular compartment.[164] The antrum also serves as a unique environment in which the cumulus–oocyte complex completes growth and maturation.

The development of the antrum requires the influx of water, which occurs primarily through a transcellular process. This may be mediated by the water channels formed by aquaporins 7, 8, and 9.[165] Because net transfer of water via the aquaporins requires an osmotic gradient, granulosa cells are believed to actively transport ions to create this gradient. Alternately, the hydrolysis of glycosaminoglycans in the antrum could increase the osmolarity of follicular fluid and support the influx of water.

Between 5 and 6 days before ovulation, the follicle undergoes rapid expansion—as a result of granulosa cell proliferation and the accumulation of antral fluid—and moves to the ovarian surface. The accelerated expansion of the follicle destined to ovulate can cause midcycle pelvic pain (mittelschmerz). The expression of the cell cycle gene, cyclin D2, is important for this expansion, because cyclin D2 null mice show impaired granulosa cell proliferation and consequently have an ovulation defect. On completion of this growth phase, the follicle, now referred to as a Graafian follicle, is prepared for ovulation.

Follicular Recruitment, Selection, and Dominance

The term "recruitment" has been used to describe the process by which the follicle departs from the resting pool to initiate growth. However, some authors also use this term to describe the engagement of a cohort of antral follicles into further growth. It has been suggested that the first situation be called *initial recruitment* and the latter, *cyclic recruitment*.[166] Cyclic recruitment, although obligatory, does not guarantee ovulation because growing follicles are vulnerable to atresia and thus may fall out from the growth trajectory. *Selection* refers to the process by which the maturing follicular cohort is reduced to a number appropriate for the species-specific ovulatory quota. This process entails negative selection against the subordinate follicles as well as positive selection of the follicles that will determine dominance.

Although traditional thinking holds that a single cohort (wave) of follicles develop during the menstrual cycle, ultrasound studies suggest that multiple waves occur. The "wave theory," supported by serial transvaginal ultrasound monitoring of normal ovulatory women and some earlier histological studies follicular recruitment, holds that two or more cohorts of 4 to 14 follicles ≥4 to 5 mm in diameter are recruited during the ovarian cycle, and that the dominant follicle that emerges in the final wave of the inter-ovulatory interval is destined to ovulate.[167]

In the early follicular phase, no gross morphological differences exist between the selected follicle and other healthy members of the cohort. However, the leading follicle can be distinguished from other members of the cohort by its size and the high mitotic index of its granulosa cells. Only the leading follicle has detectable levels of FSH in its follicular fluid. The leading follicle also contains significant levels of estradiol, a hallmark of the chosen follicle. Selection does not guarantee progression to ovulation, but given its temporal proximity to this event, ovulation usually does occur.

FSH is required for the transition of secondary preantral follicles to the antral stage. It also is a survival factor for antral follicles, and its withdrawal triggers programmed cell death in the absence of local factors that sensitize the follicle to FSH action or amplify its effects.[168,169] Follicular maturation initiated at the start of a new menstrual cycle is driven by an increase in FSH levels in the late luteal phase as progesterone, estradiol, and inhibin A levels fall. Preantral follicles apparently require a threshold FSH concentration to sustain growth, and this threshold level is reached during the late luteal phase. Remarkably, the threshold can be crossed with as little as a 10% to 30% increment in FSH, indicating that granulosa cells have a highly sensitive, but still mysterious, detection system with which they interpret circulating FSH levels. FSH can induce follicular growth to the preovulatory size of at least 17 mm in the virtual absence of LH. Although estradiol production is impaired under these circumstances, inhibin production is induced-reflecting the normal response of granulosa cells to FSH.

Granulosa cell division is promoted by FSH, possibly by an indirect mechanism. This action is mediated by growth factors produced by either somatic cells or the oocyte. For example, in rodents, estrogens produced in response to FSH are an important mitogen for granulosa cells. FSH also increases the number of gap junctions as well as the amount of junctional membrane between granulosa cells. An early sign that a dominant follicle has been selected is that its granulosa cells proliferate at a greater rate than the cells in the nondominant follicles. The differential mitotic rates can be detected in the late follicular phase.

One of the major actions of FSH is the induction of aromatase in granulosa cells (Fig. 9.6). Thus, little or no estrogen can be produced by FSH-unprimed granulosa cells,

even if they are supplied with aromatizable androgen precursors. FSH also induces expression of cytochrome P450 reductase, which transfers electrons to aromatase, and by type 1 17β-hydroxysteroid dehydrogenase (17BHSD1), the "estrogenic" 17β-hydroxysteroid dehydrogenase that reduces estrone to estradiol.

Follicle-stimulating hormone induces LH receptors in the granulosa cells of the preovulatory follicle. *LHR* mRNA is detectable in antral follicles (3–10 mm in diameter) and reaches maximal levels in granulosa cells in preovulatory follicles. In contrast, *FSHR* mRNA levels decline in granulosa cells as follicular diameter increases. Consequently, in the late stages of follicular maturation, LH can subserve FSH's function in propelling follicular maturation.[170] This attribute may allow the dominant follicle to complete its maturation cycle in the face of declining FSH levels; in addition, the dominant follicle is prepared to respond to the ovulatory LH surge.

The importance of FSH in follicular development has been documented by the discovery of mutations that inactivate the FSH β subunit and the FSH receptor in humans, and by targeted deletions of these genes in mice.[171,172] Women who are homozygous for FSH receptor mutations have the features of hypergonadotropic hypogonadism, with absent or poor development of secondary sexual characteristics and high FSH and LH levels. The ovarian phenotypes of humans with these mutations and those of FSH receptor and FSH β subunit knockout mice are similar. In the absence of functional FSH β subunit or FSH receptor, the ovaries are small and follicular development generally goes no further than the preantral stage. The genotype–phenotype correlation in humans with FSH receptor mutations is mirrored by the mouse knockout model; mice heterozygous for a FSH receptor gene mutation have accelerated loss of oocytes resulting in premature reproductive senescence.

Mutations in the transmembrane helices of the FSH receptor and the extracellular domain cause spontaneous ovarian hyperstimulation syndrome.[173] Mutations in the transmembrane helices result in ligand promiscuity, allowing the receptor to respond to endogenous human chorionic gonadotropin (hCG) and thyroid stimulating hormone (TSH), whereas mutations in the extracellular domain display increased specificity and sensitivity to hCG, but not to TSH.

Dominance refers to the status of the follicle destined to ovulate, and its role in regulating the size of the ovulatory quota. The follicle destined to ovulate attains dominance 5 to 7 days after the demise of the corpus luteum of the previous cycle.[174] This conclusion is supported by the observation that the levels of estradiol in the ovarian vein are remarkably different between ovaries by day 5 to 7 of the cycle, attesting to the emergence of the dominant follicle. This follicle continues to thrive under circumstances that it has made inhospitable for competing follicles in both ovaries.

The control of the temporal sequence of events leading up to follicular dominance has been elucidated by ablation studies in infrahuman primates, and also in women in which the dominant follicle or corpus luteum was destroyed or removed. Destruction of the largest follicle on day 8 to 12 in the primate ovary delays the next preovulatory surge of pituitary gonadotropins. Conversely, luteectomy in the midluteal phase (days 16 to 19) advances the gonadotropin surge. In women, the interval from ablation of the dominant follicle or corpus luteum to the next ovulation is 14 days. These findings are consistent with the notion that the cyclic structures of the dominant ovary (i.e., the ovary containing the dominant follicle or corpus luteum) are the timekeepers of the menstrual cycle. The 28-day menstrual cycle is thus the result of the intrinsic life span of the dominant follicle (follicular phase) and corpus luteum (luteal phase), not timing dictated by the brain or pituitary. These elegant experiments were conducted prior to the use of ultrasound monitoring of follicular growth, so it is not possible to reconcile these observations with sequential ultrasound studies of follicular dynamics in cycling women (i.e., follicular waves).

Studies on primate ovaries indicate that the selection of the follicle destined to ovulate has already occurred as early as day 8 of the cycle.[175] No other member of the follicular cohort is competent to serve as a surrogate for a destroyed follicle, and a timely mid-cycle gonadotropin surge is not achieved. In the case of the corpus luteum, the next round of follicular growth occurs only after its interference is removed—either naturally (luteolysis) or artificially (luteectomy). Studies on hormone-replaced luteectomized primates suggest that progesterone is the principal agent responsible for the inhibition of follicular growth in the luteal phase.[176] However, inhibin A secreted by the corpus luteum may also play a role in the suppression of FSH and thus follicular maturation. Also central to the process of follicular development is its vasculature. Inhibition of the action of vascular endothelial growth factor (VEGF) blocks follicular maturation secondary to an attenuation of follicular vascular density or reduced vascular permeability, which may limit access of critical growth factors or hormones necessary for follicular growth.[177]

Endocrine Characteristics of Follicles on the Way to Dominance

Follicles with a diameter of less than 8 mm have a relatively low intrafollicular estrogen-to-androgen ratio, but from the midfollicular phase onward, this ratio is reversed (Fig. 9.13). The "chosen" follicle is able to synthesize estradiol in sufficient quantities to result in appreciable passage of this hormone into the general circulation, and asymmetry in ovarian estrogen secretion as early as day 5 to 7 of the cycle.[178,179] In the late follicular phase, the intrafollicular concentrations of estradiol are directly correlated with follicular size and achieve concentrations of approximately 1 μg/mL at a time when circulating estradiol levels reach their peak.[179-182] After the ovulatory surge of LH, intrafollicular concentrations of estradiol decline and there is a parallel decrease in the concentration of androstenedione. Concurrently, progesterone and 17α-hydroxyprogesterone concentrations increase, reflecting early granulosa cell luteinization.

Inhibin A concentrations in follicular fluid increase with follicular maturation, whereas inhibin B, activin A, and free follistatin do not show variations with follicular size. Thus, as follicles mature, there is a switch from an environment dominated by activin to one dominated by inhibin A. The increase in inhibin A levels is correlated with increased expression of inhibin A α and β subunit mRNAs in granulosa cells.[181]

The IGFs are members of a family of low–molecular-weight, single-chain polypeptide growth factors named for

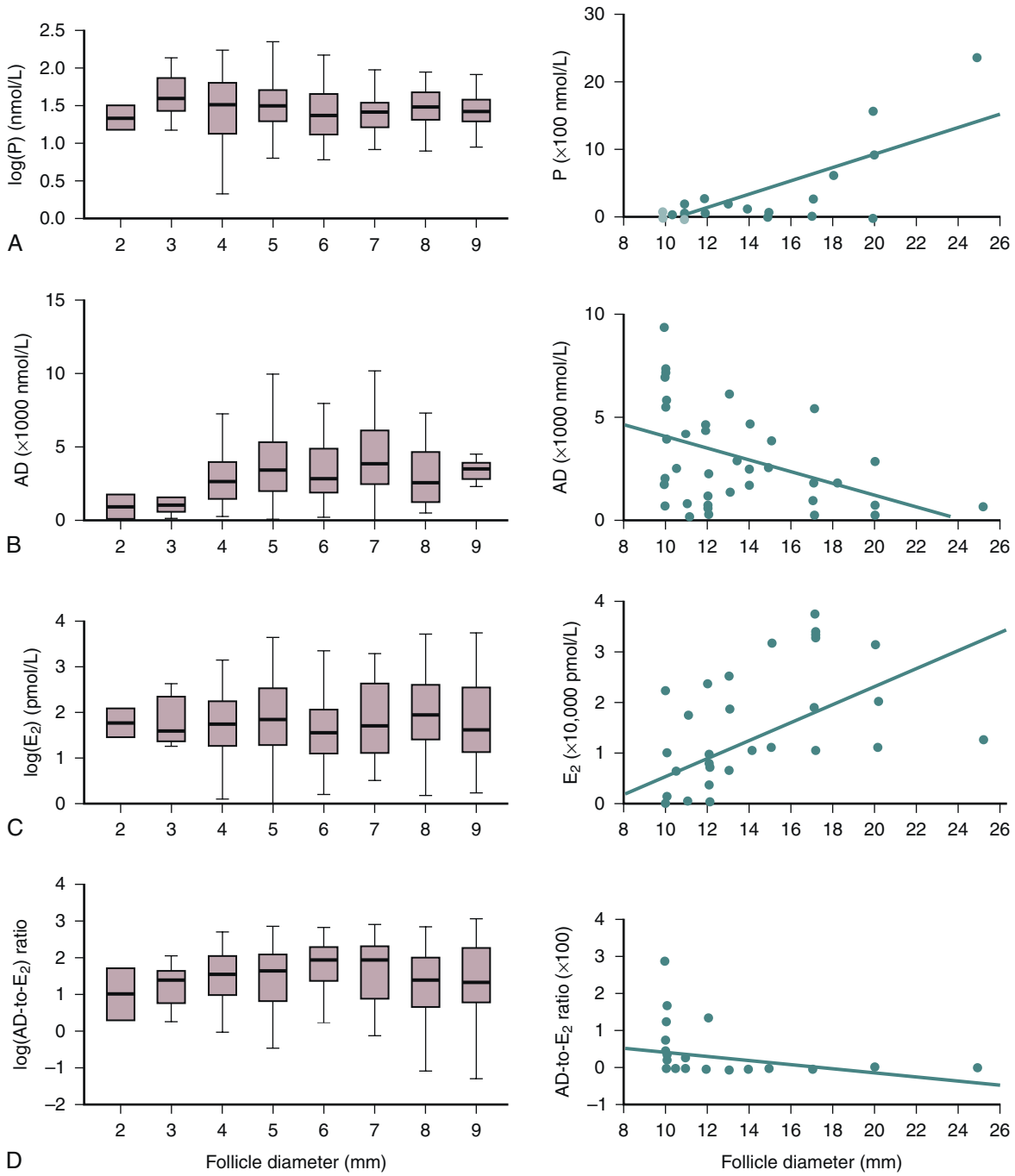


FIGURE 9.13 Follicular fluid steroid levels and follicular diameter. AD, androstenedione; E_2, estradiol; P, progesterone. *(From Van Dessel HJ, Schipper I, Pache TD, et al. Normal human follicle development: an evaluation of correlations with oestadiol, androstenedione, and progesterone levels in individual follicles.* Clin Endocrinol *44:191–198, 1996, with permission.)*

their structural and functional similarity to insulin (Fig. 9.14). Both IGF-1 and IGF-2 are present in human follicular fluid.[183,184] Follicular fluid IGF-1 is most likely derived predominantly from plasma. IGF-2, however, is produced by the theca and perifollicular vessels of all follicles, and the granulosa and theca cells of small antral follicles, and is abundantly expressed by preovulatory granulosa cells. In mice lacking IGF-1, follicular maturation is arrested; in addition, the animals are infertile, and granulosa cell proliferation in the basal state and in response to estrogen is impaired.[185] In women with Laron dwarfism, a disease characterized by IGF-1 deficiency, ovulation induction with human menopausal gonadotropins after administration of a GnRH analog resulted in development of mature follicles and fertilizable oocytes. This finding indicates that, in the human, IGF-1 is not essential for normal follicular development. IGF-2, however, would be present in this situation. Infusion of IGF-1 at levels that raise blood concentrations twofold above normal have no effect on ovarian function in Rhesus monkeys.[186] Thus, the locally produced IGFs appear to be sufficient to promote normal ovarian activity, and elevated IGF levels do not disrupt ovarian function.

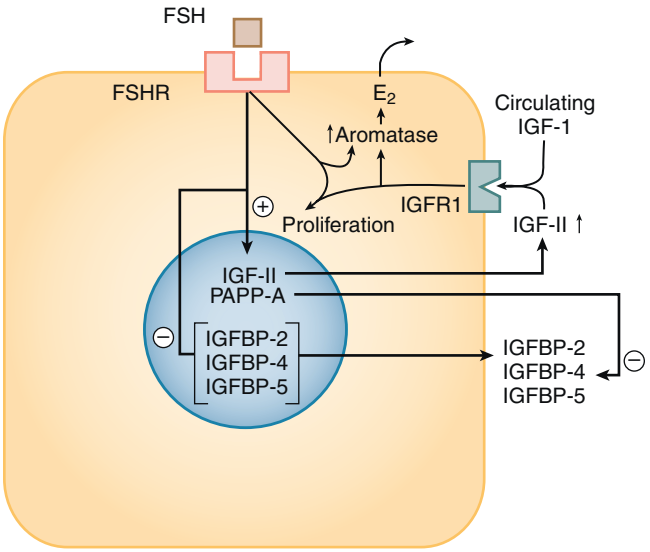


FIGURE 9.14 Intraovarian role of insulin-like growth factors (IGFs) and IGF-binding proteins (IGFBPs) in regulating granulosa cell function. E_2, estradiol; FSH, follicle-stimulating hormone; FSHR, follicle-stimulating hormone receptor; IGFR1, IGF receptor type 1; PAPP-A, pregnancy-associated plasma protein A.

In situ hybridization studies showed that IGF-1 receptors, which are activated by both IGF-1 and IGF-2, are present in the granulosa cells of dominant follicles. IGF-2 receptors, which probably are not involved in signaling, are found in theca and granulosa cells. IGF-1 and IGF-2 stimulate DNA synthesis, granulosa cell proliferation, and steroidogenesis by cultured human granulosa and granulosa–lutein cells. However, these *in vitro* experiments were conducted in restricted culture medium, so the addition of general trophic factors might be expected to increase cellular function.

The action of IGFs is modulated by the local elaboration of binding proteins. Of the seven IGF-binding proteins (IGFBPs) that have been described to date, at least five are expressed in the human ovary. The IGFBPs bind IGFs and neutralize their activity, and they also may have direct actions on ovarian cells. IGFBP-1, -2, -3, -4, and -5 have been identified either in follicular fluid or by analysis of mRNA from granulosa cells. Of these binding proteins, IGFBP-4 is of particular interest because it is a potent antagonist of FSH-stimulated estradiol production by human granulosa cells. IGFBP-4 also is present in atretic follicles, implicating this protein in the pathway leading to follicular atresia.

Secretion of IGFBPs is inhibited by gonadotropins and IGFs, resulting in enhanced IGF bioavailability and gonadotropin action. The proteolytic cleavage of IGFBPs is another mechanism controlling IGF bioavailability. IGFBP-4 protease expression is restricted to healthy follicles and corpora lutea.[187] The metalloproteinase activity of pregnancy-associated plasma protein A (PAPP-A), a large dimeric glycoprotein, degrades IGFBP-4 into inactive fragments. Granulosa cells from small follicles secrete low levels of PAPP-A, whereas granulosa cells from dominant follicles secrete high levels of this protein. The significance of this proteolytic mechanism is underscored by the fact that mice lacking PAPP-A have a reduced number of ovulated oocytes in a stimulated ovulation protocol, which is associated with lower serum estradiol levels, lower serum progesterone levels, and reduced expression of ovarian steroidogenic enzyme genes.

Insight into the importance of estrogens in ovarian function in women comes from the study of subjects in whom estrogen synthesis is impaired. Limited studies have been performed on women with 17α-hydroxylase/17,20 lyase (CYP17) deficiency who are incapable of producing thecal androgens to support granulosa cell estradiol synthesis. Promotion of follicular growth to the preovulatory stage in an estrogen-impoverished environment is possible in these individuals with exogenous gonadotropins after pituitary desensitization. The same is true in severely hypogonadotropic women given exogenous FSH. Follicles grow, but in the absence of exogenous LH, estradiol synthesis is minimal. Moreover, the development of follicular cysts with low estrogen levels is common in women with steroidogenic acute regulatory protein (STARD1), 17α-hydroxylase/17,20 lyase, and aromatase deficiency.[188] Hence, the high levels of estrogen associated with normal follicular maturation are not required for the growth of follicles to the size equivalent to the preovulatory stage.

Whether oocytes that are recovered from estrogen-deprived follicles are endowed with the properties that can lead to successful embryonic development after fertilization is less certain based on limited experience in humans with defects in ovarian estrogen biosynthesis. Animal studies with aromatase inhibitors suggest that estrogens are important for oocyte function.[189] Rhesus monkeys treated during follicular maturation with doses of an aromatase inhibitor that substantially reduces circulating estradiol levels showed no effects on follicular growth. However, a greater proportion of the oocytes recovered from follicles of the aromatase-treated animals were in prophase I, and there was retarded completion of maturation to MII. Whether this is a direct reflection of estradiol deficiency, a consequence of the suicide aromatase inhibitor (1,4,6-androstatrien-3,17-dione), or the result of compensatory changes in endocrine status due to the decline in estradiol is not known. However, clinical experience with competitive aromatase inhibitors like letrozole indicates that aromatase inhibition during follicular growth does not have a major negative impact on oocyte maturation.

The human ovary expresses the receptors that allow a variety of cells to respond to estradiol, but the physiological roles of estrogen in follicular health, maturation, and luteal function remain to be elucidated. Although estrogen receptor α and β1 and β2 are expressed, with the latter two receptor types being more prominent in luteinized granulosa cells, the extremely high levels of estradiol reached in the antrum of the preovulatory follicle (approximately 1 μg/mL) raise questions as to the function of the classical estrogen receptor system, which would be fully saturated by ligand. Moreover, follicle growth per se does not require high levels of estradiol as noted above.

Androgens have a number of effects on the primate ovary.[190-192] Administration of testosterone or 5α-dihydrotestosterone to Rhesus monkeys promotes accumulation of primary follicles as well as follicle survival, suggesting a folliculotropic action. In this model, androgen receptors are abundant in the granulosa cells of healthy preantral and antral follicles, with lesser expression in the theca

and stroma. Moreover, androgen receptors were positively correlated with a marker of cell proliferation (Ki-67) and negatively correlated with apoptosis. These observations contrast with the view that androgens are atretogenic, a concept that emerged primarily from studies on the rodent ovary in which androgens block granulosa cell proliferation *in vitro* in some systems and promote follicular atresia.[193]

Evidence that androgens have a detrimental effect on human follicular function includes the observation that follicular fluid enriched in 5α-dihydrotestosterone and poor in estradiol is characteristic of atresia. However, this steroid profile may be a consequence rather than a cause of atresia. Favoring a causal relationship are reports that high follicular concentrations of 5α-reduced androgens, such as 5α-dihydrotestosterone, act as competitive inhibitors of granulosa cell aromatase activity.[194] In this regard, follicles from patients with PCOS have greater 5α-reductase activity than follicles from normal ovaries. Thus, androgens may exert both positive and negative effects on follicular growth and function in a stage-dependent manner through androgen receptors as well as by nonreceptor-mediated mechanisms.

Ovulation

As mid-cycle approaches, the rise in estrogen emanating from the dominant follicle initiates an LH surge and, to a lesser extent, an FSH surge. This triggers the resumption of meiosis, ovulation, and luteinization (Fig. 9.15).[195]

In a normal menstrual cycle, the FSH-induced appearance of LH receptors on preovulatory granulosa cells allows LH to take over FSH's functions in the terminal stages of follicular maturation. These receptors also enable the granulosa cells to become competent to respond to the LH surge that initiates the resumption of meiosis, ovulation, and subsequent luteinization of the granulosa and theca cells. These events are triggered only when a threshold concentration of LH is achieved and not before that time. Notably, granulosa cells respond to FSH with activation of adenylate cyclase, but genes or events that constitute the program for ovulation and luteinization are not induced. The necessity for some LH to stimulate thecal androgen production and synergize with FSH for follicular maturation, together with the potential for high levels of LH to promote premature luteinization and possibly atresia of follicles that have not reached the Graafian stage, has led to the notion of an "LH window" for follicular maturation. This concept has pharmacological and clinical relevance with respect to ovulation induction.[196] Levels of LH that are capable of stimulating maturation of the dominant follicle retard the growth of smaller follicles and suppress aromatase activity.[170] This effect has led to the theoretical possibility of minimizing multifollicular development by using LH or hCG to drive terminal stages of follicular maturation.[197,198]

The threshold for activation of the ovulation–luteinization program may be sensed by granulosa and theca cells as a result of the intensity of the signal (i.e., the magnitude of the increase in cAMP); it may also be sensed by the activation of adjunctive signal transduction cascades that supplement the increase in cAMP. LH receptor activation of cAMP and IP3 signaling are dependent on LH dose, with a 10- to 100-fold higher level of LH needed to activate phospholipase C. These pathways influence expression of noncoding

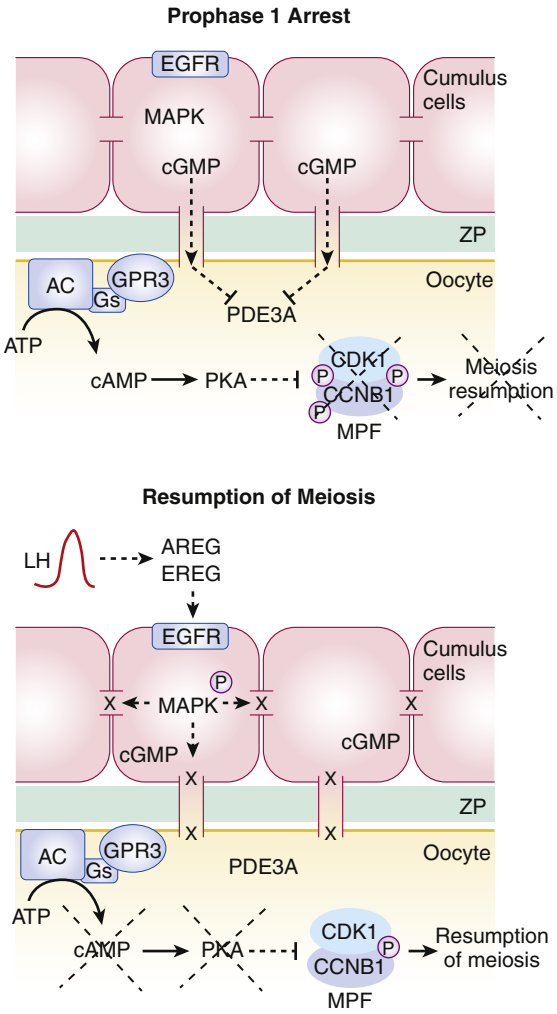


FIGURE 9.15 Meiotic arrest and resumption are controlled by cyclic nucleotides through regulation of maturation promoting factor. A. Prophase I arrest. The constitutively active Gαs-coupled receptor GPR3 stimulates adenylyl cyclase to produce cAMP. cAMP levels in the oocyte remain high because cGMP generated in granulosa and cumulus cells is transported into the oocyte via gap junctions and inhibits the activity of the cAMP phosphodiesterase, PDE3A. cAMP activates PKA, which indirectly prevents dephosphorylation and activation of MPF. B. Resumption of meiosis. The pituitary LH surge promotes expression of EGF-like factors, AREG and EREG, predominantly in the mural granulosa cells. These factors act through EGF receptors on cumulus cells to activate the MAPK pathway, resulting in closure of gap junctions. Additional unknown signaling pathways downstream of the LH surge cause a large drop in cumulus cell production of cGMP. Together with gap junction closure, this results in a dramatic decrease in oocyte cGMP and relief of inhibition of PDE3A. PDE3A then degrades cAMP, reducing PKA activity, indirectly resulting in dephosphorylation of CDK1 inhibitory sites and activation of MPF. MPF phosphorylates targets including lamins, histones, and other cellular components leading to resumption of meiosis.

RNAs, which appear to play a key role in coordinating gene expression patterns in the development of the ovulatory follicle and the response to the ovulatory LH surge.

LH initiates the sequence of events resulting in ovulation in part through the intermediacy of mitogen-activated protein kinases (MAPK). Studies in mouse double mutants for *Mapk1* and *Mapk3* revealed a phenotype of sterility

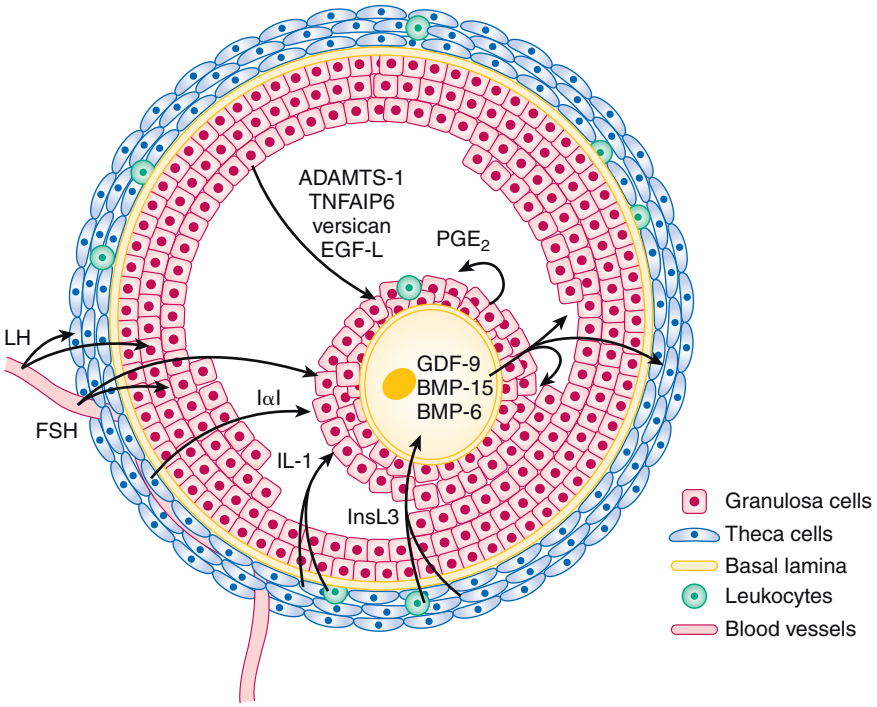


FIGURE 9.16 Mediators of ovulation. Theca cells or leukocytes within the theca layer secrete IL-1, a cytokine that stimulates expansion of the cumulus cells, and InsL3, a peptide that can trigger a reduction in oocyte cAMP levels. The mural granulosa cells secrete EGF family members that promote the cumulus cell responses leading to expansion. The mural granulosa cells also secrete proteins like versican and ADAMTS-1, which are incorporated into the cumulus cell matrix. Inter-α-trypsin inhibitor enters from the blood stream and is also incorporated into the matrix. Induction of prostaglandin synthesis enzymes leading to the formation of prostaglandin E2 by the cumulus cells creates an autocrine loop that drives cumulus expansion through promotion of expression of hyaluronan synthase 2, which leads to deposition of hyaluronan in the matrix, and tumor necrosis factor α-induced protein 6 (TNFAIP6), which catalyzes the covalent crosslinks between hyaluronan and inter-α-trypsin inhibitor. Pentraxin 3 also interacts with inter-α-trypsin inhibitor to stabilize the matrix. Oocyte-derived factors including GDF-9, BMP-6 and BMP-15 modulate granulosa cell responses.

associated with defects in cumulus expansion, ovulation, luteinization, and meiotic maturation.[70] Moreover, granulosa cells continued to proliferate and aromatase expression was maintained, indicating that the differentiation process triggered in granulosa cells by the LH surge involves the MAPK signaling pathway.

LH receptor knockout mice have a relatively normal theca layer around developing follicles.[199] However, follicular maturation is arrested at the early antral stage, and there are no signs of ovulation or luteinization. This phenotype is similar to that found in women with homozygous inactivating mutations in the LH receptor gene *(LHCGR)*. The clinical phenotype of affected women includes normal primary and secondary sexual characteristics, amenorrhea, and elevated circulating FSH and LH. The ovaries contain follicles in stages of development from primordial to antral, with well-developed theca layers, but no preovulatory follicles or corpora lutea. This phenotype supports the notion that LH is required for normal estrogen production by follicles, for ovulation, and for luteinization, but not for the formation of the theca layer.

Activating mutations of the human LH receptor are also informative regarding the role of LH in ovarian function.[199a] Women with these mutations have no obvious reproductive phenotype, in contrast to the precocious puberty found in men. One might have anticipated a hyperandrogenemic state in females, mimicking polycystic ovary syndrome with thecal hyperplasia. However, follicular compartments evidently develop in a coordinated fashion such that thecal responses to LH receptor activation do not occur prematurely. Moreover, levels of cAMP or other second-messenger molecules required for luteinization must be different from those generated by the constitutively active mutant receptor because premature luteinization of follicles does not occur.

The preovulatory LH surge precedes follicular rupture by as much as 38 hours. Before rupture, a number of critical changes take place in the granulosa cells and oocytes, including the suppression of transcription of genes that control granulosa cell proliferation; the loss of gap junction function, which uncouples the electrophysiological syncytium of the granulosa cells and the oocyte; and the induction of genes essential for ovulation in granulosa cells, including the genes encoding the epidermal growth factor–like factors amphiregulin, epiregulin, and betacellulin (Fig. 9.16). In mice, the latter growth factors activate EGF receptors, resulting in the induction of the gene encoding cyclooxygenase-2 (COX-2; *Ptgs2*) in granulosa cells and, consequently, prostaglandin E_2 synthesis. These act in concert with the EGF-like factors to trigger the cumulus cells to elaborate a hyaluronan-rich matrix that causes expansion of the cumulus. As described later, mice deficient in *Ptgs2* and the prostaglandin receptor EP2 *(Ptger2)* have ovulation defects that are related to an abnormality in cumulus expansion.[195,200]

Alterations in the extracellular matrix that promote expansion of the cumulus, a critical process for ovulation,

are mediated by genes encoding hyaluron synthase 2 *(Has2)*, required for production of the hyaluron backbone; the hyaluron-binding proteoglycan versican *(Cpg2)*; TSG-6 *(Tnfaip6)*; and pentraxin 3 *(Ptx3)*.[195,200] The matrix supporting cumulus expansion is composed of a meshlike network of hyaluronan chains that bind TSG-6, which transfers to hyaluronan the heavy chains of serum-derived inter-α-trypsin inhibitor, a complex macromolecule consisting of two heavy chains that are covalently bound to chondroitin sulfate and bikunin, a trypsin inhibitor. Pentraxin 3, a protein that forms pentamers, is another component that organizes the hyaluronan matrix. Mice deficient in TSG-6 and bikunin, one of the components of inter-α-trypsin inhibitor, have an ovulation defect associated with failed cumulus expansion.

A conical stigma eventually rises on the surface of the protruding follicle in preparation for rupture. The rupture of the stigma is accompanied by gentle (rather than explosive) expulsion of the egg and follicular fluid, which suggests that the fluid is not under high pressure.

In primates, ovulation is believed to alternate between ovaries due to the local actions of progesterone produced by the corpus luteum on follicular dynamics. However, this belief has not been supported by definitive evidence. Although some studies suggest that ovulation occurs with equal frequency in the right and left ovaries, others suggest that right-sided ovulation is more frequent.[201-203]

Requirements for Progesterone

An early action of LH in the ovulatory process, which occurs within hours of the surge, is the induction of progesterone receptors in granulosa cells.[204] hCG up-regulates progesterone receptor expression in cultured human granulosa cells in a protein kinase A-dependent fashion. The functional importance of progesterone receptor up-regulation is documented by the fact that progesterone receptor antagonists and drugs that inhibit progesterone synthesis prevent ovulation in laboratory animals and the Rhesus monkey. Moreover, administration of the progesterone receptor antagonist, ulipristal, in the immediate pre-ovulatory period delays follicular rupture in women.

Ovulation does not occur in mice with targeted deletions of the progesterone receptor gene, more specifically, the A form of the receptor. Consistent with a critical role for progesterone in ovulation, mice lacking the transcription factor liver receptor homolog 1 (NR5A2), which is thought to play a role in induction of the steroidogenic machinery required for progesterone synthesis, do not ovulate. Mice lacking a functional *PPARγ* gene, another gene regulated by the progesterone receptor, also show ovulatory defects. Finally, the nuclear receptor interacting protein RIP140 (NRIP1), which was originally described as a transcriptional repressor, is important for ovulation in the mouse. Mice lacking this nuclear protein are anovulatory, and many LH-induced genes are reduced in the mutant ovaries. The mutant mice show defects in cumulus cell function and the expression of genes controlling expansion of the cumulus–oocyte complex.

These observations indicate that progesterone regulates the expression of ovulation genes by a classic mechanism. However, the A form of the progesterone receptor is generally a repressor of transcription, raising the possibility that an important part of progesterone's action in the preovulatory period includes suppression of gene expression. A number of candidate genes that appear to be dependent on periovulatory progesterone have been identified, including a metalloproteinase and cathepsin L. COX-2 expression in progesterone receptor–deficient mice is normal, suggesting that prostanoids are not part of the progesterone-regulated ovulation program.

Requirements for Prostaglandins

The LH surge stimulates prostaglandin biosynthesis by ovarian follicles as a result of induction of the COX-2 enzyme in the preovulatory granulosa cells. COX-1 is not expressed by granulosa cells, and levels of this enzyme in the Graafian follicle do not change in response to an ovulatory stimulus.[205]

The requirement for prostaglandins in the process of ovulation has been revealed through pharmacological studies and gene targeting in mice.[206] Inhibitors of prostaglandin synthesis, administered systemically or locally into the antrum, inhibit ovulation in laboratory animals and Rhesus monkeys and lead to luteinized, but unruptured follicles. Evidence for a role for prostaglandins in human ovulation derives from studies in which rofecoxib, the orally administered selective COX-2 inhibitor, delayed the time of ultrasonographic signs of follicular rupture by more than 48 hours after the LH peak. Signs of rupture were evident within 36 hours in a placebo-treated group.[207,208]

Mice with targeted mutations in the *Ptgs2* gene have defects in ovulation that can be overcome by administration of exogenous prostaglandin E_2, which is believed to be the key prostaglandin involved in ovulation. One of the abnormalities in *Ptgs2* knockout mice is a failure of the cumulus to expand. Mice deficient in the EP2 receptor for prostaglandin E_2 also have an ovulation defect and a postovulatory abnormality in cumulus expansion. These observations implicate prostaglandin E_2 in the process of ovulation and cumulus expansion. Progesterone receptors are induced in the follicles of COX-2–deficient mice, providing further evidence that the roles of progesterone and prostaglandins in ovulation are distinct.

Requirement for EGF-like Factors

Luteinizing hormone stimulation induces the rapid, transient and sequential expression of the EGF-like growth factors amphiregulin, epiregulin, betacellulin, and neuregulin 1. These are synthesized as granulosa cell integral membrane proteins that are released by proteolytic cleavage of the extracellular domain. Upon release they bind to EGF tyrosine kinase receptors (ERBB2 and ERBB3). Incubation of rodent follicles *in vitro* with these growth factors recapitulates the morphological and biochemical events triggered by LH, including the necessary process of cumulus expansion and oocyte maturation. Mice with mutations in *Are* (amphiregulin) or *Ereg* (epiregulin) have an ovarian phenotype consistent with an *in vivo* role of these proteins in that they show reduced cumulus expansion in response to exogenous gonadotropins. Thus, the EGF-like growth factors are essential mediators of the follicular response to LH leading to ovulation.[209,210]

Mechanisms of Follicular Rupture

Several hypotheses have been advanced to explain the process of follicular rupture. An increase in hydrostatic pressure

is evidently not involved, because direct measurements have demonstrated that intrafollicular pressure is low in periovulatory follicles.[211,212] An increase in colloid osmotic pressure has been described, in part due to changes in concentrations of granulosa cell–derived proteoglycans. However, a cause-and-effect relationship between the altered composition of antral fluid and the enlargement and rupture of the follicle remains to be established.

Stigma formation and rupture also reflect the actions of enzymes working locally on the follicle wall. Instillation of protease inhibitors into the antral fluid inhibits ovulation. Among the candidate proteases proposed to be involved in ovulation are the plasminogen activators and members of the matrix metalloproteinase (MMP) family. Plasminogen activator has been localized in increasing concentrations in the walls of rat ovarian follicles just before ovulation.[195,213] But studies in knockout mice deficient in urokinase, tissue plasminogen activator, and plasminogen indicate that plasmin is not necessary for follicular rupture or, for that matter, for the activation of other proteases that might participate in ovulation.[213]

Mice deficient in MMP-3 (stromelysin-1), MMP-7 (matrilysin), MMP-9, and MMP-11 (stromelysin-3) reproduce normally, suggesting that, individually, these enzymes do not have an obligatory role in ovulation. The involvement of other members of the MMP family (e.g., MT1-MMP, ADAM-17) cannot yet be determined from existing knockout mice, because the mutant animals die either *in utero* or shortly after birth.

Members of the A disintegrin and metalloproteinase and thrombospondin motifs (ADAMTS) family appear to play a key role in ovulation. ADAMTS1 is induced in granulosa cells of the preovulatory follicle, but not in progesterone receptor knockout mice. Consequently, this gene has been implicated as a progesterone-regulated factor involved in ovulation.[195,200,213] Targeted deletion of the *Adamts1* gene in mice results in defects in follicular growth, ovulation, and consequently, female infertility, possibly as a result of abnormal cumulus expansion or release of active growth factors. ADAMTS4 may have a related role. Cathepsin L is another progesterone-regulated gene that degrades type I and type IV collagens, elastin, and fibronectin, all components of the follicle wall, and consequently, it could play a role in ovulation.

Rhesus monkey dominant follicle MMP1, MMP10, and MMP19, ADAMTS4, ADAMTS9, and ADAMTS15, cathepsin L, and urokinase-type plasminogen activator mRNA levels were up-regulated 12 hours after administration of an ovulatory dose of hCG. Moreover, a metalloproteinase inhibitor (GM6001) injected into the preovulatory follicle at the time of hCG administration prevented stigmata formation.

Oocyte Maturation

It has been known for decades that a follicle-derived oocyte maturation inhibitor was likely to be responsible for the maintenance of meiotic arrest because removal of an oocyte from its intrafollicular environment results in the spontaneous resumption of meiosis in culture. Thus, meiotic inhibition requires the intermediacy of the surrounding granulosa cells. Although the full biochemical nature of the oocyte maturation inhibitor remains a mystery, experiments in mouse models have identified many of the downstream mediators responsible for controlling meiotic arrest.

As in somatic cells, the oocyte cell cycle is controlled by alterations in the levels and activities of proteins now known as *cyclins* and *cyclin-dependent kinases*.[214,215] One of these proteins, maturation-promoting factor (MPF), was defined in bioassays as an activity that induced resumption of meiosis when microinjected into oocytes. MPF later was found to be a heterodimer of two proteins: cyclin B and cyclin-dependent kinase-1, or CDK1.[216-218] MPF is present in fully grown oocytes, but prior to the ovulatory LH surge its activity is restrained by the actions of two cyclic nucleotides, cyclic AMP (cAMP) and cyclic GMP (cGMP) (Fig. 9.15).

In prophase I-arrested oocytes, cGMP generated in the granulosa cells continuously enters the oocyte via gap junctions. Once in the oocyte, cGMP inhibits cAMP phosphodiesterase 3A (PDE3A), preventing breakdown of oocyte-derived cAMP.[219,220] The majority of oocyte cAMP is generated because of the presence of a constitutively active receptor, GPR3, at the plasma membrane. GPR3 couples to the stimulatory G protein Gs, which activates adenylyl cyclase and results in constant production of cAMP. Oocytes from *Gpr3* knockout mice fail to generate cAMP and therefore resume meiosis independently of an increase in LH.[221] A similar receptor expressed in rat oocytes, GPR12, may have this role. The steady state oocyte cAMP level is a balance of cAMP generated at the plasma membrane by adenylyl cyclase and cAMP degradation by cytoplasmic PDE3A activity.[222-224]

Oocyte-derived cAMP maintains meiotic arrest by stimulating activity of the cAMP-dependent protein kinase (PKA).[225,226] PKA phosphorylates at least three different proteins, WEE1B, MYT1, and CDC25B, resulting in inhibition of MPF activity and prevention of meiosis resumption.[227,228] As long as oocyte PDE3A activity is blocked by cGMP derived from granulosa cells, the amount of cAMP generated by the constitutively active Gs-linked receptor is sufficient to stimulate PKA activity and thereby maintain MPF in an inactive state. The requirement for cGMP to enter the oocyte from granulosa cells explains the spontaneous resumption of meiosis that occurs when the oocyte is removed from the follicle.

Resumption of Meiosis

The mid-cycle LH surge is responsible for initiating a series of events in the mature antral follicle that collectively result in activation of MPF and resumption of the meiotic cell cycle. Nuclear maturation is first visible morphologically when the germinal vesicle breaks down as a result of disruption of the nuclear lamins. With exposure to the cytoplasm, the chromatin condenses and moves toward the cortical region, and the meiosis I spindle forms. Progression through the remainder of meiosis I, with extrusion of the first polar body containing half of the oocyte's chromosome complement, soon follows (Fig. 9.17 and Table 9.2). The meiotic cell cycle proceeds immediately to metaphase of meiosis II and then becomes arrested at this stage; the oocyte is now referred to as a *secondary oocyte* or *metaphase II–arrested egg* (Fig. 9.17). Arrest in metaphase II occurs before physical release of the egg from the follicle during ovulation.

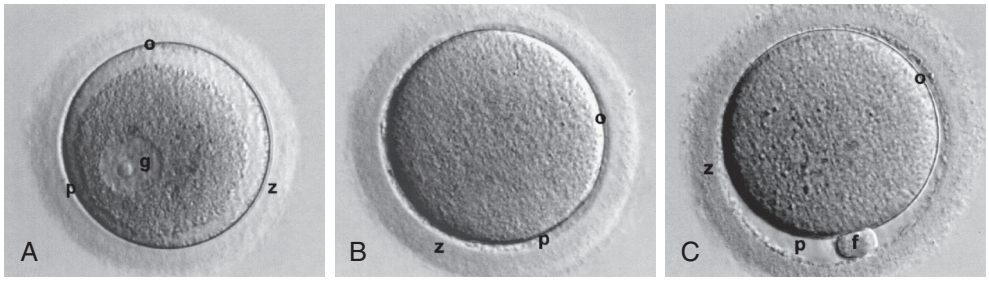


FIGURE 9.17 Appearance of human oocytes during meiotic maturation. **A,** Immature, germinal vesicle–intact stage. The chromatin is arrested in prophase of meiosis I; meiotic maturation has not begun. **B,** Germinal vesicle breakdown stage. Meiosis I has resumed, with breakdown of the germinal vesicle; the chromatin is progressing through the remainder of meiosis I. **C,** Mature oocyte. The first polar body has been emitted, and the remaining chromatin is arrested in metaphase of meiosis II. f, first polar body; g, germinal vesicle; o, oolemma (oocyte plasma membrane); p, perivitelline space; z, zona pellucida (200×). *(Adapted from Veeck LL. An Atlas of Human Gametes and Conceptuses. New York: Parthenon Publishing, 1999.)*

Table 9.2 The Acquisition of Meiotic and Developmental Competence in the Oocyte

	Primordial Oocytes	Primary	Preantral	Antral	Fully Grown Oocytes	MII-Arrested Eggs
Growth	Resting	Early growth	Mid growth	Late growth	Complete	Complete
Transcription	Limited	Active	Active	Active	Limited	Limited
GVBD	Incompetent	Incompetent	Competent	Competent	Competent	Complete
MII	Incompetent	Incompetent	Incompetent	Competent	Competent	Complete
Embryogenesis	Incompetent	Incompetent	Incompetent	Limited	Limited	Competent

GVBD, germinal vesicle breakdown; MII, metaphase.

Completion of meiosis, with extrusion of the second polar body, does not occur until fertilization.

Modulation of the levels of cGMP and cAMP within both the follicle cells and the oocyte are critical factors regulating meiosis. Midcycle LH surge-mediated stimulation of Gs-coupled LH receptors on follicular cells results in the activation of Gs and generation of cAMP by follicle cell transmembrane adenylyl cyclases. This cAMP subsequently activates transcription factors such as cAMP-response element binding protein 1 (CREB1) and cAMP-response element modulator (CREM) that induce or repress the transcription of specific genes important in modulating follicular cell function during oocyte maturation and ovulation.

Key events downstream of follicle cell LH receptor activation are decreased generation of cGMP in the cumulus cells and closure of gap junctions between the oocyte and cumulus cells, which together result in a precipitous drop in oocyte cGMP levels.[219] In response, oocyte PDE3A becomes active and breaks down cAMP, resulting in a loss of the PKA activity required to restrain MPF from inducing meiotic maturation. The LH-induced gap junction closure is mediated in part by synthesis and release from mural granulosa cells of the epidermal growth factor receptor (EGFR) ligands amphiregulin and epiregulin.[229] These ligands cause an increase in EGFR kinase activity that is required for gap junction closure. Follicle cells may also secrete other paracrine factors that promote oocyte maturation. For example, theca cells secrete insulin-like 3, which activates the inhibitory G protein (Gi)-coupled receptor, LGR8, found at the oocyte plasma membrane, diminishing oocyte cAMP production.[230]

There is clear evidence in lower species that steroid hormones are responsible for inducing oocyte maturation. Although steroids have been proposed to induce meiotic maturation in mammals, inhibition of follicular steroid production or action does not prevent resumption of meiosis in response to LH, so steroids cannot be obligatory for this process.

In addition to triggering alterations in oocyte MPF activity, the LH surge also induces translation of stored maternal mRNAs that are sequestered from the translational apparatus until meiotic maturation begins. One of these newly translated mRNAs encodes the serine-threonine kinase MOS, which is a component of a biological activity known as *cytostatic factor*, defined by its ability to induce metaphase arrest when microinjected into an actively dividing cell.[231] MOS indirectly activates a mitogen-activated protein (MAP) kinase that is responsible, at least in part, for the arrest of the oocyte cell cycle at metaphase of meiosis II.[232-234] Mice deficient in MOS are subfertile because their oocytes do not arrest at metaphase II.[235] Instead, the oocytes continue to divide without being fertilized (parthenogenetic activation) and as a result, the mice develop teratomas. With sufficient MOS production, eggs remain arrested in metaphase II until fertilization. The fertilizing sperm induces calcium oscillations that cause cyclin destruction and degradation of MOS protein, resulting in resumption of meiosis II and extrusion of the second polar body.

A family of C29 4,4-dimethylsterol intermediates in the biosynthetic pathway from lanosterol to cholesterol has been found to induce oocytes to resume meiosis.[236] One of these sterols, 4,4-dimethyl-5α-cholest-8,14,24-triene-3β-ol, was extracted from human follicular fluid and named follicular fluid meiosis–activating substance (FF-MAS). A related compound, 4,4-dimethyl-5α-cholest-8,24-diene-3β-ol, was isolated from bull testis and called T-MAS. These compounds are synthesized from lanosterol by P450 14α-demethylase, which is encoded by the *CYP51* gene.

FF-MAS and T-MAS are present in micromolar concentrations in preovulatory follicle follicular fluid: 1.6 μM for FF-MAS and about half that for T-MAS.

The accumulation of FF-MAS and T-MAS in mature follicles may be the result of increased synthesis as well as inhibition of cholesterol synthesis at steps beyond the formation of FF-MAS and T-MAS. Gonadotropins have been reported to cause a several-fold increase in *Cyp51* gene expression in rodent ovaries, which could contribute to enhanced MAS formation.[237] Additionally, progestins at concentrations found in follicular fluid in the preovulatory period block cholesterol synthesis at late steps, which would result in an accumulation of FF-MAS and T-MAS.

When perfused into rodent ovaries, FF-MAS can induce maturation of cumulus cell–deprived oocytes or oocyte maturation. However, experiments using various inhibitors of sterol synthesis—including drugs that block 14α-demethylase and those that inhibit enzymes that metabolize MAS—have yielded conflicting results. Inhibitors of 14α-demethylase block gonadotropin-stimulated, but not spontaneous meiosis in rodents, whereas drugs that block MAS metabolism generally result in germinal vesicle breakdown of cumulus-enclosed oocytes. Consequently, the physiological roles of FF-MAS and T-MAS in oocyte maturation, if any, remain uncertain. The pharmacological value of FF-MAS and T-MAS also is uncertain. Some, but not all, studies on *in vitro* oocyte maturation suggest effects of these compounds on maturation by stimulating progression to metaphase II or increasing the survival of oocytes without affecting maturation.

Cytoplasmic Maturation

A process of "cytoplasmic maturation," less obvious than the morphological changes of nuclear maturation, also occurs subsequent to the LH surge and is critical for successful egg activation and preimplantation embryo development should the egg be fertilized. At the ultrastructural level, there are changes in the distribution of the organelles, with movement of the endoplasmic reticulum, mitochondria, and cortical granules toward the oocyte cortex.[237a] The organelle movement is mediated by both microtubules and microfilaments, and depends on the presence of a fibrous structure in the oocyte cytoplasm known as "cytoplasmic lattices."[238,239] The Golgi complex breaks down, explaining the extensive decline in the ability of the mature egg to synthesize new proteins. With movement of chromatin to the cortical region, the oocyte becomes highly asymmetric. The actin cytoskeleton is altered, with a thickening of cortical actin overlying the metaphase II spindle. This region of the plasma membrane is devoid of microvilli—unlike the rest of the oocyte plasma membrane, which is enriched with microvilli. This loss of microvilli may reduce the chance of sperm entering the region of the metaphase II spindle, potentially interfering with the normal progression of meiosis.

The transition metal zinc is an essential element that serves both structural and catalytic roles within numerous cellular proteins such as zinc-finger transcription factors and metalloenzymes. Extensive zinc accumulation occurs during meiotic maturation, with an overall increase in oocyte zinc content of about 50%.[240] Reduction of available zinc in prophase I-arrested, germinal vesicle intact oocytes causes resumption of meiosis by perturbing the function of the zinc-binding protein FBXO43 (previously known as EMI2), and also causes abnormalities in cortical reorganization leading to diminished cell polarity.[241,242] The mechanisms mediating zinc uptake and storage in the oocyte are not yet defined, but likely involve plasma membrane zinc transporters and sequestration in subcellular compartments.

At the molecular level, cytoplasmic maturation is accompanied by the recruitment of specific dormant maternal mRNAs that are translated into protein. In rodents, examples of these recruited mRNAs are *Mos*, tissue plasminogen activator *(Plat)*, and the inositol 1,4,5-triphosphate receptor, type 1 *(Itpr1)*. As mentioned above, translation of *Mos* is critical for blocking cell cycle progression beyond metaphase II. Studies in the mouse suggest that the maturation-associated increase in IP3R-I protein is important for successful egg activation by increasing the ability of the egg to exhibit calcium oscillations.[243] The role of tissue plasminogen activator has yet to be clarified.

The molecular mechanism by which maternal mRNAs are recruited is cytoplasmic polyadenylation. Specific nucleotide sequences in the 3′ untranslated region of these mRNAs, known as *cytoplasmic polyadenylation elements*, direct the binding of poly(A) polymerase to those mRNAs and the addition of poly(A) tracts. Among the proteins that regulate this process are cytoplasmic polyadenylation binding element protein 1 (CPEB1) and deleted in azoospermia-like (DAZL).[244,245] Polyadenylation leads to the association of specific maternal mRNAs with polysomes, translation of the mRNAs, and consequently to increases in the levels of the encoded proteins.

Post-translational modification of cytoplasmic proteins also occurs during oocyte maturation. For example, microtubules undergo alterations in acetylation during the transition from metaphase I to metaphase II.[246] Microtubule acetylation is essential for proper organelle positioning and movement, possibly by affecting interactions between tubulin and cytoplasmic lattices.[239] In addition, phosphorylation and dephosphorylation of cytoplasmic proteins, particularly those involved in regulating the cell cycle, are required for successful cytoplasmic maturation.

Atresia

Atresia occurs at all stages of follicular development, spontaneously or in response to environmental factors or drugs.[247,248] Spontaneous atresia is primarily a reflection of the absence of essential trophic factors at critical times in follicular formation or maturation (e.g., FSH, IGFs, and other growth factors).[249,250] In fetal ovaries, apoptotic cell death is primarily responsible for elimination of germ cells. In adult quiescent follicles, the oocyte and granulosa cells are both involved in apoptotic death, whereas in growing follicles it is the granulosa cells that first undergo changes leading to atresia.[251] In growing follicles undergoing atresia, apoptotic granulosa cells begin to accumulate, whereas apoptosis is not evident in the oocyte or theca.[253]

The importance of apoptosis in the control of follicular dynamics is illustrated in the phenotypes of mutant mice. Mice lacking acid sphingomyelinase, an enzyme that produces the proapoptotic signaling molecule, ceramide, have

an enlarged oocyte reserve and are resistant to oocyte depletion by anticancer drugs and radiation. Fas-deficient mice (*lpr/lpr* mice) have increased numbers of secondary follicles, reduced numbers of large antral follicles, and defective oocyte and granulosa cell death in response to FAS ligand. The pattern of expression of FAS antigen in the human ovary, with abundant staining in oocytes of atretic primordial and primary follicles and granulosa cells of atretic antral follicles, is consistent with a role for Fas in atresia.[169] In mice lacking the proapoptotic protein, BAX, oocyte reserve is augmented due to reduced postnatal apoptosis and defective granulosa cell apoptosis.[252] Conversely, mice lacking the antiapoptotic protein, BCL-2, have a diminished oocyte reserve. The same is true for mice lacking *Bcl-w* or mice engineered with a hypomorphic *Bcl-x* allele. Mice lacking the death effector enzymes caspase-2, caspase-9, and caspase-11 have enlarged oocyte reserves as a result of attenuated fetal germ cell apoptosis. Caspase-12–deficient mice are resistant to anticancer drug–induced germ cell death and caspase-3–deficient mice display aberrant atresia as a result of defective granulosa cell apoptosis. In addition, TNF-α and TNF-α-related apoptosis inducing ligand (TRAIL) are implicated in apoptosis of granulosa cells in follicles undergoing atresia (Fig 9.18).

The kinases, PI3K and AKT, have emerged as central anti-apoptotic signaling molecules. They exert their influence through phosphorylation of the forkhead transcription factors FOXO1 and FOXO3, which when phosphorylated are excluded from the nucleus. When dephosphorylated, these factors activate transcription of pro-apoptotic genes including FAS ligand and pro-apoptic proteins of the BCL2 family, ultimately leading to activation of caspases (caspases 8, 9, and 3).[253]

Spontaneous Twinning and Spontaneous Ovarian Hyperstimulation Syndrome

Spontaneous dizygotic twinning, a result of multiple follicular maturation, is associated with elevated FSH levels, is more prevalent in older women, and is also a genetic trait.[254] The highest rate of spontaneous dizygotic twinning has been recorded in the Yoruba people of Nigeria, with an incidence fourfold greater than in white individuals.[255] The genes governing this trait, which presumably control either FSH levels or follicle sensitivity to FSH, have not yet been identified. Putative mutations and variants in several obvious candidate genes—including the inhibin α subunit, and the BMPRIB receptor—have been excluded from being major contributors to familial dizygotic twinning. However, a potential functional variant in the 5′-untranslated region of the FSH receptor gene that segregated with the dizygotic twinning has been reported. Rare genetic variants/mutations in the *GDF9* gene have been reported in mothers of dizygotic twins, but a causal link has not been established. There is no evidence that mutation in *BMP15* or its cognate receptors are associated with dizygotic twinning in women.[254, 256-258] However, this finding has yet to be confirmed, and more recent studies suggest complex inheritance patterns for dizygotic twinning, at least in Caucasian populations.[259]

Spontaneous ovarian hyperstimulation syndrome is exceedingly rare. It is associated with mutations in the FSH receptor gene that result in a nonsynonymous amino acid

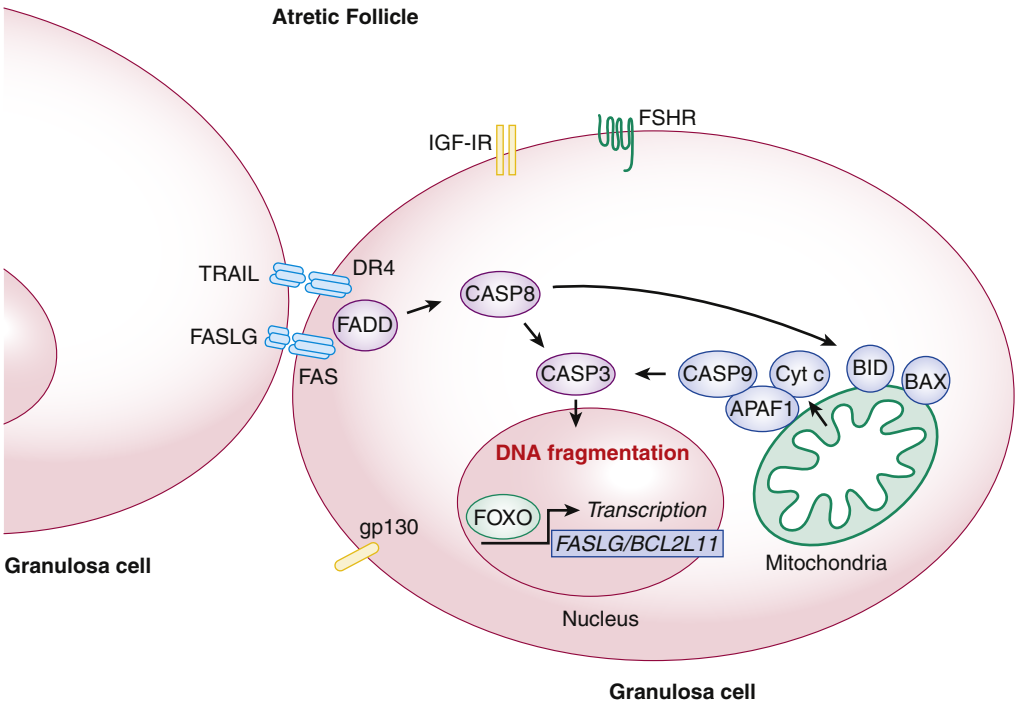


FIGURE 9.18 Factors involved in granulosa cell apoptosis. When cell survival signals such as FSH or insulin-like growth factos (IGF) decline, forkhead transcription factors (FOXO) are dephosphorylated and enter the nucleus where they stimulate production of pro-apoptotic genes including Fas ligand (FASLG) and BCL2LII. Death ligand receptors (FASLG-FAS and TRAIL-DR4) expression on the plasma membrane is increased. This triggers a cascade leading to activation of caspases (CASP) including via the release of cytochrome C from mitochondria, resulting in nuclear DNA fragmentation. *(Modified from Matsuda et al.* J Reprod Develop *58: 44–50, 2012.)*

substitutions (e.g., D576N) that broaden its ligand specificity to include hCG and TSH. Five mutations have been identified, all located in the seven transmembrane domains of the receptor.[260]

Corpus Luteum Formation, Function, and Demise

Initial Stages of Corpus Luteum Formation

After ovulation, the ruptured follicle is reorganized into the corpus luteum. The process of luteinization and formation of a corpus luteum is associated with significant alterations in gene expression, encompassing hundreds of different genes in the granulosa cells alone.[261] A prominent feature of this reorganization is the establishment of a rich vascular network. The hemorrhage into the ovulatory cavity associated with follicular rupture is accompanied by proliferation and penetration of capillaries and fibroblasts from the surrounding stroma. The resulting neovascularization of the developing corpus luteum makes it possible for large blood-borne molecules, such as LDL, which provides cholesterol substrate for progesterone production, to reach the granulosa and theca–lutein cells, and for secretory products to be efficiently transported into the circulation. The development of the corpus luteum blood supply thus parallels progesterone production. By the time the corpus luteum is fully formed, endothelial cells make up approximately 50% of the cellular content.

The vascularization of the corpus luteum is directed by angiogenic factors, including VEGF and basic FGF, which are triggered by LH.[262-264] There is a sixfold rise in VEGF in monkey follicular fluid within 6 hours of an ovulatory stimulus, with sustained elevations in VEGF concentrations lasting up to 36 hours. The granulosa cells appear to be a primary source of VEGF. Because there are no significant changes in VEGF mRNA during this time, the rise in VEGF protein appears to be a consequence of a post-transcriptional mechanism.

Evidence for an important role for VEGF in the development of the corpus luteum vascular network includes the finding that soluble VEGF receptor (FLT-1) administered to gonadotropin-treated rats almost completely suppresses luteal angiogenesis. The Notch ligand, delta-like ligand 4, which negatively regulates VEGF-mediated vessel sprouting and branching, plays a role in the development of the corpus luteum vasculature since *in vivo* neutralization of this factor results in enhanced angiogenesis and vascular density.

Moreover, as noted previously, VEGF appears to play an important role in follicular development before the LH surge because inhibition of VEGF by neutralizing antibodies or truncated FLT-1 interrupts preovulatory follicle development. The angiopoietins and the TIE-2 receptor expressed on endothelial cells also appear to contribute to the development and maintenance of the luteal vascular network, based on their spatial and temporal patterns of expression.

The mural granulosa cells undergo significant morphological changes in response to the LH surge, collectively referred to as *luteinization*. The mitotic potential of these cells is lost, as reflected in changes in the genes involved in granulosa cell proliferation: cyclin D2 expression is terminated, whereas cell cycle inhibitors *p21cip* and *p21kip* are increased.[265] The expression of genes encoding proteins involved in progestin synthesis (including *STARD1*, and *3BHSD2*) is dramatically increased as well.

The human steroidogenic cells of the corpus luteum are heterogeneous in size and function.[266-268] Luteinized granulosa and theca cells are both present. These two different cell types have different functional characteristics, as defined by immunohistochemistry and studies of their steroidogenic activity after purification. The granulosa–lutein cells display greater basal production of progesterone and are the presumed sites of luteal estrogen synthesis because they express aromatase. The theca–lutein cells have 17α-hydroxylase/17,20 lyase activity. These cells presumably produce the androgen precursors that are aromatized by granulosa–lutein cells, and they are probably the main sites of luteal 17α-hydroxyprogesterone production. Thus, a two-cell system for estrogen synthesis exists in the corpus luteum as it does in the follicle.

With respect to progesterone production, there are differences in the characteristics of cells of different sizes within the corpus luteum and their *in vitro* responses to trophic stimulation. These variations have been shown by studies on dispersed cell preparations purified on density gradients.

Relaxin, a hormone that may play a role in facilitating decidualization of the endometrium, suppression of myometrial contractile activity, and the maternal adaptation to pregnancy is produced by the large luteal cells of the corpus luteum from one of the two genes encoding this hormone (*RLN2* gene).[269] Immunohistochemical studies show progressive accumulation from the early to late luteal phases, with corpora lutea in the late luteal phase containing the most intensely stained cells. The highest circulating levels of relaxin are achieved in the first trimester, and levels then decrease by approximately 20% and remain constant throughout pregnancy.

The Role of Luteinizing Hormone

In addition to inducing ovulation and luteinization, LH has a central role in the maintenance of corpus luteum function. Long-term withdrawal of LH support in a variety of experimental circumstances almost invariably results in luteal regression. Withdrawal of LH during the monkey luteal phase, either by passive immunization or by termination of gonadotropin-releasing hormone (GnRH) infusions in animals dependent on GnRH to drive gonadotropin secretion, leads to a pronounced decline in progesterone and other steroid hormone levels.[270] Administration of a GnRH antagonist to women in the luteal phase also causes a marked fall in peripheral progesterone levels within 6 hours of treatment (Fig. 9.19). In the monkey model, luteal progesterone production is restored when LH levels recover if the LH deprivation has not been prolonged. The trophic role of LH in controlling luteal progesterone secretion is evident during the human mid- and late luteal phases, when LH secretion is characterized by distinct pulses and a corresponding pulsatile pattern of progesterone secretion is observed.[271]

It is notable that the monkey corpus luteum can be deprived of LH support for several days but can recover its endocrine function when LH is restored, remembering its temporal history and surviving for the usual 14-day duration.[270] This intrinsic memory suggests that the process

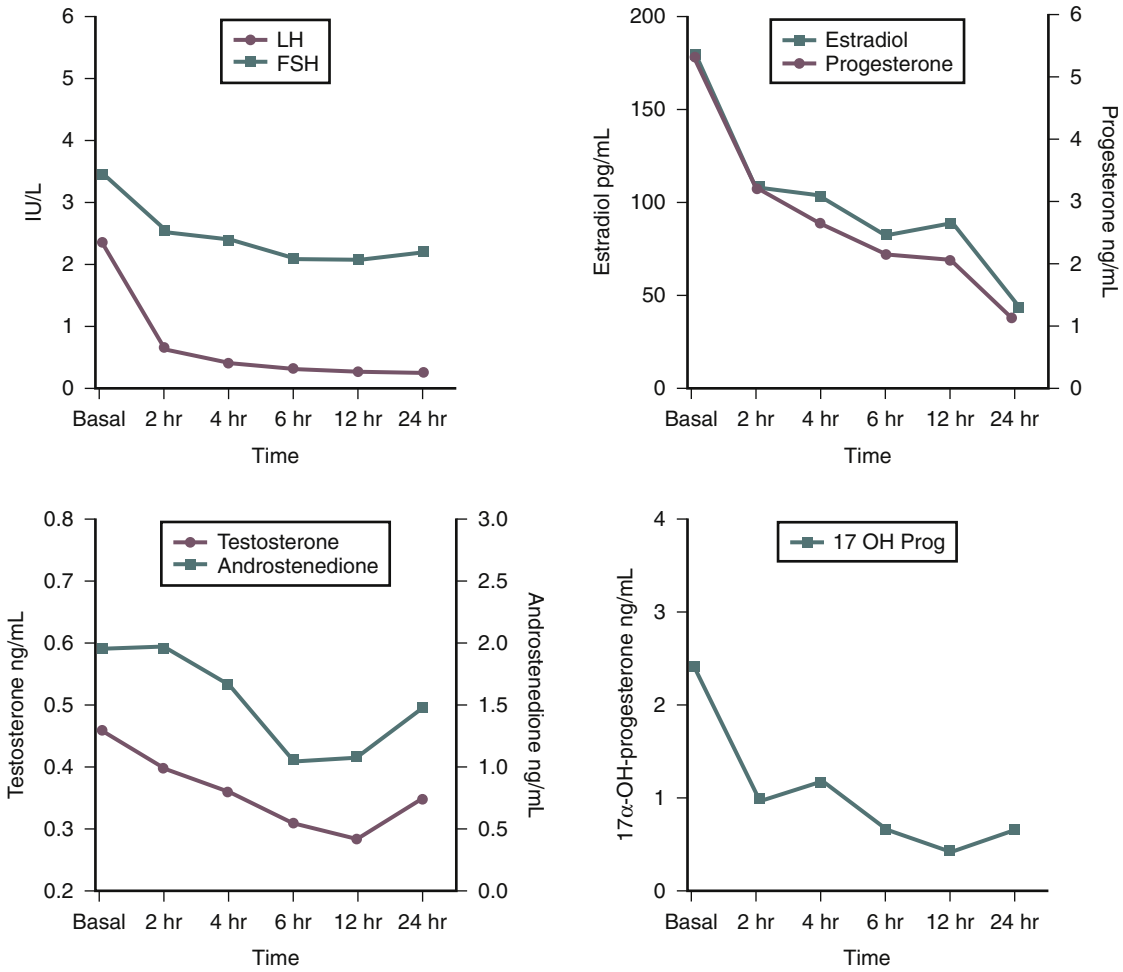


FIGURE 9.19 Effects of the gonadotropin-releasing hormone receptor antagonist cetrorelix (2 mg subcutaneously) on function of the midluteal phase corpus luteum. Plasma gonadotropin and steroid concentrations in a woman receiving cetrorelix in the midluteal phase. FSH, follicle-stimulating hormone; LH, luteinizing hormone.

of luteinization triggers a preprogrammed life cycle that will play out in the absence of conception. The molecular and cellular mechanisms of this clock remain to be elucidated. One hypothesis is that the timekeeping is the result of sequential events that include steroid modulation of infiltration of leukocytes and immune cells that eventually suppress luteal function through the elaboration of cytokines.

Levels of LH/CG receptors in corpus luteum membranes rise progressively during the human luteal phase and then decline, but remain detectable by binding assays, even in the late luteal phase. This receptor is evidently fully engaged immediately after the endogenous LH surge, because injection of 10,000 IU hCG within the first few days after ovulation does not elicit a substantial increase in progesterone production.[272] However, by the mid- and late luteal phases, the corpora lutea show substantial steroidogenic responses to exogenous hCG. LH and hCG receptor mRNA expression tends to follow the same pattern as LH and hCG binding, with an increase in transcript abundance from the early to midluteal phases and a decline at the time of menses.[273,274] However, LH and hCG receptor transcripts are maintained should pregnancy occur.[275] In the Rhesus monkey, LH receptor mRNA expression is maintained in the late luteal phase and only declines after menstruation.[276-279]

Progesterone as a Luteotropin

The human corpus luteum produces 25 to 50 mg of progesterone daily. The luteal cells also appear to respond to this steroid, so it has both an endocrine and an intracrine role in reproduction. In the Rhesus monkey, the progesterone receptor antagonists mifepristone and HRP2000 inhibit hCG-stimulated progesterone and relaxin secretion by human granulosa–lutein cells.[280] Moreover, the reduction in the expression of STARD1 in the Rhesus monkey corpus luteum following administration of a GnRH-antagonist can be prevented by treatment with the progestin, R5020. Thus, the actions of LH and hCG on STARD1 expression may be, in part, indirect and attributable to the action of progesterone.[281]

Progesterone receptors, both the A and B forms, are present in the Rhesus monkey and human corpus luteum, with progesterone receptor mRNA increasing from the early to the midluteal phase. The ratio of progesterone receptor B to progesterone receptor A increases from the early to the late luteal phase. The above-noted actions of progesterone receptor antagonists on lutein cell steroidogenesis are

presumably a reflection of altered transcription regulated by these nuclear receptors.

Luteolysis

The functional life span of the corpus luteum in a nonfertile cycle is normally 14 ± 2 days. Unless pregnancy occurs, the corpus luteum is transformed into an avascular scar referred to as the *corpus albicans*. Regression of the corpus luteum, known as *luteolysis*, encompasses functional changes (i.e., endocrine changes, most prominently a decline in progesterone production) as well as structural changes (i.e., apoptosis and tissue involution).

Withdrawal of LH and a decline in LH receptors do not account for luteolysis in primates. However, there is a postreceptor loss of LH and hCG signaling efficiency that is reflected by the reduced response of the primate corpus luteum to hCG stimulation.[277-279] This reduced signaling efficiency in the late luteal phase leads to a decline in progesterone production associated with a fall in expression of the *STARD1* gene, in terms of both mRNA and protein.[272]

The decline in *STARD1* gene expression precedes a fall in expression of other steroidogenic enzymes. Administration of a large dose of hCG in the late luteal phase restores *STARD1* mRNA and protein levels to those found in the midluteal phase and causes a dramatic increase in plasma progesterone levels.[272] Infusion of exponentially increasing doses of LH or hCG also prolongs the life of the monkey corpus luteum.[278] These observations suggest that one important feature of functional luteolysis in the human is a decline in *STARD1* expression. High levels of hCG can prevent this decline, maintaining progesterone-producing capacity.

Microarray studies of the Rhesus monkey corpus luteum in which luteal regression was induced by a variety of methods (i.e., GnRH antagonist, prostaglandin F2α[PGF2α]) and compared to corpora lutea undergoing natural luteolysis revealed no consistent expression signature, although mRNA abundance for genes involved in cholesterol uptake and synthesis tended to be reduced.[282]

Structural regression of the corpus luteum is brought about by two processes: apoptosis and autophagolysis. Early corpora lutea show no evidence of DNA fragmentation, whereas midluteal and late luteal corpora display DNA fragmentation; the frequency of apoptotic cells is increased in the regressing corpus luteum compared with the midluteal phase. In contrast, the corpora lutea of early pregnancy show no detectable apoptotic DNA fragmentation.

The factors that control cell survival and death in the human corpus luteum remain a subject of debate.[279-289] BCL2, a cell survival factor, has been localized in granulosa–lutein cells, theca–lutein cells, endothelial cells, and blood vessels. Some researchers find no evidence of changes in BCL2 levels during the normal luteal phase or after hCG administration; however, others describe substantial changes with a decline in the late luteal phase. The proapoptotic protein BAX has been reported to remain unchanged throughout the luteal phase—and to increase from low levels in the midluteal phase to high levels in regressing corpora lutea, while being undetectable in corpora lutea of pregnancy. As described later in this section, expression of FAS and FAS ligand increases at the time of luteal regression. The existing data indicate that apoptosis is an important feature of human luteal regression, and some reports describe reciprocal changes in the expression of cell survival (BCL2) and proapoptotic (BAX and FAS) genes; however, morphological studies strongly suggest that autophagy contributes to luteal regression. These two processes may, of course, coexist in the regressing corpus luteum.

What triggers the diminished sensitivity of the primate corpus luteum to LH and subsequent luteal regression in a nonfertile cycle? Although PGF2α is a recognized luteolysin in animals, its role in regulating primate corpus luteum regression is less certain.[283] *In vitro*, PGF2α reduces gonadotropin-stimulated progesterone secretion by luteinized granulosa cells. PGF2α also suppresses *STARD1* gene expression in cultured human luteal cells. *In vivo*, infusion of PGF2α transiently reduces progesterone levels during the luteal phase in humans, and intraluteal injection of PGF2α causes a fall in progesterone production and tissue involution. Human corpora lutea in the late luteal phase express higher levels of the receptor for PGF2α and have higher PGF2α contents than in the early luteal phase. Moreover, the inhibitory effects of PGF2α on hCG-stimulated progesterone production are most notable in the late luteal phase. Collectively, these observations speak for a potential role for PGF2α in the initiation of human luteolysis, perhaps in the inhibition of luteal progesterone production. However, this is probably not the only mediator of primate corpus luteum regression.

Unlike domestic species, in which PGF2α of uterine origin is involved in triggering luteal regression, hysterectomy in the human has no effect on luteal life span.[283] Thus, if prostanoids are involved in human luteal regression, they are not of uterine origin. The corpus luteum itself may be the source. In the monkey, estrogen promotes luteolysis and also raises PGF2α levels in ovarian blood. The luteolytic action of estrogens is reportedly blocked by indomethacin; however, other researchers have suggested that the luteolytic action of estradiol in the primate is mediated by effects on gonadotropin secretion.

There is good evidence that cytokines of the TNF-α superfamily and interferon γ play roles in human luteolysis.[283] The temporal and spatial expression of FAS and FAS ligand mRNA and protein is strongly correlated with luteolysis in animal and human corpora lutea. The FAS-FAS ligand system can trigger apoptotic cell death. During the late luteal phase, the expression of FAS protein increases, diminishing only as the structure transitions into a corpus albicans.

Tumor necrosis factor-α inhibits steroidogenesis by human luteal cells *in vitro*.[283,284,286] TNF-α, derived from macrophages and leukocytes and also possibly endothelial cells, increases in the late luteal phase corpus luteum.[286] TNF-α–producing macrophages and leukocytes accumulate in the late luteal phase in regressing corpora lutea, probably due in part to up-regulation of monocyte chemoattractant protein-l (MCP-1) derived from endothelial cells.[283,287] Other proinflammatory cytokines produced by leukocytes could also contribute to the inhibition of steroid production. Interferon γ inhibits gonadotropin-stimulated progesterone production by human luteal cells *in vitro* and also induces cell death. It is another product of macrophages and leukocytes that could contribute to functional and structural luteolysis. As noted in the earlier discussion of ovarian leukocytes and lymphocytes, abundant regulatory T lymphocytes may be critical to sustaining luteal function and an anti-inflammatory state,

and a decline in this population of lymphocytes is thought to promote an inflammatory environment in which cytokines suppress luteal cell steroidogenesis.[127]

The vascular component of the corpus luteum may contribute to luteal regression through production of factors directly or indirectly involved in the luteolytic mechanism, including TNF-α, endothelin-1, and MCP-1.[283] Effects of luteolytic substances on endothelial cell function, including endothelial cell survival, can affect luteal perfusion. The endothelial cells may also be targets of PGF2α, and their role in the luteolytic process, best exemplified by studies in domestic species, has yet to be comprehensively evaluated in the human corpus luteum.

Reactive oxygen species produced by leukocytes infiltrating the corpus luteum are another potentially important factor in luteolysis.[283,289] Immunosuppressive levels of dexamethasone block luteolysis in a rat model. Both human and rat luteal cells respond to H_2O_2 with a rapid reduction in progesterone secretion and gonadotropin responsiveness. The action of H_2O_2 appears to be mediated by OH^-, which inhibits protein synthesis, depletes ATP, and induces DNA damage. The LH receptor also is uncoupled from adenylate cyclase by H_2O_2, and there is impaired mitochondrial use of cholesterol for steroidogenesis.

Rescue of the Corpus Luteum in the Cycle of Conception

In a cycle of conception, the human corpus luteum is rescued from luteolysis by the appearance of trophoblast-derived hCG.[290] In the corpus luteum of late pregnancy, hCG suppresses apoptosis, with a lesser effect on autophagy, allowing structural maintenance of the gland and sustaining expression of the *STARD1* gene.[198] The progressively rising concentrations of this luteotropin, first detectable in peripheral blood 8 days after ovulation, both stimulates steroidogenesis and prevents structural involution of the gland, which is the major source of progesterone for the first 10 weeks of gestation. The corpus luteum doubles in volume during the first 6 weeks of gestation as a result of hypertrophy of the luteinized granulosa and theca cells and accumulation of connective tissue and nonsteroidogenic cells, particularly endothelial cells.[279] Experimentally, a protocol of ever-increasing doses of hCG rescues both the human and monkey corpus luteum. Recent observations suggest that hCG stimulates luteal 11β-hydroxysteroid dehydrogenase type 1 expression, resulting in increased intraluteal generation of cortisol, which is proposed to act through the lutein cell glucocorticoid receptor to promote corpus luteum survival in the cycle of conception.[291]

The corpus luteum is essential for the first few weeks of pregnancy, and luteectomy results in miscarriage if performed before 7 weeks of gestation. Its secretory function, however, is not sustained at high levels throughout gestation, despite the presence of hCG. This characteristic has been documented by monitoring levels of 17α-hydroxyprogesterone, a steroid that is not produced by the placenta, and therefore largely reflects corpus luteum function. Levels of 17α-hydroxyprogesterone rise to peak concentrations at 6 weeks of pregnancy and then decline. Part of the decline in steroidogenic activity is due to the fact that the early hypertrophy of the corpus luteum is later followed by shrinkage. The biochemical changes underlying the functional and structural changes in the corpus luteum of pregnancy have not been elucidated.

The corpus luteum of pregnancy also secretes protein hormones, including inhibin A and relaxin. Relaxin may function to promote the decidualization of the endometrium and suppress uterine smooth muscle contractile activity, as well as the maternal adaptation to the pregnant state.

Luteoma of Pregnancy and Hyperreactio Luteinalis

Luteomas of pregnancy, rare benign tumors, which usually occur bilaterally from pockets, luteinized cells that develop under the influence of hCG. They appear to be more frequent in women with polycystic ovary syndrome. Androgen production by these tumors can range from modest to significant, causing maternal symptoms of hyperandrogenism and virilization of female fetuses. Progesterone and estrogen production has also been described. These tumors spontaneously regress after pregnancy.

Hyperreactio luteinalis, enlargement of the ovaries caused by theca-lutein cysts, usually in the third trimester, results from high levels of hCG associated with multiple gestations, molar pregnancies or choriocarcinoma. Approximately 30% of cases are associated with maternal symptoms of hyperandrogenism but the fetus appears to be protected through placental aromatization.

Ovarian Aging

With age, there is a decline in both the quantity and quality of the pool of follicles and oocytes.[292] Linear extrapolation of follicular depletion in women with regular menses predicts that, by 50 years of age, each ovary would contain 2500 to 4000 primordial follicles. Because the menopausal ovary is largely devoid of follicles, it has been thought that follicular depletion accelerates in the last decade of reproductive life. Recent re-analysis of the existing data suggests that the loss of follicles is best described by a simple power function with progressive increasing rates of follicle loss (Fig. 9.20). Below a critical number of some thousand follicles, which is reached at a mean age of 45 to 46 years, menstrual irregularity occurs. Thus, if oogonial stem cells truly exist in the human ovary, their capacity to contribute new follicular units is also exhausted. The median age of natural menopause in Caucasian women is 50. In some studies, unilateral oophorectomy and nulliparity are associated with an earlier occurrence of menopause and increasing parity with later menopause. One percent of women experience menopause below the age of 40, and 0.1% below the age of 30. Menopause occurring before the age of 40 is defined as premature ovarian failure.

The postmenopausal ovary, weighing less than 10 grams, is a lusterless structure with a wrinkled surface.[293,294] Morphologically, the main changes in the aging ovary are a decrease in volume and increasing fibrosis of the stroma, with accumulation of connective and scar tissue. A few primordial follicles and follicles undergoing maturation and atresia may be found up to 5 years after the last menses. A reduced vascular network, with smaller vessel lumens and thickening or sclerosis of the vessel walls, is characteristic, leading to diminished ovarian stromal blood flow, as assessed by Doppler ultrasonography.

With aging, the ovarian surface epithelium changes. Papillae and crypts are seen less frequently, and the surface epithelial cells become flatter, with fewer and shorter microvilli. Increased numbers of apoptotic and necrotic cells are observed.

Mechanisms of Natural Ovarian Aging

Ovarian aging reflects the combined interaction of genetic factors that determine the follicular complement, the additive effects of insults secondary to normal cellular metabolism, and environmental factors that affect follicular viability.[295-298] The importance of genetic factors is highlighted by the positive correlation in age of menopause between mothers and daughters, sisters, and monozygotic twins. Studies of sister pairs suggested that approximately 85% of the variation in age of menopause could be attributed to genetics, and as described below, candidate loci for age at natural menopause have been identified. Pre-menopausal hysterectomy has been associated with a twofold risk of ovarian failure. It is not yet known whether the surgery, and possible vascular compromise to the ovaries is the cause, or

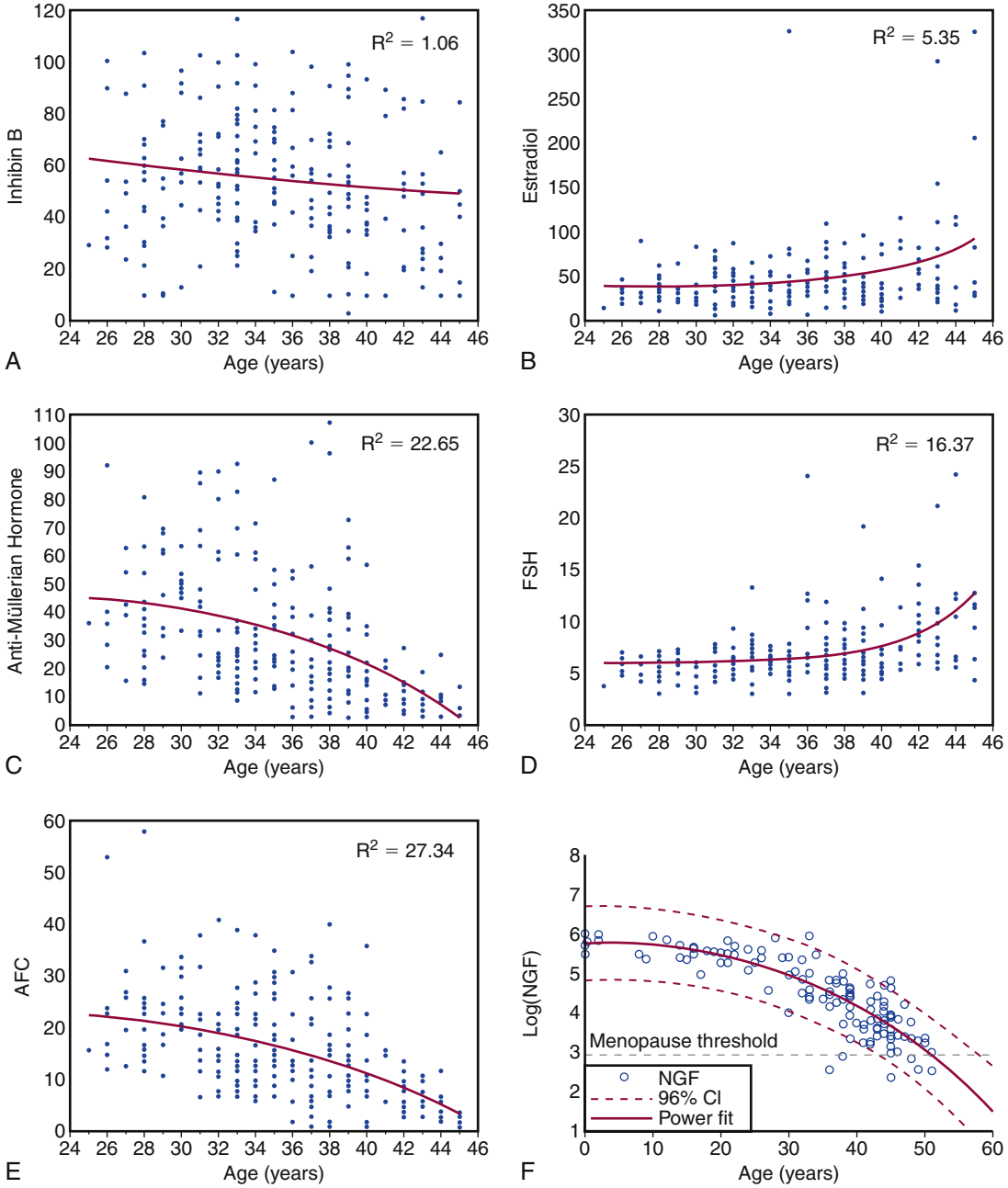


FIGURE 9.20 The power model relationships between ovarian reserve markers and age, with follicle counts of histological ovarian specimens. All but one of the serum markers has a relationship with age that mirrors the histological data (**F**). Serum markers and antral follicle counts (AFCs) show a gradual acceleration of oocyte loss with age, and FSH and estradiol (E2) levels exhibit an upturn with age, but this upturn occurs later than the downturn in follicle number in histological specimens. Inhibin B does not parallel the histological curve. *(Modified from Rosen et al.* Fertility & Sterility *97: 238–243, 2012.)*

whether the association is due to the pathology leading to the hysterectomy.

Oocyte aging is associated with increased meiotic nondisjunction.[296] The explanation for this phenomenon is not yet known, although oocytes apparently become more error-prone with respect to meiosis with age, probably because of a decline in chromosome cohesion. A two-hit model has been offered, which holds that there is a reduced frequency and pattern of recombination in a proportion of oocytes from the beginning; a second hit associated with an increased frequency of nondisjunction results from age-related damage due to oxidative stress and impaired microcirculation around the selected follicles. Hypo-oxygenation of the preovulatory follicle, assessed by the dissolved oxygen content of follicular fluid and hypoxia-responsive genes (e.g., VEGF), is associated with a high frequency of oocyte cytoplasmic defects, impaired postfertilization cleavage, and chromosome segregation defects. Centromeres have been reported to undergo a putative premature division at meiosis I in oocytes collected from older women. Moreover, examination of oocytes from older women at meiosis II showed a diffuse spindle and lack of bipolarity, with chromosomes irregularly and loosely attached to the spindle. These observations suggest that irregularities in the meiotic apparatus, including loss of key proteins in the spindle assembly checkpoint and chromosome cohesive ties, predispose aging oocytes to nondisjunction.[296]

Spontaneous deletions in the mitochondrial genome accumulate in muscle during aging, accelerating between the ages of 30 and 40 years. These deletions have been associated with a number of diseases and pathological processes, possibly arising as a consequence of damage resulting from the oxidative activity of mitochondria that is not adequately defended by cellular antioxidant mechanisms. Luteinized granulosa cells collected from women older than 38 years have a greater frequency of mitochondrial DNA deletions than those from women younger than 38 years of age.

Accumulation of these deletions in the oocyte mitochondria is another potential cause of ovarian aging. Indeed, the common delta mtDNA4977 deletion is found in 33% to 50.5% of oocytes, but in a smaller number of embryos (8% to 32.5%). This finding suggests that oocytes harboring these mitochondrial DNA deletions have an impaired ability to support embryonic development. Moreover, oocytes from older women have been found to be more likely to contain mitochondrial DNA deletions than those of younger women in some studies. Oxidative stress is hypothesized to be a cause of these DNA deletions, as well as induction of apoptosis. However, oxidative stress markers assessed in follicular fluid (e.g., conjugated dienes, lipid hydroperoxides, thiobarbituric acid–reactive substances) have not been correlated with the reproductive potential of oocytes in the setting of an *in vitro* fertilization/embryo transfer program. Shortened telomeres have also been found in women who experience early menopause.

Cigarette smoking represents an environmental factor that is associated with increased ovarian aging.[292,297] Smoking reduces the size of the ovarian follicular pool and advances the age of menopause by 2 years. It is also associated with reduced spontaneous fertility, poorer outcomes in assisted reproduction, and an increased incidence of trisomy 21. Follicular fluid cotinine levels, measures of a woman's exposure to cigarette smoke, are positively correlated with lipid peroxidation and negatively correlated with antioxidant activity.[299] Because inhibitors of oxidative stress mimic the ability of FSH to prevent follicular apoptosis, the shift in peroxidant and antioxidant activities in the follicular fluid of smoke-exposed women suggests a mechanism underlying smoking-associated ovarian aging. Smoking also may lead to the accumulation of mitochondrial DNA deletions as a result of oxidative stress. In an animal model, polycyclic aryl hydrocarbons, a component of cigarette smoke, up-regulated expression of the proapoptotic gene *Bax* in the ovary, resulting in follicular atresia.[300] Double-stranded genomic DNA breaks, as caused by chemotherapeutic agents may also contribute, which is consistent with genetic evidence for DNA repair pathways having a major role in controlling age of menopause.[301]

Genes Influencing the Age at Natural Menopause

Recent genome-wide association studies have revealed genetic loci associated with age of natural menopause as well as early menopause/ovarian insufficiency. Initial reports described loci on 20p12.3, 19q13.42, 13q34, 5q35.2, and 6p24.2. A subsequent meta-analysis of 22 genome-wide association studies confirmed some of these and added additional candidate loci with a statistical significance meeting genome-wide threshholds. Among the candidate genes identified to date are those involved in DNA replication and damage repair, hormone production and action, and immune function (Table 9.3). These include *EXO1*, *HELQ*, *UIMC1*, *FAM175A*, *FANCI*, *TLK1*, *POLG*, *MCM8*, and *PRIM1* in the realm of DNA repair, and *IL11*, *NLRP11*, and *PRRC2A* in the domain of immune function. Some of these candidates were previously identified in earlier genome-wide association studies *(MCM8)* or implicated in primary ovarian insufficiency, early menopause, or ovarian development (e.g., *FANC1*, *POLG*). The signaling pathways encompassed in a network analysis of the candidate genes included

Table 9.3 SNPs Strongly Associated with Age at Menopause

SNP	Chromosome	Gene	Feature
rs4246511	1	*RHBDL2*	Intron
rs1635501	1	*EXO1*	Intron
rs2303369	2	*FNDC4*	Intron
rs10183486	2	*TLK1*	Intron
rs4693089	4	*HELQ*	Intron
rs890835	5	*RNF44*	Intron
rs365132	5	*UIMC1*	Coding (synonymous)
rs2153157	6	*SYCP2L*	Intron
rs1046089	6	*PRRC2A*	Missense
rs2517388	8	*ASH2L*	Intron
rs12294104	11	-	-
rs2277339	12	*PRIM1*	Missense
rs3736830	13	*KPNA3*	Intron
rs 4886238	13	*TDRD3*	Intron
rs2307449	15	*POLG*	Intron
rs10852344	16	-	-
rs11668344	19	*TMEM150B*	Intron
rs12461110	19	*NLRP11*	Missense
rs16991615	20	*MCM8*	Missense

Adapted from Stolk et al., *Nature Genetics* 44: 260–268, 2011.

inflammation (NF-κB) and mitochondrial dysfunction, both of which participate in cell death.[302-308]Although these findings represent a significant advance in our understanding of heritable factors determining reproductive lifespan, the functional genetic variants that contribute to the age of menopause in the candidate genes noted above, their mechanism of action, and their interactions with environmental factors and gonadotoxic agents, that affect ovarian reserve, remain to be determined.

Genes Implicated in Primary Ovarian Insufficiency

Primary ovarian insufficiency, also known as premature ovarian failure, is a complex, multifactorial disorder defined as cessation of ovarian function below the age of 40 in association with hypergonadotropic (elevated FSH levels) hypogonadism (diminished estrogen levels). It can be familial, with variable expression of the ovarian phenotype, syndromic (e.g., Turner Syndrome, autoimmune polyglandular syndrome 1, galactosemia), nonsyndromic, as well as iatrogenic. Approximately 25% of cases of primary ovarian insufficiency are iatrogenic, attributable to gonadotoxic therapies, and 50% are idiopathic.

Genes on the X Chromosome

Normal ovarian development requires the activity of autosomal genes and the presence of two functional X chromosomes. In a study of a cohort of more than 500 Chinese women with premature ovarian insufficiency, 12.1% were found to have X chromosome structural abnormalities, X-autosome translocations and X aneuploidy.[309] Although the requirement for two X chromosomes for ovarian development has long been recognized, the essential X-linked genes and their functions are largely unknown. Cytogenetic studies showed that terminal deletions from Xp11 to Xp22.1 are associated with primary amenorrhea, and deletions from Xq13 to Xq27 usually coincide with primary amenorrhea or primary ovarian insufficiency.[310] The latter stretch of the X chromosome is considered to be the critical region for normal ovarian function. Based on the study of interstitial deletions and breakpoints, this critical region has been subdivided into two domains encompassing Xq13-21 and Xq23-Xq27.[311] Deletions in these critical regions were thought to cause primary ovarian insufficiency because of haploinsufficiency of critical genes. However, with the exception of reports of rare mutations, definitive evidence for a key role for genes in or near disrupted loci is lacking. One example of an implicated X chromosome gene is *POF1B*, located at Xq21, which encodes a nonmuscle actin binding protein. Ovarian insufficiency was observed in members of a family homozygous for a point mutation in the actin-binding region of POF1B. In the absence of strong documentation of disrupted function of specific genes in the critical region, thinking has turned to the possibility that these structural abnormalities interfere with ovarian function as a result of aberrations in chromosome dynamics, or positional or epigenetic effects on flanking genes, including autosomal genes in translocations.[310-313]

A "premutation" in the fragile X syndrome gene *(FMR1)* on Xq27.3, which encodes an RNA-binding protein, FMRP, that controls mRNA stability, is associated with nonsyndromic primary ovarian insufficiency. The underlying mechanism of ovarian dysfunction resulting from the premutation has yet to be determined, although recent findings suggest that the premutation RNA causes a reduction in the number of growing follicles acting through Akt and mTOR.[314,315] The *FMR1* premutation is an expansion of a cytosine-guanine-guanine (CGG) repeat in the 5′-untranslated region from 56 to 200 copies, which reduces translation of the FMR1 mRNA. Approximately 20% of women carrying the premutation have primary ovarian insufficiency. Interestingly, when there are greater than 200 copies of the CGG repeat, FMR1 expression is silenced as a result of methylation of the CpG islands, causing fragile X syndrome, but not primary ovarian insufficiency. Population studies suggest that the number of premutation CGG repeats is not linearly correlated with the extent of ovarian dysfunction, and carriers of 80 to 100 CGG repeats are at higher risk of primary ovarian insufficiency. Other genes and environmental factors such as smoking are thought to influence the ovarian phenotype in premutation carriers. Microdeletions in the *AFF2 (FMR2)* gene on Xq28, approximately 600 kb distal to *FMR1*, have been associated with primary ovarian insufficiency, although the frequency of these mutations in women with ovarian insufficiency and the pathophysiological mechanism underlying this association are not known.[316] Some researchers have suggested that analysis of *FMR1* alleles could identify women with reduced ovarian reserve, but this approach is not recommended as a primary screening tool.[317]

Genes lying on the short arm of the X chromosome that may have a role in human ovarian function include *BMP15*, which lies on Xp11.2. It is a candidate gene for ovarian insufficiency, based on its involvement in follicular arrest in the sheep. A number of rare variants in *BMP15*, possibly mutations, causing nonconservative amino acid substitutions, have been identified in women with primary ovarian insufficiency or dizygotic twinning. Many of these variants affect the dimerization and post-translational processing of BMP-15 into the bioactive mature protein.[318-321] The affected individuals have been heterozygous for the variants/mutations and it is postulated that these variants might disrupt BMP-15 or GDF-9 signaling by affecting dimerization. However, some of these variants have been identified in normal individuals, so there is uncertainty about the pathophysiological roles of these variants or mutations. The zinc finger gene *ZFX*, on Xp22.1, which is important in ovarian development in the mouse because heterozygous and homozygous mutations are associated with a reduced germ cell number, is another candidate gene for ovarian failure, but little is known with respect to human ovarian biology.[322]

Autosomal Genes

A number of autosomal genes are associated with mostly rare forms of abnormal ovarian—differentiation or ovarian insufficiency due to either failure of normal follicular development, increased loss/destruction of follicles, or inability of follicles to respond to tropic hormones.

Mutations in the *GALT* gene encoding galactose-1-phosphate uridyltransferase, which cause galactosemia, are associated with syndromic primary ovarian insufficiency in almost all women homozygous for inactivating mutations. The loss of germ cells is thought to be due to the toxic effects of accumulated galactose metabolites or deficiency

of galactose-containing glycoproteins or glycolipids, and this occurs despite dietary control.[323]

Primary ovarian insufficiency is associated with the ataxia-telangiectasia resulting from mutations in the *ATM* gene, which functions in DNA repair and cell cycle control. Mutations in *TRIM37* cause mulibrey (muscle-liver-brain-eye) nanism, a peroxisomal disorder characterized by severe intrauterine growth restriction, cardiomyopathy, hepatomegaly, and premature ovarian failure.[324]

Forkhead transcription factor gene *FOXL2* mutations cause a syndromic form of premature ovarian insufficiency, blepharophimosis–ptosis–epicanthus inversus syndrome (BPES) type 1.[325a,326] This phenotype reflects the involvement of FOXL2 in granulosa cell function, including expression of genes involved in ovarian steroidogenesis, apoptosis, detoxification of reactive oxygen species, and cell proliferation. In mice lacking FOXL2, the ovaries are small and disorganized, with abnormal primordial and primary follicle formation, reflecting a granulosa cell abnormality. Oocytes are not initially affected but after birth, there is massive early germ cell atresia. Interestingly, a unique somatic mutation in the *FOXL2* gene is found in more than 95% of adult granulosa-cell tumors, the most common type of malignant ovarian sex cord-stromal tumor.

Mutations in the mitochondrial DNA polymerase gamma *(POLG)* are a rare autosomal dominant cause of sydromic ovarian insufficiency associated with progressive external ophthalmoplegia disorder or Parkinson's disease.[327,328] The *POLG* gene was also identified as a candidate for controlling natural age at menopause.

Mutations in the autoimmune regulator gene *(AIRE)* cause autoimmune polyendocrinopathy syndrome 1, which is associated with ovarian insufficiency due to immune cell-mediated depletion of ovarian follicles.[329-331]

Other rare syndromic forms of primary ovarian insufficiency include Demirhan syndrome, characterized by abnormal skeletal development caused by mutations in *BMPR1B*, encoding the GDF5 receptor; pseudohypoparathyroidism type 1a, caused by mutations in adenylate cyclase imprinted gene *GNAS1*, which occurs when a mutant allele is inherited from the mother leading to generalized hormone resistance; and ovarian leukodystrophy caused by mutations in the *EIF2B* gene.[332]

Genitourinary malformations, including sex reversal and streak gonads, are characteristic of syndromes associated with heterozygous mutations in the *WT1* and *NR5A1* genes.[333,334] Girls homozygous for a mutation in the *PSMC3IP* gene that affects the protein's ability to serve as a coactivator of estrogen receptor-mediated transcription have XX gonadal dysgenesis.[335] This observation provides a possible link between estrogen action in the ovary and follicular function/survival.

Mutations in the follicle-stimulating hormone receptor gene *(FSHR)* represent an infrequent cause of primary ovarian insufficiency with a unique gonadal phenotype. Women with these mutations have absent or poor secondary sexual development, elevated FSH and LH levels, with primordial follicles as well as early growing follicles present in their ovaries. Although some authors have reported that common polymorphisms in the *FSHR* gene are associated with reduced responsiveness to exogenous gonadotropins in stimulated cycles, this association has not been validated in studies on larger cohorts.

Mutations, again extremely rare, in genes known to be essential for ovarian development and folluculogenesis in mice or other animals also cause primary ovarian insufficiency in humans. The genes identified to date include the homeobox gene *NOBOX* and the basic helix-loop-helix transcription factor gene *FIGLA*.[325,332] *GDF9* mutations have been described as a cause of non-syndromic mutations in humans.[336-338] Conversely, mothers of dizygotic twins have an increased frequency of *GDF9* variants and missense mutations in *GDF9* have been found in Chinese women with polycystic ovarian syndrome.[339,340] However, a relationship between *GDF9* variants and twinning and polycystic ovary syndrome has not been replicated, nor has causality between these *GDF9* genetic variants and the phenotypes of twinning and polycystic ovary syndrome been established.

Polymorphisms in the gene encoding the inhibin α subunit (*INHA* 769G>A, A257T) have also been reported by some to be associated with premature ovarian failure, but others dispute this claim.[341,342] It is notable that the genome-wide association studies for natural age at menopause did not detect signals in most of the genes noted above.

Assessment of Ovarian Reserve

A variety of tests of ovarian reserve have been proposed including those that assess basal ovarian function (e.g., antral follicle counts, AMH levels) and those that assess response to a stimulus/challenge (e.g., clomiphene challenge test, GnRH agonist stimulation test) (Fig. 9.20). There is good evidence that some of these tests can predict ovarian response and treatment outcome in the context of assisted reproduction.[292,343-345] Although different measures are employed in some of these tests, the benefit of combining tests that have a strong correlation with ovarian response and clinical outcome is debated.

Fluid-filled follicles ranging in size from 2 to 10 mm (antral follicles) can be detected by transvaginal ultrasonography. The number of these follicles (defined as either those of 2 to 5 mm in size or 2 to 10 mm in size) parallels the number of primordial follicles and early growing follicles that are too small to visualize with current technology. The antral follicle count is highly correlated with chronological age and ovarian response to controlled ovarian stimulation including oocyte yield. However, ultrasonography cannot distinguish normal from atretic follicles.

Elevated day 3 FSH and estradiol levels are predictive of reduced ovarian reserve.[346] Generally accepted criteria for normal ovarian reserve include a cycle day 3 FSH level of less than 10 mIU/mL and an estradiol level of less than 80 pg/mL. However, there are wide variations FSH, with considerable overlap among values measured in older and younger women. Moreover, elevated FSH levels reflect primarily the egg quantity that can be expected in stimulated cycles. This is not directly linked to egg quality, which is primarily affected by aging, although egg quantity and egg quality are frequently linked in older women.[347-350]

Granulosa cell products inhibin B and AMH have been used as biomarkers of ovarian reserve. Inhibin B levels do not show a gradual decline with aging, and the decline when detected appears to be a better late marker of reduced follicle number. Consequently, basal inhibin B levels are not

recommended as a predictor of poor ovarian response to ovarian hyperstimulation.[292]

AMH is produced by granulosa cells of small follicles, and does not show major fluctuations during the cycle. Moreover, because AMH production appears to be independent of gonadotropin secretion, its levels show low intercycle and intracycle variability. Thus, AMH levels can be informative when sampled at random times during the cycle.[292]

Levels of AMH decline with aging, and are highly correlated with the number of antral follicles observed by ultrasonography. Of all of the potential biomarkers of ovarian reserve, AMH is the earliest to change with age, which has led some to propose that AMH levels can predict the time of natural menopause or reproductive potential.[351] In addition, AMH levels have consistently been found to be elevated in women with polycystic ovary syndrome, even in adolescents displaying phenotypes predictive of the disorder. Consequently, AMH has emerged as a valuable marker for ovarian aging, and can be used to predict both hyper- and hypo-responsiveness to controlled ovarian stimulation and ovarian function after gonadotoxic therapies in cancer survivors as AMH levels correlate with extent of gonadal insult.[352,353] Unfortunately, standardized assays for AMH with established normative values are not yet available to clinicians.[354]

Several challenge tests have been developed in an attempt to improve assessment of ovarian reserve and predict response to controlled ovarian stimulation and pregnancy rates. These provocative tests are not recommended for use in routine screening because their accuracy has not been demonstrated to be superior to basal assessments, particularly antral follicle counts and measurement of AMH levels.

The clomiphene citrate challenge test is a provocative assessment of ovarian reserve in which a cycle day 3 FSH level is determined, followed by administration of clomiphene citrate (100 mg/day) on days 5 to 9. A second FSH determination is obtained on cycle day 10. The test indicates reduced ovarian reserve if either initial or final FSH values are elevated. Although this is more cumbersome than "basal function" tests, it can reveal reduced ovarian reserve not detected using basal (day 3) FSH levels.[292]

Another provocative test based on the evaluation of FSH-sensitive follicles 24 hours after administration of a GnRH agonist (GnRH agonist stimulation test) has been evaluated in a small number of studies. This test can be predictive of poor ovarian response and pregnancy in the context of IVF.[292]

A third provocative test, also studied in a limited number of centers, is the response to a single dose of FSH with measurement of estradiol and inhibin B. Some reports suggest a high correlation with the size of the FSH-sensitive follicle cohort and antral follicle counts.[292]

Endocrine Activity of the Postmenopausal Ovary

Although devoid of follicles, the menopausal ovary is not a completely defunct endocrine organ in that it has a variable capacity to produce androgens.[355-357] The postmenopausal ovary is believed to be a source of testosterone, although there may be considerable variation among individuals with respect to androgen production (Fig. 9.21). This may be a reflection of variable numbers or activity of hilar cells. The circulating level of testosterone in postmenopausal women is only slightly lower than that observed in premenopausal women. Serum testosterone decreases approximately 50% after oophorectomy, and there is a significant level of testosterone in the ovarian vein compared with the peripheral blood in postmenopausal women.[358]

The postmenopausal ovary probably contributes no more than 20% of the daily production of androstenedione, with the adrenal being the predominant source. This proposal is supported by the following observations: (1) Serum androstenedione is minimally reduced after oophorectomy; (2) blood androstenedione has a diurnal rhythm (suggesting a substantial adrenal contribution); (3) serum androstenedione is markedly reduced after treatment with dexamethasone; (4) levels of androstenedione increase after the systemic administration of adrenocorticotropic hormone (ACTH), but not of hCG; and (5) the difference between androstenedione levels in the ovarian veins of menopausal women and levels in peripheral blood is of a lesser magnitude than that found in premenopausal women.

Some authors have concluded that the postmenopausal ovary is not a major site of androgen biosynthesis. Cauley and coworkers[359] examined hormone levels in postmenopausal women with and without ovaries and observed no statistically significant differences in circulating levels of testosterone and androstenedione between the two groups. Couzinet and colleagues[360] reported that plasma androgen levels were very low in all postmenopausal women with adrenal insufficiency and were similar between oophorectomized and nonoophorectomized postmenopausal women with normal adrenal function. These investigators also showed that dexamethasone dramatically suppressed plasma androgen production, whereas hCG treatment had no effect on plasma sex steroid levels, findings that are consistent with an adrenal source of androgens.

Estrogens in postmenopausal women arise almost exclusively from extraglandular aromatization of androstenedione.[361] Oophorectomy results in no substantial reduction

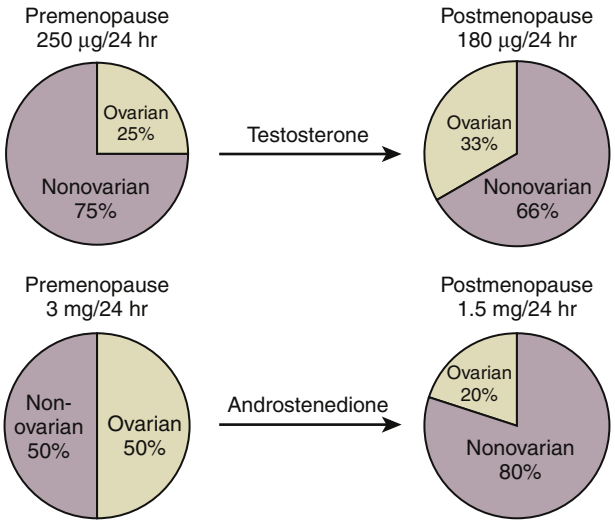


FIGURE 9.21 Sources of androgens in pre- and postmenopausal women.

in urinary estrogen excretion by postmenopausal women. However, adrenalectomy after oophorectomy virtually eliminates measurable estrogens from the urine. Longcope and colleagues[362] found a significant estradiol concentration gradient across the postmenopausal ovary in less than 20% of the women they studied. From *in vitro* studies, researchers concluded that the postmenopausal ovarian stroma is unable to aromatize androgens.[363]

Incubation of postmenopausal ovarian stromal slices with pregnenolone yielded progesterone, dehydroepiandrosterone, and testosterone. Incubation of strips of ovarian hilar tissue from postmenopausal women showed a steroidogenic pattern similar to that of the postmenopausal ovarian stroma. However, the overall amount of steroid produced was substantially greater compared with stroma. These findings imply that the hilar cells have greater importance than the stromal cells in the overall steroidogenic potential of the postmenopausal ovary. Consistent with this view, immunohistochemical studies of most postmenopausal ovaries show that stromal cells are rarely positive for expression of P450scc, 3β-hydroxysteroid dehydrogenase, and P450c17, three steroidogenic enzymes necessary for biosynthesis of androgens. Expression of steroidogenic enzymes involved in androgen biosynthesis is greatest in women with endometrial cancer or endometrial hyperplasia.[364]

There is some evidence that ovarian androgen production in postmenopausal women is gonadotropin-dependent. Administration of hCG to postmenopausal women results in a small increase in the circulating levels of testosterone.[365] Daily injection of hCG causes hyperplasia of the ovarian hilar cells and histochemical evidence suggesting active steroidogenesis.[366] The administration of hCG, but not ACTH, resulted in increased production of androgen, but not estrogen, by the ovaries.[367] Treatment of postmenopausal women with a long-acting GnRH agonist caused a decrease in circulating levels of testosterone, as well as a 22% decline in the levels of estradiol. The fall in serum estradiol levels was presumed to be the consequence of the reduction in serum testosterone levels. Taken together, these observations suggest that ovarian androgen biosynthesis is at least partially gonadotropin-dependent.

As suggested from the summaries of these studies, the main site of steroidogenesis may be the hilar cells. Binding sites for both LH and FSH were identified in the cortical stroma and in hilar cells.[368] Addition of hCG to hilar cells results in increased cAMP formation and steroid biosynthesis, indicating preserved responsiveness to gonadotropins.

Stromal hyperplasia can occur in the postmenopausal ovary, with the ovary enlarging with hyperplastic stromal nodules consisting of lipid-rich, luteinized cells that resemble theca interna. The ovaries with stromal hyperplasia produce large amounts of androstenedione, resulting in hirsutism and virilization.[369] Hilar cells can give rise to functional neoplasms (i.e., hilar cell tumors).[370,371] These tumors usually produce excess amounts of androgens, leading to virilization, but signs and symptoms of estrogen excess can also be evident when there is significant peripheral aromatization.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 10

The Structure, Function, and Evaluation of the Female Reproductive Tract

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The fallopian tubes, endometrium and myometrium, and uterine cervix function in concert to receive gametes, facilitate fertilization, support embryo growth, and ultimately orchestrate the timely expulsion of the mature fetus. The preparation of a receptive reproductive tract is programmed by ovarian steroid hormones acting directly on their cognate receptors and indirectly through the intermediacy of various steroid-regulated growth factors and cytokines. This chapter describes the structural and biochemical changes in the human reproductive tract during the normal menstrual cycle and pregnancy, the clinical evaluation of reproductive tract function, and the pathophysiology of some relevant disorders (see also Chapters 26 and 27).

The components of a receptive endometrium include the luminal epithelium, which undergoes apical surface specialization expressing cell adhesion molecules that permit adherence of the blastocyst; glandular epithelial cells, which secrete substances that support blastocyst development; decidual cells and large granular lymphocytes that modulate trophoblast function and endometrial angiogenesis through the secretion of growth factors, growth factor-binding proteins, angiogenic factors, and cytokines; a stromal extracellular matrix that facilitates trophoblast invasion. The combined actions of the locally acting molecules and extracellular matrix promote trophoblast proliferation and penetration of the endometrium, while controlling excessive penetration.

Innate and adaptive immune systems under the regulation of steroid hormones collectively defend the reproductive tract environment, but are also regulated to allow the fetal semiallograft to be accepted by the maternal host. The vascular system nourishes the endometrium in the initial receptive phase and is subsequently remodelled by invading trophoblasts to establish the placental blood supply. The coordinated contractile activity of the myometrium promotes sperm migration in a cycle of conception as well as facilitating embryo transport prior to attachment. During pregnancy, the myometrium experiences hormone-directed hyperplasia and contractile quiescence that accommodate fetal growth. In the absence of conception, the endometrium is shed through a controlled inflammatory-like reaction involving matrix metalloproteinases (MMPs), the production of vasoactive substances, and uterine contractions that, respectively, promote remodelling, hemostasis, and the expulsion of the shed endometrial lining. Compartmentalized and specialized endometrial cells at the menstrual interface provide remarkable regenerative capacity, assuring that a new intact luminal surface is prepared for the next round of oocyte release and potential fertilization.

Structure and Morphology

Morphogenesis of the Female Reproductive Tract

The female reproductive tract is derived from the urogenital ridge, which, during week 6 of gestation, gives rise to paired mesodermal (paramesonephic) tubes (the müllerian ducts) formed from longitudinal invaginations of the coelomic epithelium.[1-3] These form the fallopian tubes, with the caudal ends fusing by week 10 of gestation to produce the primordial uterus and upper portion of the vagina. A thin septum remaining after fusion eventually resorbs, yielding single cavities of the uterus and vagina. The primordial uterus is initially lined by a simple cuboidal epithelium that subsequently becomes columnar and pseudostratified. Beneath this epithelium lies a condensed mesenchyme that yields the endometrial stroma and the surrounding myometrium. Glandular epithelium buds from the luminal epithelium, invaginating into the stroma. By week 22 of gestation, the uterus has taken on the structure of the adult organ. Glandular secretory activity, glycogen accumulation, and stromal edema are evident by week 32 under the influence of placentally-derived steroid hormones. After delivery, with the precipitous fall in placental estrogens and progesterone, the endometrium regresses to an atrophic state, containing a few small-calibre glands and a poorly vascularized stroma.

The Role of the Wnt Family and Homeobox Genes

The embryonic events described above are driven, in large measure by the expression of secreted ligands of

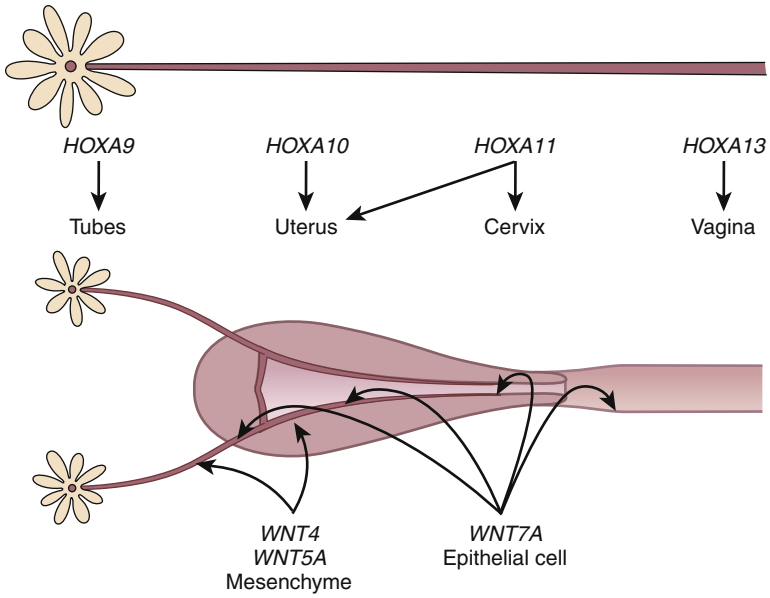


FIGURE 10.1 Expression patterns of *HOX* and *WNT* genes in the female reproductive tract during development. *(Adapted from Taylor HS. The role of HOX genes on the development and function of the female reproductive tract.* Semin Reprod Med *18:81–89, 2000.)*

the wingless-type MMTV integration site (Wnt) family (WNT4, WNTa, WNT7a) and transcriptional regulators of the homeobox (HOX) gene family (Fig. 10.1).[4-6] This morphogenetic program can only be played out in the absence of anti-müllerian-hormone (AMH), a member of the transforming growth factor β family made by the sertoli cells of the fetal testes. In the absence of testosterone, the müllerian ducts elongate and develop into the fallopian tubes, uterus, cervix, and upper part of the vagina. The elongation phase of the müllerian ducts requires a number of factors. Given their common embryonic origin, early development in the mouse of the kidneys, ureters, and reproductive tract are tightly linked and involve other specific genes including *Pax2*, *Lim1*, *Emx2*, as well as the members of the Wnt and abdominal-B HOXA families of genes.[7] *Lim1* encodes a transcription factor that along with PAX2 is essential for urogenital tract development. *Lim1* null mice lack uteri and oviducts.[8] *Pax2* null mice lack kidneys, ureters, and genital tracts.[9] Caudal elongation of the paramesonephric duct is absent. EMX2 is another transcription factor of the homeodomain gene family that appears to be essential for urogenital tract development.[10] EMX2 is highly expressed in the adult uterus and its expression is correlated with cell proliferation and appears to be inhibited by the homeobox gene, *HOXA10*. There is decreased expression of PAX2 and LIM1, and mesenchymal segmental polarity gene product, WNT4, is also absent in mice lacking EMX2, suggesting the essential role for this transcription factor.

The study of mice with targeted inactivation of *Wnt* genes revealed the importance of these signalling molecules in the development of the reproductive tract.[4] The müllerian ducts are absent in female mice lacking *Wnt4*, a gene expressed in the mesenchyme.[5] Moreover, female mice lacking *WNT4* are partially sex-reversed due to the retention of the wolffian ducts. Cases of *WNT4* null mutations associated with müllerian duct regression and a phenotype, including hyperandrogenemia, resembling that of the *WNT4* knockout mouse have been reported in women.[11] *WNT9b* is expressed in the wolffian duct epithelium and is necessary for müllerian duct extension.[12] Deficiency of *Wnt5a*, a gene expressed in the genital tubercle and genital tract mesenchyme, results in mice with stunted genital tubercles and the absence of external genitalia.[4] *WNT7a* expression is found in the luminal epithelium of the müllerian duct[13] and involved in paracrine signalling to the endometrial mesenchyme. Although *WNT7A* mutations have not been found in women with müllerian anomalies,[14] in mice lacking *WNTta*, the oviduct is not clearly demarcated from the upper uterine horn, and the uterus develops cellular characteristics that are similar to the vagina (including a stratified epithelium without uterine glands), and the uterine smooth muscle is disorganized.[8-9] Postnatal expression of *HOXA10* and *HOXA11* in the uterus is also lost. Mesenchymal beta-catenin appears to be the essential down-stream effector of the WNT7A pathway, and mediates its effects on the oviduct and proper development of the uterus.[15] The Wnt family of genes, including receptors and downstream signalling molecules, are also expressed in a regulated fashion in the adult reproductive tract, indicating that they have additional roles beyond those involved in early morphogenetic events including the regulation of steroid hormone action in adult tissues (discussed later in the chapter).[4-6,16-19]

The *HOX* genes encode an evolutionarily conserved family of transcription factors that contain a signature 60 amino acid deoxynucleic acid (DNA)-binding homeodomain.[6] They play critical roles in organizing cells along the anterior-posterior axis and directing them to select a particular pathway of development. Mammalian *HOX* genes are arranged in four different clusters, designated A through D, with each cluster organized in a linear arrangement that parallels the order of expression along the anterior–posterior body axis. The expression of *HOXA* genes in the human and mouse reproductive tract is conserved, with HOXA9 being expressed in the fallopian tubes, *HOXA10* and *HOXA11* in the uterus, *HOXA11* in the cervix, and *HOXA13* in the upper vagina.[6] Although there is a consistent regional distribution of HOX gene expression along the reproductive tract, there is evidence for some functional redundancy among

the adjacent genes. Like the *WNT* genes, the *HOXA* genes are also expressed in the adult uterus and their expression is under steroid hormone (estrogen and progesterone) regulation. *HOXA10* and *HOXA11* have both been implicated in the process of implantation.[20]

The importance of the homeobox gene family in reproductive tract function has been demonstrated through targeted deletions in specific *HOXA* genes. Another significant discovery was that hand–foot–genital syndrome and Guttmacher syndrome, autosomal dominant conditions that affect bones in the hands and feet and cause reproductive tract abnormalities (including bicornuate uterus), are caused by mutations in the *HOXA13* gene.[21,22] However, to date, mutations in *HOXA7* to *HOXA13* and HOX gene cofactor pre B-cell leukemia homeobox1 (PBX1) have yet to be found in subjects with congenital absence of the uterus and vagina. Mice with targeted deletions in the *HOXA10* and *HOXA11* genes have subtle abnormalities in uterine morphology, including transformation of the upper uterine segment into an oviduct-like histology (*HOXA10* mutants); reduced endometrial stromal development and expression of leukemia inhibitory factor (LIF) are seen in *HOXA11* mutants.[20,23] Notably, both *HOXA10* and *HOXA11* nullizygous females are infertile due to a uterine factor, implicating these genes in the implantation process in the adult. Mice lacking H6 homeobox 3 *(Hmx3)*, another homeobox domain gene product, are also infertile due to an implantation defect associated with perturbations in *WNT* and *LIF* gene expression.[24]

Müllerian anomalies represent a complex and rare collection of developmental defects occurring in 5% of the general population. Depending on the stage of development at which they occur, these defects in the reproductive tract can be mild (including a uterine septum) or severe with complete absence of the cervix, uterus, and fallopian tubes. These can be associated with infertility, endometriosis, and miscarriage but also can require surgical correction and are often discovered at the time of puberty, if not before.[25] Given the close developmental interaction between the müllerian and urinary system, it is not surprising that combined renal and müllerian duct agenesis occur.[26] The patterns and genetics of müllerian anomalies provide further insight into the embryology of the reproductive tract morphogenesis.

Mayer-Rokitansky-Küster-Hauser syndrome (MRKH) is the congenital aplasia or severe hypoplasia of the adult derivatives of the müllerian ducts, including the fallopian tubes, uterus, cervix, and upper vagina. Although müllerian hypoplasia can be a feature of many other specific genetic syndromes,[27] in MRKH, müllerian hypoplasia is the most prominent anomaly. MRKH has an incidence of at least 1 in 4500 women and is commonly subclassified into type I, isolated müllerian hypoplasia and type II, also known as müllerian Renal Cervico-thoracic Somite (MURCS association), involving renal, skeletal, and hearing defects, as well as occasional cardiac and digital anomalies.[11] The more common renal anomalies include unilateral agenesis, ectopia of kidneys and horseshoe kidney; the more common skeletal anomalies include fused vertebrae (commonly cervical) and scoliosis. Fusion of at least two cervical segments, short neck, low hairline, and restriction of neck motion, known as Klippel Feil syndrome, is seen.[11] The genetic basis of these syndromes is largely unknown, but a few gene defects have been associated. Copy number variants (CNVs), due to microdeletions and microduplications are relatively common,[28] especially at 16p11.2 and 17q12. In addition, CNVs involving the short stature homeobox *(SHOX)* gene at Xp22 were detected in some MRKH case series,[29] but not others.[30] Studies of candidate genes, suggested by mouse and human mutation phenotypes have been unrewarding, with failure to associate defects in AMH receptors, Wilms Tumor 1 *(WT1)*, *PAX2*, galactose-1-phosphate uridyl transferase *(GALT)*, cystic fibrosis transmembrane conductance regulator *(CFTR)*, or the *HOXA* cluster genes with MRKH syndrome.

More recently, a third distinct entity, MRKH associated with hyperandrogenism, has been reported and a clear association with four different defects in the *WNT4* gene have been confirmed.[31-34] Studies of the mutated WNT4 proteins in cell culture and WNT4 deficiency in mice suggest that the hyperandrogenism is a result of failure of WNT4-mediated repression (via beta catenin stabilization) of both *CYP17A1* and *HSD3B2* or only *CYP17A1*. The failure of müllerian duct formation appears to result from the requirement for WNT4/beta-catenin signaling in müllerian development. Multiple case series of MRKH without hyperandrogenism have failed to find any association with *WNT4* mutations, further strengthening the hypothesis that MRKH with hyperandrogenism is a clinically and genetically distinct disorder.[29,35-38]

A clinical approach to MRKH patients should include evaluation of pelvic anatomy imaging, renal anatomy imaging, measurement of plasma follicle stimulating hormone (FSH), estradiol, and testosterone. Depending on the associated symptoms and signs, adjunctive tests may include an electrocardiogram (ECG), audiogram, skeletal x-ray and pelvic laparoscopy. Dilator treatment or neovagina creation may also be necessary to allow sexual intercourse. Since surrogacy in a MRKH patient would allow her to have a genetically related child, genetic testing or pre-implantation genetic screening may be indicated.

The morphogenesis of the female reproductive tract does not require the action of estrogen. The oviducts, uterus, cervix, and vagina form in mice with inactivating mutations of both nuclear estrogen receptors (ERα and ERβ).[39] Despite this independence from maternal or fetal estrogens, normal differentiation of the female reproductive tract can, paradoxically, be disrupted by exogenous estrogens.[40] Diethylstilbestrol (DES), a synthetic estrogen that causes uterine and cervical anomalies in exposed females (discussed later in this chapter), and polychlorinated biphenyls suppress expression of *WNT7a* and alters the pattern of expression of *HOXA9* and *HOXA10* in the murine reproductive tract through ERα.[39-41] This suggests that alterations in *HOXA* and *WNT* gene expression are the likely molecular mechanism underlying the anatomical defects observed in human females exposed to DES in utero. Postnatal progesterone may also inhibit normal endometrial gland development, as shown in the neonatal ewe.[42] Using this model, it appears that aglandular endometrium involves disruption of the WNT system,[43] and illustrates that, while normal development may be independent of steroid hormones, exposure at the wrong time can alter the normal developmental pathways.

Steroid Hormone Regulation of Female Reproductive Tract Growth and Differentiation

Steroid Hormone Action in the Reproductive Tract

Many, but not all, of the reproductive tract responses to steroid hormones are mediated by nuclear receptors. These nuclear transcription factors undergo striking spatial and temporal changes in expression during the primate menstrual cycle.[44-47] The uterine response to steroid hormones is determined by the amount of bioavailable hormone, which is influenced by hormone production rates as well as local steroid metabolism; the repertoire of steroid receptors, coactivators, and corepressors expressed; and the action of growth factors and cytokines that modulate the action of steroid hormone receptors. The discovery of multiple forms of steroid hormone receptors with different transcriptional activities and cell and tissue-specific patterns of expression revealed new levels of complexity in the control of reproductive tract function. The patterns of expression of coactivators and corepressors add another level of intricacy, which is now being explored.[48,49] The presence of membrane bound receptors with specific recognition of steroid receptors helps coordinate the paracrine, autocrine, and juxtacrine cellular mechanisms and provides an explanation for rapid effects of steroid hormones as well as the relationship between steroid hormones and growth factor responses.[50,51] Rapid effects of estradiol are mediated by at least two distinct receptors, a membrane associated form of ERα and a recently described integral membrane receptor known as both G protein coupled estrogen receptor (GPER) and G protein coupled receptor 30 (GPR30).[50] GPR30 has been characterized as an estrogen receptor and subsequent development of a GPR30-specific agonist, G-1, and antagonist, G-15, have revealed important functions of this non-classical estrogen receptor in multiple physiological and pathophysiological processes.[52,53] In the endometrium, this receptor is present in the endometrial epithelium during the late proliferative phase and transitions to the stroma and decidua in the latter stages of the menstrual cycle and pregnancy.[54] Given the down-regulation of classical ERα in response to progesterone at this time, this receptor may provide alternative opportunities for estrogen to act and maintain progesterone receptor and other essential estrogen actions. Together, both steroid hormones provide a diversity of responses that modulate an exquisite complexity required for establishment of a successful pregnancy.

Estradiol is the primary trophic hormone for the uterus. The uterine growth response is mediated by ERα, whose concentrations are highest during the proliferative phase of the cycle and decline after ovulation (Fig. 10.2).[55] These changes correlate with the proliferative activity of the endometrium. Immunohistochemical studies demonstrate estrogen receptors in the nuclei of epithelial, stromal, and myometrial cells during the proliferative phase, with the epithelial cell staining being most prominent.[45,46] After progesterone levels rise in the luteal phase, estrogen receptor staining is restricted to the deep basal glands and vascular smooth muscle. *In situ* hybridization studies to distinguish patterns of expression of ERα and ERβ reveal that

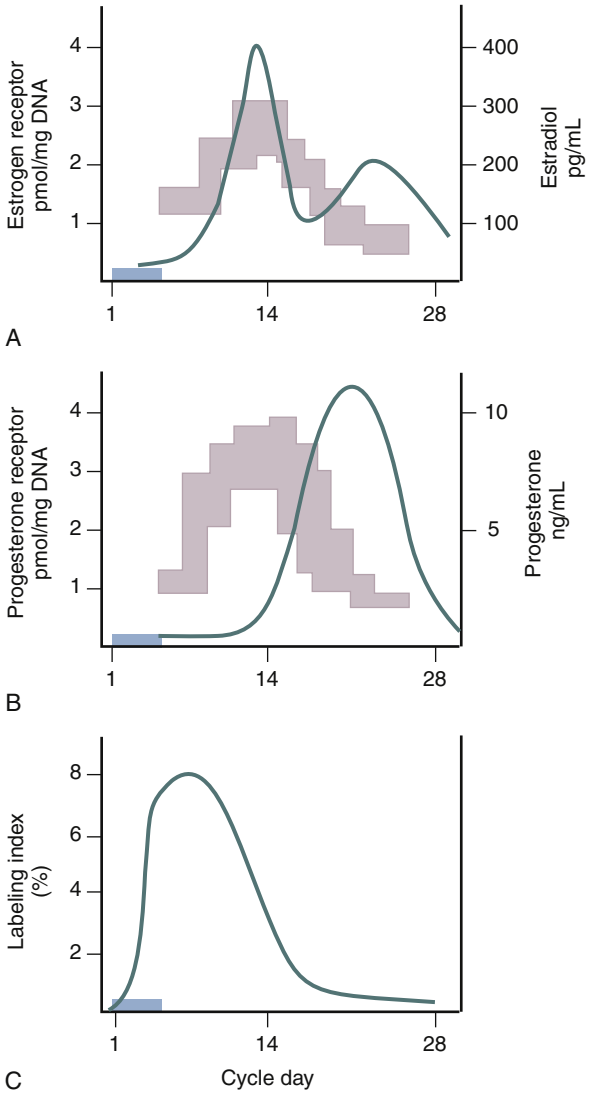


FIGURE 10.2 Estrogen and progesterone receptors in endometrial cells during the normal menstrual cycle. The concentrations of estrogen receptor (shaded area, **A**) and progesterone receptor (shaded area, **B**), mean values of plasma estradiol and progesterone (green lines, **A** and **B**), and endometrial proliferation as assessed by incorporation of [^{3}H]thymidine *in vitro* (labeling index, **C**). The black bar indicates menses. *(Modified from Frolich M, Brand EC, van Hall EV. Serum levels of unconjugated aetiocholanolone, androstenedione, testosterone, dehydroepiandrosterone, aldosterone, progesterone, and oestrogens during the normal menstrual cycle.* Acta Endocrinol [Copenh] *81:548–562, 1976.)*

transcripts for both receptor forms are expressed in the epithelial, stromal, and smooth muscle cells at all stages of the cycle.[56] However, ERα expression is more abundant in the endometrium. In the proliferative phase, ERα messenger ribonucleic acid (mRNA) is detectable in epithelial cells and stroma, while ERβ mRNA is predominantly in the glandular epithelium and is the only estrogen receptor found in vascular endothelium of the endometrium.[57] Expression of both transcripts falls in the secretory phase, with the decline in ERα expression being least in the basalis—consistent with the previously noted results of immunocytochemical studies. This fall in ERα at the time of implantation may be physiologically important and is a common finding across many

species.[46,47,58] Failure to down-regulate estrogen receptors indicates an imbalance in regulatory mechanisms of steroid hormone interactions, and is associated with endometrial dysfunction, endometriosis, and infertility [59] Endometriosis (discussed in detail later in the chapter) is also associated with increased local endometrial estrogen production (P450 aromatase over-expression)[60] and resistance to the down-regulatory effects of progesterone.[61]

A variant of ERβ, ERβcx/β2, formed by alternative splicing of the eighth exon of the "classical" ERβ (ERb1) transcript has been described with distinct ligand and coactivator affinity.[62] These isoforms have different selectivity for ligands and affinities coactivators. The patterns of ERβ1 and ERβ2 protein expression in the endometrium differ: ERβ1 expression appears to be more intense than ERβ2; ERβ1 protein levels are unchanged throughout the menstrual cycle while ERβ2 levels decline in the glands of the functionalis layer but not the basalis layer in the midsecretory phase. The physiological significance of these ERβ isoforms in the endometrium remains to be determined.

Studies on mice with targeted deletions in the ERα and ERβ genes have provided clues as to the roles of the two molecules in mediating uterine responses to estrogen.[39] ERα is responsible for the main uterotrophic actions of estradiol, as the uteri of mice lacking this receptor are hypoplastic and do not show the classical cell proliferation, increase in uterine wet weight, and edema in response to estradiol.[63] In mice lacking ERβ there is an exaggerated response to estradiol, including increased accumulation of uterine secretions and uterine dilatation.[64] This suggests that ERβ may function to attenuate the transcriptional activity of ERα in the uterus.[65] Severe uterine hypoplasia is observed in mice lacking both ERα and ERβ.[39] Progesterone receptors, which are up-regulated by estrogen, are reduced in the uteri of estrogen receptor knockout mice, but this reduction does not prevent the induction of progesterone-regulated genes. A role of ERβ has been suggested in endometriosis where ERβ is overexpressed. A high ERβ/ERα ratio may promote a reduction in estrogen-induced progesterone receptor, as a cause of progesterone resistance seen in this condition.[66]

Other members of the estrogen receptor family include estrogen receptor related alpha (ERRalpha) and beta (ERRbeta), orphan receptors with homology to the classical ERα. ERRalpha and its coactivator peroxisome proliferator-activated receptor gamma coactivator-1 alpha were found to show dramatically increased expression in the decidua, and stimulates metabolical pathways for energy generation perhaps in preparation for implantation.[67] ERRbeta is seen throughout the endometrium, including uterine natural killer cells, and a precise role for this receptor has yet to be determined in the endometrium.[68]

Is there a threshold dose of estrogen required to elicit a uterine growth response? Key and Pike proposed a threshold estrogen level of approximately 50 to 100 pg/mL at which point endometrial proliferation is triggered, and above which there is no further stimulation of endometrial proliferation. This estimation was made on the basis of comparing endometrial proliferation assessed through *ex vivo* thymidine incorporation into endometrial explants from different stages of the menstrual cycle with corresponding estradiol levels at the different days of sampling.[69] This hypothesis finds relevance in postmenopausal estrogen therapy where effects on bone, cardiovascular, and endometrial function are different and have different risk/benefit profiles.[70]

Progesterone antagonizes actions of estrogen in the endometrium and promotes glandular and stromal differentiation via progesterone receptors. The antagonism of the uterotrophic actions of estradiol involves several events, including alterations in estrogen receptor expression, inhibition of estrogen-induced translocation of the cell-cycle regulators, and induction of enzymes that catabolize estradiol.[71-73] These effects are mediated by specific cognate nuclear receptors that are induced by estrogen.

Three forms of the progesterone receptor (PR), designated PR-A, PR-B, and PR-C, have been identified.[74] These isoforms are derived from a single gene through the use of alternative promoters and alternate translation start sites. The PR-A form lacks a 164-amino acid N-terminal sequence present in the PR-B form. The PR-C form, the smallest of the progesterone receptors, also appears to be derived from an alternative translation start site. The A and B forms of the progesterone receptor have different functions, and the differential expression of these two isoforms determines tissue responses. The B form of the receptor is a strong transcriptional activator of several progesterone-responsive genes that do not respond to the A form. Further, PR-B contains a motif that interacts with the Src homology 3 (SH3) domain of the intracytoplasmic signaling component, Src; rapid activation of Src provides a mechanism for mitogen activated protein (MAP) kinase signaling.[75] When PR-B was tagged with a simian virus 40 (SV40) nuclear localization signal causing it to be translocated into the nucleus, it no longer was able to interact with Src or to activate MAP kinase signalling pathways.[76] In addition, the extended size of PR-B provides a binding site for the melanoma antigen A11 (MAGE-11),[77] a cycle-regulated endometrial coactivator protein;[78] MAGE-11 is required for isoform-specific PR-B up-regulation of FK506 binding protein 5 (FKBP5) immunophilin, which functions to stabilize the PR-B receptor complex.

The PR-A receptor functions as a transcriptional repressor of the PR-B and other steroid receptors, including the estrogen receptor. The C form, of which relatively little is known, binds progesterone but has no evident transcriptional activity. The C form may act as a repressor or modulator of progesterone receptor A and B activity through heterodimerization, or it may serve as a progesterone-binding "sink." Differential subcellular localization of PR-B outside the nucleus and PR-A inside the nucleus may also dictate isoform-specific activities of these too isoforms.[79,80]

Interestingly, a class of membrane receptors, with high affinity, low-capacity binding to P, but completely structurally distinct from the classical PRs has been described. This family of non-classical, membrane progesterone receptors, termed mPRα (PAQR VII), mPRβ (PAQR VIII), and mPRγ (PAQR V) was first described in fish and subsequently in mammals and amphibians.[81] Each mammalian mPR has been shown to specifically bind progesterone and can activate G-protein coupled signaling pathways.[82] Expression of each has been shown in human endometrium and myometrium, as well as placenta, but their role in progesterone regulation of uterine function remains unknown.[83]

Classical endometrial progesterone receptors peak at the time of ovulation, localized to both epithelial and stromal

cells, then decline. The rise is a reflection of the ability of estradiol to induce progesterone receptor expression while the decline after ovulation is caused by progesterone down-regulation of its own receptor.[44,46,84] By 4 days after ovulation, progesterone receptor staining of the epithelial cells declines markedly in the epithelial compartment and remains weak or absent during the remainder of the secretory phase. In contrast, staining of stromal cells remains moderate to strong. The PR-A form predominates in the stroma, while the PR-B is more abundant than the A form in epithelial phase of the secretory phase of the cycle.[85] Progesterone receptors have not been detected in vascular endothelial cells or vascular smooth muscle, but they are abundant in perivascular stroma. Consequently, the effects of progesterone or its withdrawal on the vasculature are likely indirect or perhaps via membrane PRs (discussed earlier in the chapter).

The significance of the different ratios of the two major progesterone receptor forms, with respect to regulation of gene expression in the human uterus, is increasingly being studied.[86-88] Clues are being provided from the study of mice with targeted deletion of the receptor isoforms. The uteri of mice lacking both the A and B form of the progesterone receptor are hyperplastic and contain inflammatory infiltrates.[72,73] Hyperplasia reflects the lack of antagonism to the uterotrophic actions of estradiol. Selective targeting of the A form of the progesterone receptor revealed that it is essential for progesterone-mediated actions on implantation and the decidual response. However, the examination of genes associated with the window of uterine receptivity that are known to be regulated by progesterone indicated that the A form controls expression of only a subset, while others appear to be under the control PR-B. Ablation of PR-A in mice uncovered an unexpected role for the B form of the receptor in inducing epithelial proliferation. Administration of estradiol to PR-A receptor–deficient mice resulted in uterine hyperplasia, but the combination of estradiol and progesterone resulted in even greater hyperplasia. It thus appears that progesterone receptor A antagonizes the uterotrophic responses mediated by both ERα and progesterone receptor B. With regards to implantation and decidualization, however, when PR-A null mice are examined, they are similar in their defects to mice that lack both PR-A and -B subunits, while PR-B knock out mice (PRBKO) were fertile.[89] Clinically, reductions in PR-B have been associated with proliferative states such as endometriosis,[90] supporting this paradigm of counter-regulatory mechanisms involving PR isoforms.

There is additional evidence for a dichotomous action of progesterone on uterine cell proliferation. DNA synthesis assessed by [^{3}H] thymidine incorporation into cells of the endometrium of the rhesus monkey *in vivo* or into explants of human endometrium *in vitro* is greatest in the epithelial cells of the functionalis and low in the deep zona basalis during the proliferative phase.[91] Labeling indices fall dramatically in the luteal phase with the exception of the basalis, where thymidine incorporation increases as progesterone levels rise. Using the double fluorescent labelling for ERα and the nuclear marker Ki-67, Okulicz demonstrated that the presence of ERα related directly with cell proliferation.[92] After ovulation, loss of ERα in the upper zona functionalis correlated with lack of cell proliferation, while in the zona basalis, where ERα persisted, proliferation was increased, even in the presence of high serum progesterone levels. This phenomenon of natural progesterone resistance is probably important for the rapid proliferation and healing that occurs after menses, providing a head start for the reestablishment of the endometrium following loss of the upper functionalis layer.

In addition to steroid hormone receptors, coactivators and chaperone proteins have an important impact on the uterine response to estrogens and progestagens.[48] The uterine expression of p160 coactivators, steroid receptor coactivator-1 (SRC1), steroid receptor coactivator-2 (SRC2), and steroid receptor coactivator-3 (SRC3) has been examined by immunocytochemistry. SRC3 levels increased in the glandular epithelium in the late secretory phase, whereas SRC1 and SRC2 expression did not change. However, in endometrial biopsies from women with polycystic ovary syndrome, SRC2 and 3, along with ERα, were elevated in stroma and glandular epithelium, demonstrating that an abnormal endocrine milieu can alter coactivator levels, which could, in turn, result in endometrial dysfunction.

Many of the effects of estrogen on epithelial proliferation and progesterone receptor expression are indirect, involving activation of estrogen receptors in the stroma, which produces paracrine substances that act on the epithelium to promote DNA synthesis.[93] Moreover, there are reciprocal paracrine interactions between the epithelium and the stroma. For example, under the influence of progesterone, the epithelium produces substances that affect response of the underlying stroma to estrogen.[94] Conversely, the stroma responds to progesterone by exerting an inhibitory effect on estrogen-induced epithelial cell DNA synthesis.[95]

The indirect actions of estradiol on epithelial proliferation have been demonstrated in elegant reconstitution and grafting experiments employing stroma and epithelium from wild-type and estrogen receptor knockout mice. Epithelial cell proliferation does not occur when stroma from ERα knockout (ERKO) mouse uterus is paired with epithelium from wild-type uterus.[93,94] Conversely, epithelial cell DNA synthesis in response to estrogen occurs when wild-type stroma is paired with epithelium from ERKO mice. Studies on human endometrial cells in culture are consistent with the mouse studies.[96] Estradiol increases epithelial cell proliferation when cocultured with stroma, but it does not increase proliferation in epithelial cells cultured in the absence of stromal cells.

What is the estrogen-stimulated signal from the stroma that promotes epithelial cell proliferation? Candidates include growth factors under the transcriptional control of the estradiol receptor, including insulin-like growth factor-1 (IGF-I), transforming growth factor alpha (TGF-α), and epidermal growth factor (EGF).[97] Alternatively, estradiol might suppress production of stromal factors that restrain epithelial cell proliferation. Among the growth factors, there has been particular interest in EGF and IGF-I. Studies using mouse models, including transplantation of uteri and vaginae from EGF receptor knockout mice, indicate that this receptor is required for the maximal fibromuscular stroma growth but not the epithelial cell proliferative response to estrogen.[98]

IGF-1 and IGF-2 both stimulate the proliferation of human endometrial stromal cells via the type 1 IGF receptor.

IGF-1 expression is greatest in the late proliferative and early secretory phase, whereas IGF-2 is most abundant in the mid-secretory endometrium and decidua of the first trimester of pregnancy. A family of binding proteins (IGFBPs) sequester IGFs, and modulate their activities in target tissues. One of the binding proteins, IGFBP-1, is a major secretory product of decidualized stromal cells, and it has been hypothesized to play a role in controlling trophoblast invasion.[99]

Estrogen is the primary regulator of IGF-1 expression in the uterus, which occurs predominantly in the stroma.[100,101] Estrogen also increases expression of IGF-1 receptors, which are primarily found on epithelial cells. The mitogenic response of the mouse uterus to estrogen is absent in mice with targeted deletion of the IGF-1 gene. Moreover, mice over expressing IGFBP-1, which results in reduced IGF-1 bioavailability, have a blunted DNA synthesis response to estrogen treatment.[102] Collectively, these observations suggest that IGF-1 produced in the uterine stroma in response to estrogen acts on the epithelial cells to stimulate DNA synthesis. However, tissue grafting experiments showed that a IGF-1 knockout mouse uterus responds to estrogen when placed into a wild-type animal, whereas a wild-type uterus placed into a IGF-1 knockout mouse showed minimal growth, indicating that systemic IGF-1 is sufficient to support estrogen-driven uterine growth.[103] These findings substantiate the importance of IGF-1 in the uterine growth response to estrogen. Although uterine growth can occur in the absence of a paracrine IGF-1 system, these studies do not preclude a role for locally generated IGF-1 as a redundant signalling mechanism or the role of other locally produced growth factors.

Progesterone Signaling

Progesterone is a vital steroid hormone that is involved in secretory preparation of the endometrium for implantation, decidualization, and suppression of myometrial contractility during pregnancy. As seen in a later section, progesterone possesses anti-inflammatory characteristics and promotes immunotolerance during implantation and pregnancy. It is also associated directly, or indirectly, with most of the secretory proteins made by the endometrium that support embryo development and implantation.

Progesterone signaling in the endometrium is a complex process involving differential actions of progesterone on each of the cellular compartments, including the epithelium, stroma, and resident bone-marrow derived immune cells, as well as the resulting paracrine communication between cells of adjacent compartments.[104,105] The complexity of these interactions is further extended by the dynamic changes in expression of the receptors for progesterone.

As discussed earlier in the chapter and in Chapter 5, both ERα and PR concentrations increase in response to circulating estradiol in the proliferative phase of the menstrual cycle. After ovulation, estrogen action may be unnecessary for normal secretory endometrial development,[106,107] and may inhibit implantation.[108] Estrogen receptors normally disappear in the endometrial epithelium at the time of implantation in most mammals studied to date, including humans.[46,109]

In mice, specific and selective deletion of ERα and PR as well as recombination studies of stroma and epithelium have yielded insight into the relative roles of these steroid receptors in each compartment. It has become increasingly clear that progesterone has both direct and important indirect roles in both stroma and epithelial function.[93,110,111] The relevance of these findings to human tissue remains to be fully understood.

During the early proliferative phase, when progesterone concentrations are low, cytoplasmic PR is shielded from degradation by heat shock proteins and by interactions with coactivator proteins (SRC1 and 2) and chaperone proteins such as FKBP4 and FKBP5. In mice with a SRC2 conditional knock *(Ncoa2 $^{-/-}$)* the females are infertile, similar to FKBP4 null mice, with each displaying implantation and decidualization failure.[112,113] In women, both FKBP4 and FKPB5 expression normally increases in the secretory phase, possibly regulated by HOXA10. Interestingly, blunted increases in FKBP4 are seen in endometrium from women with infertility and endometriosis,[114] regulated in part by changes in microRNA that regulate degradation of mRNA coding for FKBP4 and other progesterone-regulated genes.[115-117] Recent evidence links SRC1 to the progression of endometriosis as well. A proteolytic fragment of SRC1, cleaved by tumor necrosis factor-alpha (TNFα)–induced MMP-9, prevents TNFα-induced apoptosis of the endometrial epithelium and promotes epithelial-mesenchymal transformation, part of the pathophysiology of endometriosis.[118]

Much of our understanding of implantation and the role of progesterone is based on studies in the mouse. The action of progesterone begins with epithelial PR binding to progesterone followed by the induction of a key target gene, Indian Hedgehog (IHH).[111] Epithelial IHH is secreted and communicates with the endometrial stroma, through the receptor Patched-1 (PTCH1) resulting in increased stromal transcription factor, chicken ovalbumin upstream promoter transcription factor II (COUP-TFII), with down-stream effectors including bone morphogenic protein-2 (BMP2) and WNT4. Similar to mice lacking FKBP4, COUP-TFII-deficient mice are infertile and have excessive estrogen action and failure of ERα down-regulation. As mentioned earlier in this chapter, WNT4 is required for müllerian development and in adults is required for decidualization. WNT4 is also thought to be responsible for shuttling Forkhead box protein 1 (FOXO1), a transcription factor essential for decidualization, from the nucleus to the cytoplasm, preventing its apoptotic actions. These signaling pathways are shown in Figure 10.3.

Some of the stromal genes expressed in response to progesterone action include *HOXA10*, heparin-binding EGF-like growth factor *(Hbegf)*, cyclooxygenase 2 (Cox-2, encoded by *Ptgs2*) and mitogen inducible gene 6 *(Mig-6)*. Mig-6 is a negative regulator of EGF receptor and transgenic knockout mice for this protein have endometrial hyperplasia, suggesting this progesterone-regulated gene is an important brake for cell proliferation in the endometrium. The homeobox gene, HOXA10 is essential for uterine development, but in the adult has been shown to regulate FKPB4[119] and is therefore also essential for progesterone action and implantation. HOXA10 also directly upregulates epithelial receptivity genes such as the αvβ3 integrin.[120] Transgenic mice lacking PR, HOXA10, Cox-2, or LIF in transgenic mice all have decidualization defects and infertility.[121]

The uterus is a target for androgens, as well as estrogens and progestins.[122] Androgen receptors (AR) are expressed

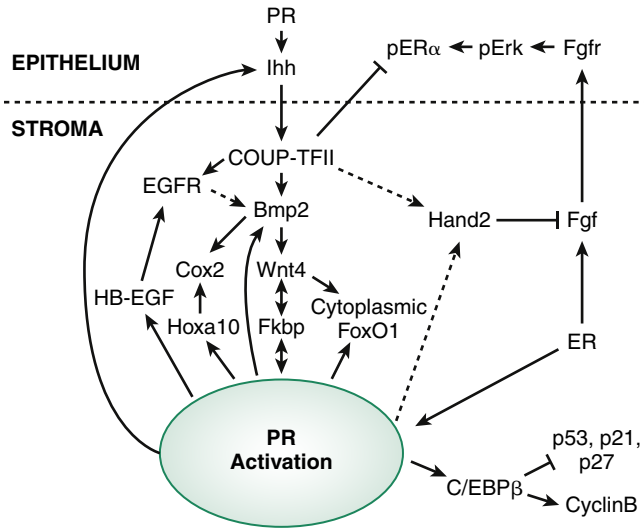


FIGURE 10.3 Progesterone signaling in the endometrium involves communication between the epithelium and the stroma, with complex interactions of the Indian Hedgehog and WNT pathways. One of the down-stream effects of progesterone is the down-regulation of estrogen signaling in both endometrial epithelial and stromal cells. *(Adapted from Large MJ, Demayo FJ. The regulation of embryo implantation and endometrial decidualization by progesterone receptor signaling.* Molecular and Cellular Endocrinology *358:155–165, 2012.)*

in the endometrium and myometrium, most prominently in the stromal cells during the proliferative phase and in the epithelial cells of the secretory phase. MAGE-11, was originally described as an AR coactivator,[123] reaches peak expression in the secretory phase and colocalizes with AR.[78] Interestingly, 5α-reductase types 1 and 2, which convert testosterone to dihydrotestosterone, are expressed in epithelial cells throughout the cycle.[124] Estradiol treatment increases androgen receptor expression in the stroma of the Rhesus monkey uterus, and estradiol in combination with either testosterone or progesterone augments epithelial and myometrial androgen receptor levels. Consistent with these observations, androgen receptor expression is elevated in the endometrium of women with polycystic ovary syndrome; this finding may explain, in part, the implantation defects and early pregnancy loss reported to be associated with this syndrome.[125] Androgens and AR may also have essential roles for reproductive tract function in females and this topic is becoming an active area of research.[126]

There is increasing interest in functional, non-traditional estrogen and progesterone receptors with roles in reproductive tract biology.[127-129] Rapid actions of estrogen have been known since the discovery of the estrogen receptor, though a thorough understanding of its mechanisms of actions has only recently been understood. Estradiol can act within minutes to stimulate second messenger generation and phosphorylation of kinases. One mediator for rapid estrogen signaing is the orphan G protein-coupled receptor, GPR30. This plasma membrane protein is thought to serve as an alternative steroid receptor to ERα. Evidence suggests that this protein mediates activation of EGF and down-stream kinase activity,[130,131] likely involving release of the growth factor EGF and MAP kinase activation.[132] Progestins may also use this pathway, adding further complexity to our understanding of estrogen/progesterone interactions.[133] It is not clear whether GPR30 is on the outer plasma membrane or within the endoplasmic reticulum.[50] During the menstrual cycle, endometrial GPR30 is maximal during the late proliferative phase, similar to traditional ERα —and like ERα, is increased in women with endometriosis.[54]

Membrane forms of PR have also been identified, including mPRα, mPRβ, and mPRγ.[134] Membrane PR have been shown to rapidly activate MAP kinase and inhibit cyclic adenosine monophosphate (cAMP).[135] Both mPRα and mPRβ have been localized to the myometrium of humans and function to inhibit cAMP with a possible role of facilitating uterine contractions at term.[136]

Steroid Hormone Metabolism in the Reproductive Tract

The level of bioactive steroid hormone in the uterus is determined in part by the activities of uterine enzymes that form active molecules from prohormones and catabolize active hormones (Fig. 10.4).[137] The enzymes that carry out transformations of steroid hormones are subject to regulation during the menstrual cycle. Estradiol taken up from the plasma can be converted into estrone by the action of 17β-hydroxysteroid dehydrogenases (17β-HSD), or converted to sulfated conjugates by estrogen sulfotransferase.[138] Three different forms of 17β-HSD capable of oxidizing estradiol to estrone have been detected in primate endometrium: the type 2, type 4, and type 8 enzymes.[139] Type 2 and type 8 enzymes are associated with microsomes; type 4 enzyme is in peroxisomes. Type 2 and type 4 enzymes use the oxidized form of nicotinamide adenine dinucleotide as a cofactor and are predominantly localized to glandular epithelium in the secretory phase. The endometrial type 2 enzyme shows the greatest change in expression during the cycle, peaking in the secretory phase. The type 8 enzyme appears to be constitutively expressed. Progesterone enhances the conversion of estradiol to estrone in endometrial cells by inducing expression of the type 2 17β-HSD and, to a much lesser degree, the type 4 enzyme. Progesterone also increases endometrial estradiol sulfation by increasing estrogen sulfotransferase activity. Thus, progesterone activates enzymatic pathways that inactivate estradiol. However, there are regional differences in the balance of systems removing and restoring estradiol in the uterus. For example, estrogen sulfatase is detected only in the glandular epithelium of the basalis, where it may function to increase the level of estradiol from estradiol sulfate.[140] The human uterus does not normally have the capacity to produce significant amounts of estrogen locally (either de novo or from circulating prohormones), but endometrial cancers, endometriotic lesions, and, to a lesser extent, eutopic endometrium of women with endometriosis aberrantly express components of the steroidogenic machinery ($P450_{arom}$) that endows the tissue with the capacity to synthesize estrogens from circulating adrenal or ovarian androgens or by *de novo* synthesis. Endometriosis lesions have been found to express StAR, the cholesterol side-chain cleavage enzyme, P450c17, aromatase, and type 1 17β-HSD in stromal cells (see Chapter 4). Estradiol produced in these lesions can enhance production of prostaglandin E2, which in turn stimulates transcription of the aromatase gene, resulting in a feed-forward mechanism for increasing local estrogen

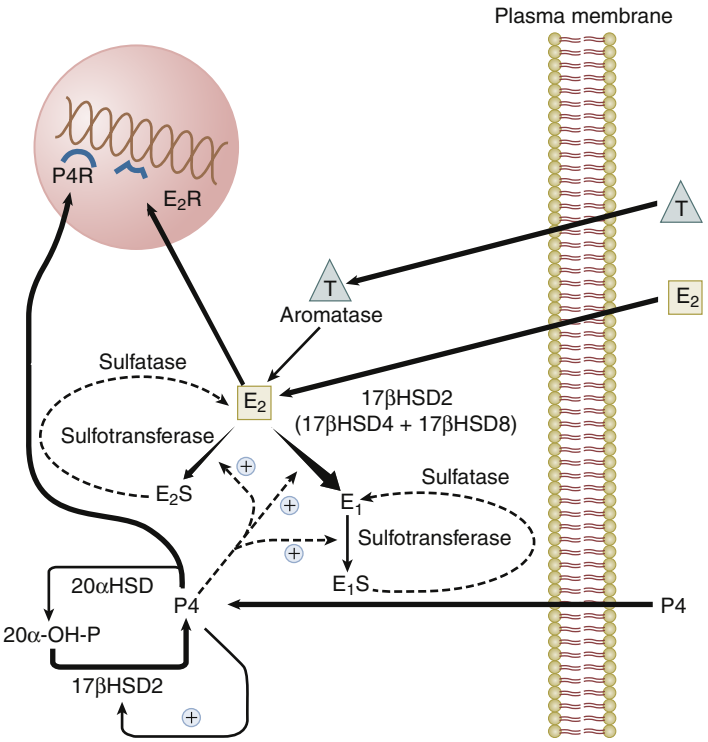


FIGURE 10.4 Steroid metabolism and action in the endometrium. (+) stimulation; (–) inhibition; E_1, estrone; E_1, S, estrone sulfate; E_2, estradiol; E_2R, estrogen receptor; P4, progesterone; HSD, hydroxysteroid dehydrogenase; T, testosterone; P_4R, progesterone receptor; E_2S, estradiol sulfate. P4 prevents the E_2-stimulated translocation of cyclin Di and Cdk4 into the nucleus while activating Cdk2, a cyclin A kinase, which inhibits the mitrogen effects of E_2.

levels. In addition, endometriotic lesions do not express the progesterone-regulated type 2 17β-HSD that converts estradiol to estrone, which effectively increases the bioavailability of estradiol. Eutopic endometrium from both endometriosis and polycystic ovary syndrome (PCOS) patients exhibit progesterone resistance as measured by decreased type 2 17β-HSD[141,142]; with the lack of deactivation of estradiol leading to increased estrogen action. Elevated estrogen may explain the increased proliferation seen in the endometrium of women with endometriosis[143] and contribute to the decrease in endometrial receptivity defects noted in these conditions.[59]

Aberrant expression of aromatase in endometriosis is thought to be due, in part, to the presence of steroidogenic factor 1 (SF-1), an orphan nuclear transcription factor that is not usually present in normal endometrium.[144] It has been proposed that SF-1 in endometriosis stromal cells displaces an inhibitory transcription factor, COUP-TFII (which is abundant in normal endometrial stromal cells) from SF-1 binding sites on the aromatase promoter, propelling assembly of the general transcription machinery and aromatase expression. In addition, by supplanting COUP-TFII, a mediator of progesterone action, may contribute to the progesterone resistance observed in this disease.[61] Other steroidogenic proteins found in endometriosis lesions, including StAR, P450scc, and P450cl7 are encoded by SF-1 (NR5A1)-regulated genes; a similar mechanism may underlie their expression in endometriosis.[145] The lack of type 2 17β-HSD in endometriosis has been attributed to abnormal expression of progesterone receptor isoforms with the A form, which generally suppresses transactivation, predominating over PR-B in endometriosis.[90,146] Studies also suggest aberrant expression of ERα[59] and ERβ,[147,148] as other features of endometriosis that promotes proliferation and cell survival.[66,149,150]

Progesterone is catabolized in the uterus to inactive 20α-hydroxyprogesterone by 20α-HSDs. The type 2 17β-HSD, which is increased in the secretory phase, is also a 20α-hydroxysteroid oxidase that converts 20α-hydroxyprogesterone back into progesterone.[137] The induction of the type 2 17β-HSD by progesterone in the secretory phase therefore not only contributes to the catabolism of estradiol, but also to the maintenance of endometrial progesterone levels.

The Adult Female Reproductive Tract and Dynamic Changes During the Menstrual Cycle

The Fallopian Tubes

The fallopian tubes facilitate gamete transport and serve as the venue for fertilization and early embryo transport. These functions are accomplished by the tubal epithelium and underlying smooth muscle. Although *in vitro* fertilization-embryo transfer has clearly demonstrated that the fallopian tubes are not essential for animal and human conception, an understanding of tubal physiology was essential to the development of the modern practice of assisted reproduction. Moreover, further elucidation of the oviductal environment could improve the outcome of assisted reproduction.

The oviduct has four anatomical regions spanning from the distal to the proximal end: the *fimbria* and *infundibulum*, the *ampulla*, the *isthmus*, and the *intramural segment*. The structures of the endosalpinx and myosalpinx in these segments are distinctive and correlated with their functional roles in gamete transport and the fertilization process.[151] In addition, the junctions between the ampulla and isthmus and the uterus and tube are important physiological sphincters that regulate the residence time of eggs and early embryos in the oviduct.

Gamete Transport

At the time of ovulation, the fimbriae become tumescent and congested, exhibiting pulsatile and sweeping movements.[152] The fimbriae and infundibulum have the highest density of ciliated cells in the oviduct, which beat in a centripetal direction in the peri-ovulatory period. The concerted movements of the fimbriae and ciliary strokes facilitate the pick-up of the ovulated egg and its entrance into the ampulla, which has a thin muscular layer and a mucosa that is thrown up in numerous folds, providing a large surface area for transport and exchange. Transport is arrested when the egg reaches the ampullary-isthmus junction, the site where fertilization occurs. Transport through the isthmus, which, in contrast to the ampulla, has only a few primary mucosal folds but a thicker myosalpinx, takes several days.[153]

The intramural segment of the tube, surrounded by abundant smooth muscle, serves as a second physiological sphincter. Activity of this sphincter appears to be regulated by adrenergic innervation, steroid hormones, and prostanoids. Gamete and embryo transport process is facilitated by contraction and relaxation of the myosalpinx. Light microscopic studies distinguish longitudinal and circular smooth muscle layers in the tube, but the ampulla appears to have a different organization, consisting of a continuous network of randomly anastomosed smooth muscle bundles that are repeatedly intertwined, branching into different orientations.[154] It has been proposed that this single network of muscular fibers generates random contractile waves that cause "stirring" of the tubal contents, a process that may facilitate fertilization and early embryo development by enhancing access of growth factors and nutrients and exchange of metabolites. Local production of prostaglandins F2α and E2 (which stimulate contraction) and prostacyclin (which causes relaxation) probably play an important role in regulating the contraction and relaxation pattern of both the longitudinal and circular muscle of the tubes.[155] Human chorionic gonadotropin (hCG) released by the early embryo can induce Cox-2 mRNA expression in tubal epithelium, which promotes increased prostacyclin production that would favour the opening of tubal sphincters.

Studies of laboratory and domestic species have shown that sperm reaching the oviduct are stored in the caudal part of the isthmic region through surface-associated sperm lectins that bind to oviductal epithelial cell glyco-conjugates.[156] These interactions appear to increase the viability of sperm and to suppress capacitation and sperm motility evidently by modifying intracellular calcium concentrations in the spermatozoa. Near the time that the egg enters into the ampulla of the tube, sperm initiate the capacitation process and are released from the oviduct epithelium in a hyperactivated state. Although there is a substantial body of literature documenting sperm binding to the oviduct in animals, there is no conclusive evidence that this occurs in humans. However, human sperm viability *in vitro* is promoted by coculture with oviductal epithelial cells despite the lack of tight binding.

The Tubal Epithelium and Tubal Fluid

The human tubal epithelium is composed of ciliated and secretory cells that undergo cyclic alterations in response to changes in ovarian steroid hormone levels.[157] In both the fimbriae and ampulla, epithelial cells attain their greatest height, extent of ciliation, and beat frequency in the late follicular phase. At the end of the luteal phase some cellular atrophy and loss of cilia is observed, particularly in the fimbriae, with hypertrophy and reciliation occurring in the subsequent follicular phase. In contrast, if pregnancy is achieved there is further atrophy and loss of cilia. These observations indicate that estrogen dominance promotes ciliogenesis while progesterone dominance leads to atrophy and deciliation. Female infertility, presumed to be of tubal origin, is a variable feature of primary ciliary dyskinesia and Kartagener syndrome, indicating that ciliary function in the female reproductive tract is important, but not absolutely essential, for *in vivo* fertilization.[158,159]

Gamete transport, fertilization, and early embryo development occur in a fluid environment created by the tubal epithelium. The production of oviductal fluid by selective transudation and secretion changes during the cycle in primates.[160,161] Tubal fluid accumulates at a rate of 1 to 3 mL every 24 hours, with a significant increase in accumulation around the time of ovulation. The primary cells responsible for fluid secretion in the oviduct are thought to be the non-ciliated cells, which maintain a transmural potential electrical potential through flux of chloride ions. The mechanisms of tubal fluid secretion and its regulation by steroid hormones, neurotransmitters, and inflammatory mediators are incompletely understood. Knowledge of tubal fluid composition has informed the development of culture media for *in vitro* fertilization and early embryo development.[161-164] Tubal fluid is enriched in bicarbonate compared with plasma, which facilitates sperm capacitation by activating a sperm soluble adenylyl cyclase; bicarbonate also promotes separation of the corona radiata of the egg. The high bicarbonate content of tubal fluid is maintained by carbonic anhydrase activity in tubal epithelial cells.

There are cyclic variations in the nutrient content of tubal fluid, which can influence human embryo development *in vitro*.[164] Such variation is exemplified by a sixfold decline in glucose, from 3.1 to 0.5 mM, from the follicular phase to the time of ovulation, and an increase in lactate from 4.9 to 10.5 mM during the same time. The levels of pyruvate in tubal fluid (0.14 to 0.17 mM), an important initial embryo energy substrate, do not change during cycle. Arginine, alanine, and glutamine are the most abundant amino acids in the tubal fluid; these and most other amino acids, except asparagine, are generally present in highest concentrations during the secretory phase. Taurine and hypotaurine are also major constituents that have been implicated in improved viability of gametes and preimplantation embryos.

Albumin is the major protein in tubal fluid that also contains secretory proteins from the epithelium. A high-molecular-weight mucin-like glycoprotein called *oviductin* (also known as oviduct-specific glycoprotein) is expressed exclusively by the oviduct epithelium under the regulation of estrogen.[165] Oviductin has chitinase-like domains that may facilitate its binding to oligosaccharides on the zona pellucida. This coating of oviductin may prevent ectopic pregnancy by forming a barrier between the embryo and the tubal epithelium. Oviducts also express a variety of growth factors and cytokines that could influence embryo growth and development.

The metabolical and secretory activity of animal and human tubal epithelial cells has been employed in human assisted reproduction. Several co-culture protocols have resulted in increased implantation and pregnancy rates, indicating a beneficial effect of a "tube-like" microenvironment. However, it is not known if any beneficial effects of co-culture are specific to oviductal epithelium as opposed to being a general salutary effect of a somatic cell layer.

Tubal Pregnancy

The fallopian tube is functionally important for transport of gametes and embryos, but ill-suited as a site of embryo implantation.[166] A period of receptivity for implantation seen in the endometrium is not observed in tubal epithelium.[167] Understanding ectopic implantation of human embryos provides further insight into the mechanisms governing endometrial implantation.[168]

The high incidence of ectopic pregnancy, about 1% to 2% of all pregnancies,[166] is often taken to suggest that the embryo's requirements for implantation depend less on the tissue into which it implants than its own intrinsic potential for attachment and invasion. However, the tight temporal control of endometrial implantation suggests a strong influence of the tissue comprising the implantation site. These apparently contradicting data may be reconciled by a more detailed understanding of ectopic pregnancies. While tubal epithelium is not normally receptive to implantation,[167] changes in tubal epithelium might lead to an altered molecular dialog or endocrine milieu that contributes to ectopic implantation.[169-173]

Tubal ectopic pregnancies, which comprise over 90% of all ectopics, are strongly associated with tubal infection and surgery. The most common single cause is thought to be a history of chlamydial infection. Chlamydial infection of murine fallopian tubes initiates an inflammatory cascade dependent on Toll-like receptor 2 (TLR2) and modulated by Toll-like receptor 4 (TLR4), leading to generation of inflammatory cytokines, including TNFα, interleukin (IL) -1beta, and IL-6, which lead to damage of fallopian tube epithelium, in part via MMP-9.[174] Interestingly, IFN-γ, unlike IFN-alpha or beta protects against tubal inflammation and destruction after murine chlamydial infection.[175,176] The factors mediating chlamydial damage in human fallopian tubes is less well understood, but expression and regulation of TLR2, TNF-alpha, IL-1beta, IL-1R, IFN-γ, and MMP-9 have been clearly documented in human tubal epithelium and MMP-9 immunoreactivity is absent in tubal epithelium of women with ectopic pregnancy.[177]

Recently, the role of estrogen signaling tubal transport of embryos was reported.[178] Since inflammatory actions of TLR2 and TLR4 are mediated via NF-kB activation and since NF-kB is noted to inhibit steroid hormone action in many cell types, the authors suggested that lack of estrogen action in the fallopian tube might lead to embryonic stasis and subsequent implantation into damaged epithelium. Thus, the role of tubal inflammation and damage in ectopic implantation provides a window to look at mechanisms governing normal embryo implantation.

The Endometrium

In discussions of structure and function, the primate endometrium is commonly described as consisting of two major layers, the *functionalis* and *basalis* (Fig. 10.5). The functionalis is a transient layer consisting of a compact zone that includes the stroma subjacent to the luminal epithelium and an intermediate spongy zone containing more

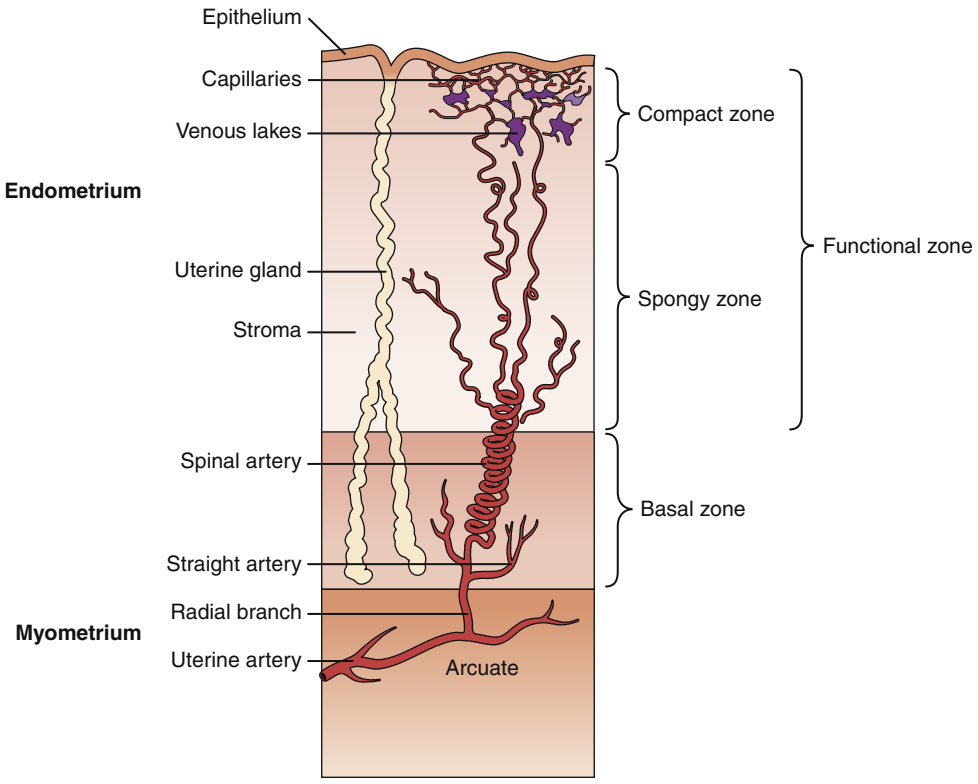


FIGURE 10.5 Histological organization of secretory-phase human endometrium. *(Modified from Weiss L, Creep RO [eds]. Histology, 4th ed. New York: McGraw-Hill, 1977, p 911.)*

densely packed tortuous glands, giving it a lacy histological appearance. The basalis, or basal layer, lies beneath the spongy zone and adjacent to the myometrium. It contains the gland fundi and supporting vasculature, and can generate the entire endometrium after the menstrual shedding of the *functionalis*. These endometrial layers are histologically definable during the secretory phase. However, in terms of function, the endometrium is best considered as a polarized gradient of cells with different phenotypes. The upper layers undergo a striking progression of histological change during the menstrual cycle, whereas the basal region shows only modest alterations. Patterns of cell proliferation, programmed cell death, and gene expression also show gradients across the layers, as described in the next sections. The majority of epithelial cell proliferation occurs in the upper regions of the functionalis during the proliferative phase of the cycle. Proliferatve activity in glands in the basalis is modest during the proliferative phase, with increased proliferative activity during the early to midsecretory phase,[179-181] maintaining both ER and PR at a time when these receptors are normally depleted in the upper functionalis epithelium.[46]

The Early Proliferative Phase

During the early proliferative phase, the endometrium is usually less than 2 mm in thickness. Proliferation of cells in the basal zones and epithelial cells persisting in the lower uterine and cornual segments result in the restoration of the luminal epithelium by day 5 of the menstrual cycle. At that time, mitotic activity is evident in both the glandular epithelium and stroma. Remarkably, this recurrent "wound healing" process does not normally produce scarring. Endometrial stem cells capable of yielding progenitors of both the stromal and epithelial components of the endometrium presumably contribute to the regenerative process.[182]

Rapid rejuvenation of the endometrium depends on many of the same factors that were involved in the ontogeny of the reproductive tract. WNT7A, is expressed solely by the luminal epithelium and is a diffusible factor that triggers cell proliferation through complex pathways.[18,19] Acting on the underlying stroma, soluble WNT7A binds to the receptor Frizzled (FZL) that phosphorylates the intracytoplasmic protein Dischevelled (DSH); (Fig. 10.6A). This protein inactivates glycogen synthase kinase beta (GSKβ) turning off the breakdown of beta catenin by ubiguitinization. Accumulation of beta catenin signals cell proliferation activities

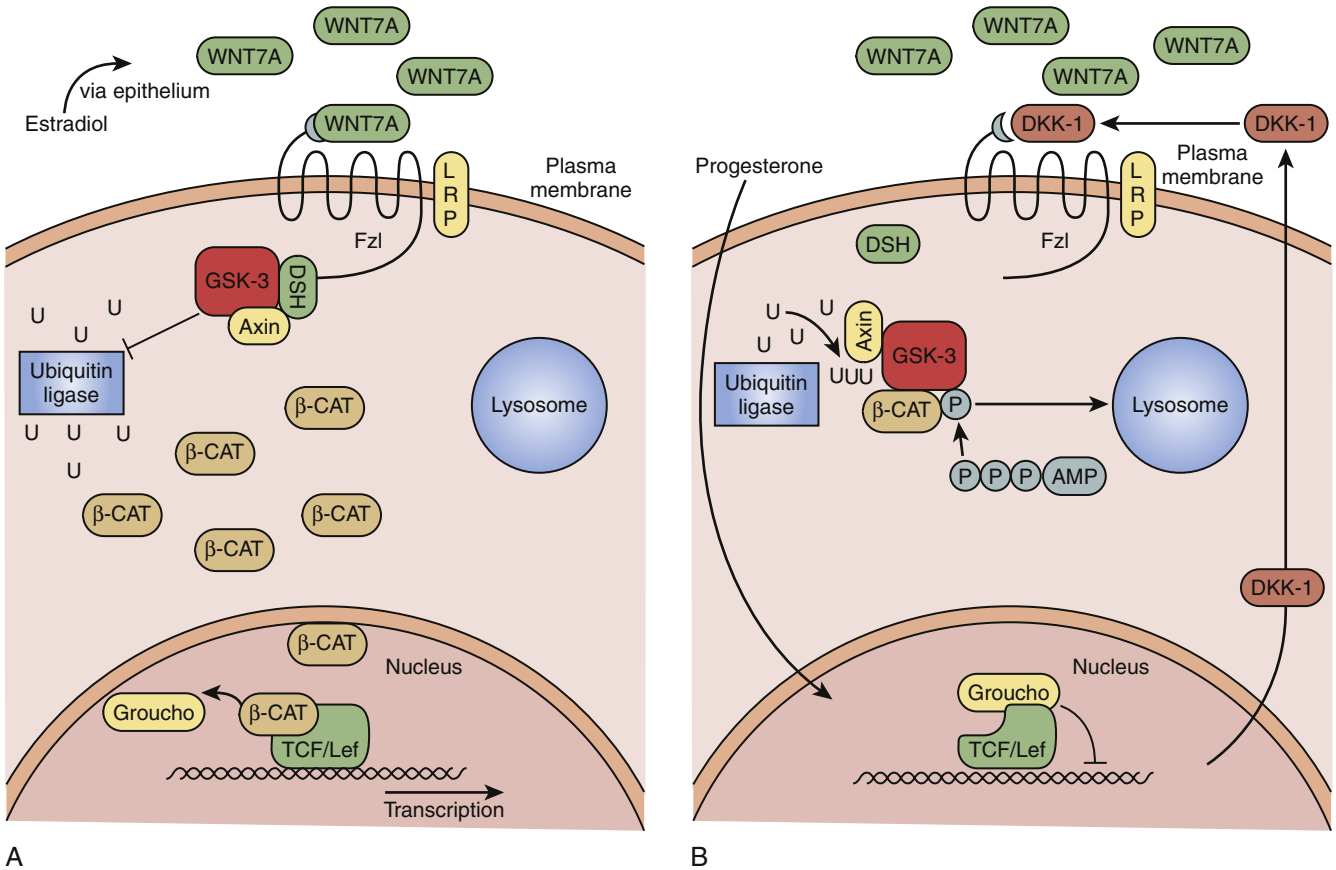


FIGURE 10.6 Wnt signaling pathways in proliferative and secretory phase of the menstrual cycle. WNT7A is a soluble factor thought to arise from the luminal epithelium. **A,** Under conditions of estrogen action, WNT7A binds to the receptor, Frizzled (FZL) initiating signaling through the intracytoplasmic protein dissheveled (DSH). Dissheveled binds axin and glycogen syntase kinase beta (GSK), allowing beta catenin (β-CAT) to accumulate and stimulate gene transcription leading to cell proliferation. **B,** When progesterone levels increase, cellular dickkopf-1 accumulate and inactivate Fzl through an accessory receptor, LRP. Without WNT7A, GSK facilitates the ubititinization of β-CAT, which makes it unavailable as a transcription factor.

associated with endometrial growth acting as a transcription factor in the nucleus. The diffusion gradient of WNT7A downward into the growing endometrium is an attractive model for self-regulatory mechanisms to determine the growth of the endometrium.

Counter-regulatory mechanisms to disable WNT7A/FZL/DSH pathways include the action of progesterone, which stimulates secretion of a protein called Dickkopf-1 (DKK-1); (Fig. 10.6B).[18,19] Dickkopf-1 binds to a coreceptor, LRP6 blocking the FZL receptor turning off the action of WNT7A by preventing beta catenin accumulation. Defects in DKK-1 production have been described in endometriosis and reflect progesterone resistance and might explain the proliferative phenotype of the endometrium in this condition.[114,183]

The glands during the early proliferative phase are narrow, straight, and tubular, lined with low columnar cells that have round nuclei located near the cell base (Fig. 10.7). At the ultrastructural level, the epithelial cell cytoplasm contains numerous polyribosomes, but the endoplasmic reticulum and Golgi complexes of these cells are not well developed.

The Late Proliferative Phase

The endometrium thickens in the late proliferative phase as a result of glandular hyperplasia and an increase in stromal extracellular matrix. The glands are widely separated near the endometrial surface and more crowded and tortuous deeper into the endometrium. The glandular epithelial cells increase in height and become pseudostratified as the time of ovulation approaches (Fig. 10.7).

The effect of steroid hormones on the proliferation and secretion within the endometrium is highly dependent on the zones (basalis vs. functionalis layers). Studies from the rhesus macaque endometrium using specific labelling techniques show proliferation during the "proliferative" phase is confined to the functionalis layer.[184] By the midsecretory phase, this proliferation has shifted to the basalis layer, presumably due to maintenance of epithelial and stromal estrogen receptors.[92,185]

The Early Secretory Phase

Ovulation marks the beginning of the secretory phase of the endometrial cycle, although it should be noted that the endometrial luminal and glandular epithelial cells also

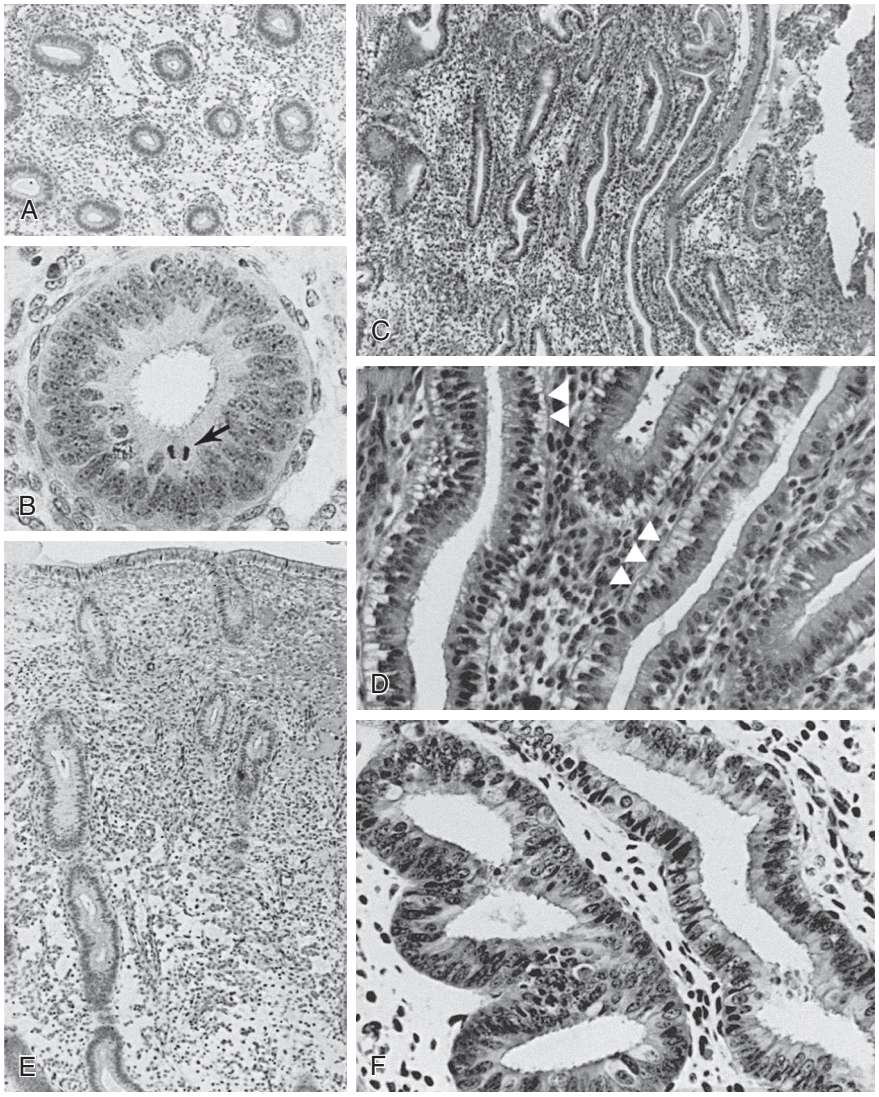


FIGURE 10.7 Histology of the endometrium during the menstrual cycle. **A** and **B,** Proliferative endometrium. Mitoses are present *(arrow)*. Nuclei in the glandular epithelium are pseudostratified. **C** and **D,** Secretory endometrium, day 18. Subnuclear glycogen vacuoles are uniformly present in glandular epithelium *(arrowheads)*. **E** and **F,** Midsecretory endometrium. Glandular secretory activity is present, and the stroma is edematous. The stromal cells in the more superficial layers as well as around vessels have become pseudodecidualized and exhibit a flattened, polygonal configuration with distinct cell borders.

display secretory activity during the proliferative phase. Mitotic activity in epithelial and stromal cells is restricted to the first 3 days after ovulation and is rarely observed later in the cycle. The nuclei of glandular epithelial cells and stromal cells develop heterochromatin in the early secretory phase (Fig. 10.6). The glandular epithelial cells begin to accumulate glycogen-rich vacuoles at their base, displacing the nuclei to the mid regions of the columnar cells. Evidence for modest secretory activity is seen in histological preparations as a light eosinophilic collection in the gland lumina. Ultrastructural studies of endometrial epithelia reveal abundant endoplasmic reticulum, and unusually large mitochondria with prominent cristae. A reticular network of argyrophilic fibers containing fibrillar collagens (collagen fiber type I and type III) is established in the stroma by the early secretory phase. Stromal edema contributes to the thickening of the endometrium at this time.

The Midsecretory Phase

A characteristic feature of this phase of the cycle is the development of the spiral arteries. These vessels become increasingly coiled as they lengthen more rapidly than the endometrium thickens. The endometrial glands are tortuous in the midsecretory and late secretory phases. Their secretory activity reaches a maximum about 6 days after ovulation, as reflected by loss of vacuoles from the epithelial cell cytoplasm (Fig. 10.7).

An ordered spherical stack of interdigitating tubules, the nucleolar channel system, appears transiently in the nucleoli of approximately 5% to 10% of the secretory phase epithelial cells between day 16 and 24 (Fig. 10.8).[186] The nuclear channel system is thought to form from an invagination of the inner nuclear membrane, providing a direct connection to the perinuclear space for transport of mRNA to the cytoplasm. Nopp140, a highly phosphorylated protein that associates with small nucleolar ribonucleoprotein particles that are required for RNA processing, appears to induce formation of this intranuclear endoplasmic reticulum.[187] The nucleolar channel system forms in response to progesterone and is an ultrastructural hallmark of the secretory phase during the expected time of implantation.

Stromal cells around blood vessels enlarge and acquire an eosinophilic cytoplasm and a pericellular extracellular matrix in the mid to late secretory phase. These changes, referred to as *predecidualization* to distinguish them from the further transformation of the stroma that occurs in a fertile cycle, subsequently spreads accentuating the demarcation between the subepithelial compact zone and the spongy zone. Unlike many laboratory animal species, an embryonic signal is not required for initiation of decidualization in the human uterus.

The fact that the predecidual changes occur first near blood vessels suggests that humoral or local factors provoke them. Among the local factors may be interactions with decidual granular lymphocytes, also referred to as uterine *natural killer* (NK) cells. NK cells encircle arterioles and closely associate with stromal cells through contacts that are remarkably similar to gap junctions.[188] At the ultrastructural level, the predecidual stromal cells display well-developed Golgi complexes and parallel lamellae of endoplasmic reticulum. Their surrounding matrix consists of laminin, fibronectin, heparan sulfate, and type IV collagen.[189,190] The profile of gene expression decidualizing human stroma cells *in vitro* has been mapped by microarray analysis and includes induction of 121 genes, down-regulation of 110 genes, and 50 genes showing a biphasic response.[191]

The stromal cells of the midsecretory and late secretory phase also express a repertoire of proteins that promote hemostasis—including tissue factor, a membrane-associated protein that initiates coagulation when it contacts blood, and plasminogen activator inhibitor type 1 (PAI-1), also known as Serpin E1, which restrains fibrinolysis.[192,193] The pattern of gene expression prevents focal hemorrhage that might result from trophoblast invasion during implantation.

The Premenstrual Phase

The main histological features of the premenstrual phase are degradation of the stromal reticular network, which is catalyzed by matrix metalloproteinases; infiltration of the stroma by polymorphonuclear and mononuclear leukocytes; and "secretory exhaustion" of the endometrial glands, whose epithelial cells now has basal nuclei. Morphological changes in the nuclei of granular lymphocytes, including pyknosis and karryorhexis suggesting apoptosis, have been proposed to be some of the first events presaging menses; these changes occur prior to the breakdown of extracellular matrix and leukocyte infiltration.[194] In the glandular epithelium, the nucleolar channel system and giant mitochondria characteristic of the early and midsecretory phases have vanished. The endometrium shrinks preceding menstruation,

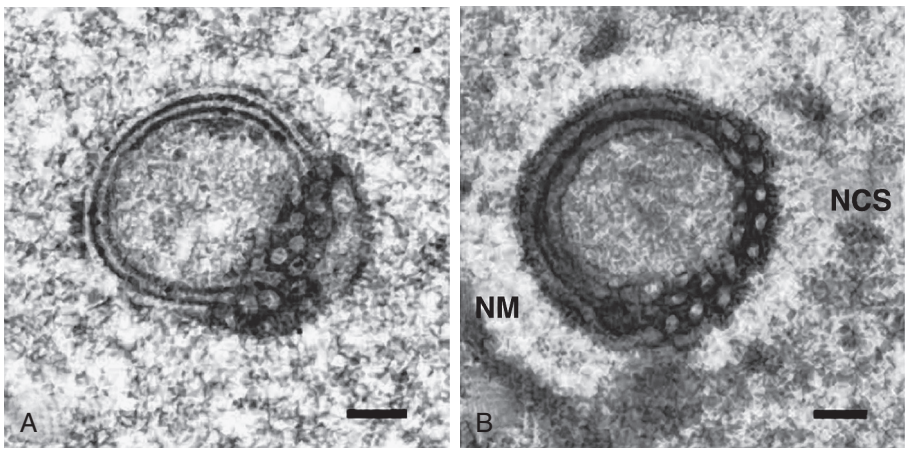


FIGURE 10.8 Images of the nucleolar channel system (NCS) at the electron microscope level. **A,** A nucleolar channel-like structure in COS cells transfected with a Nopp140 construct. **B,** A nucleolar channel system induced by progesterone in endometrial cells. NM, nuclear membrane. *(Reprinted with permission from Isaac C, Pollard JW, Meier UT. Intranuclear endoplasmic reticulum induced by Nopp140 mimics the nucleolar channel system of human endometrium.* Cell Sci *114:4253–4264, 2001.)*

partly as a result of diminished secretory activity and the catabolism of extracellular matrix.

Menstruation

Menstruation, caused by progesterone and estrogen withdrawal, marks a failure to achieve pregnancy and the need to shed the specialized uterine lining that results from spontaneous decidualization. The uniqueness of this process is highlighted by the fact that, although circulating progesterone and estrogen levels decline with corpus luteum regression in non-fertile cycles in all mammals, menstruation only occurs in human females and some Old World primates. In menstruating species, moreover, tissues that respond to estrogen and progesterone such as the fallopian tubes, vagina and breast do not shed as ovarian steroid levels decline. The molecular mechanisms triggered by progesterone withdrawal include activation of the NF-κB transcriptional pathway (a major target of cytokines) and the resulting expression of genes, like endometrial bleeding—associated factor (EBAF; AKA Lefty), an anti—TGF-β cytokine that interferes with the action of other members of the TGF-β family that promote endometrial integrity. This orchestrated blockade of the actions of TGF-β appears to initiate many of the subsequent events of menstruation, including the elaboration of MMPs.[195]

Menstruation is a complex process of changes initiated by progesterone withdrawal.[196] The functional zone of the human endometrium is supplied by spiral arterioles that, in contradistinction to the radial and basal arteries that feed them, are highly sensitive to steroid hormones. In the classic studies of Markee,[197] the structure of autologous endometrial transplants into the anterior eye chamber was observed during menses, suggesting that an ischemic phase caused by vasoconstriction of the arterioles and spiral arteries precedes the onset of menstrual bleeding by 4 to 24 hours. It has been proposed that bleeding occurs after the arterioles and arteries relax, leading to hypoxia or reperfusion injury.

These findings of Markee form the basis for the vasoconstriction model of menstruation. However, the notion that menstruation is a consequence of generalized vasoconstriction and hypoxia/anoxia in the functionalis is not supported by studies of endometrial perfusion that have failed to reveal any significant reduction in endometrial blood flow in the perimenstrual phase and during menstruation.[198] In addition, an analysis of expression and localization of hypoxia-inducible factor (HIF), a heterodimeric transcription factor induced by hypoxia and thus a biochemical marker of reduced oxygen availability, was unrevealing. No up-regulation of the two HIF component subunits, HIF-lα and HIF-lβ, was observed, and no nuclear localization of HIF subunits took place in the peri-menstrual human endometrium.[199] These studies do not, however, exclude the possibility of localized regions of vasoconstriction and hypoxia.

An alternative hypothesis to the vasoconstriction model is that menstruation is an inflammatory response engendered by the withdrawal of progesterone. The inflammation hypothesis is supported by two features: the prominent accumulation of leukocytes in the endometrium in the premenstrual phase,[188] and the release of matrix-degrading enzymes characteristic of an inflammatory response.[200-203] The hypothesis of progesterone withdrawal—induced inflammation is supported by the uterine inflammation observed in mice lacking progesterone receptors. Apoptotic cell death, which can be triggered by inflammatory mediators, occurs in the late secretory phase first in stromal cells and then gradually spreading throughout the functionalis.[204-206] Rescue from apoptosis has been shown to occur *in vivo* with the administration of progesterone or exogenous hCG.[207]

Changes in proteins involved in apoptosis appear to contribute to the regional programmed death in the endometrium. The anti-apoptotic protein, B cell CLL/lymphoma 2 (Bcl-2), is prominently expressed in the glandular epithelium during the proliferative phase; expression declines in the secretory phase to reach low levels in the late secretory phase when apoptosis occurs.[206] Studies report an inverse pattern of expression of survivin, a recently discovered inhibitor of apoptosis. Survivin binds to and blocks the effector cell death proteases, caspase-3 and -7. The activities of caspase-3, -8, and -9, are higher in the secretory phase. Low rates of survivin expression in glandular epithelium are found in the proliferative phase, rising to peak expression in the late secretory phase. The protein was localized to the nuclei of cells in the functionalis and the cytoplasm in cells in the basalis. This differential distribution may indicate that survivin is not capable of suppressing apoptotic death in the functionalis in the late secretory phase but performs this role in the basalis, consistent with the observed patterns of apoptotic cell death. Elevated levels of survivin in endometriotic lesions correlates with reduced apoptotic death of cells in these lesions.[208]

Although the vasoconstriction and inflammation hypotheses of menstruation might appear to be distinct, there are several overlapping biochemical features of hypoxia and inflammation, including the release of pro-inflammatory cytokines and apoptotic cell death that tend to blur the distinctions between these models. The vascular changes in the endometrium in the perimenstrual phase, resulting either from ischemia-hypoxia or inflammatory reaction, lead to extravasation of blood. Autophagy and heterophagy are evident, as is apoptotic cell death. The superficial endometrial layers become distended by the formation of hematomas; fissures subsequently develop, leading to the detachment of tissue fragments and the ultimate shedding of the functionalis. The resulting menstrual effluent contains the fragments of tissue mixed with blood, liquefied by the fibrinolytic activity of the endometrium. Clots of varying size may be present if blood flow is excessive.

The inflammatory components of menstruation may be essential for the rapid restoration of tissue integrity that occurs following endometrial sloughing.[196] The withdrawal of progesterone, as a well-known anti-inflammatory mediator of the secretory phase, likely participates in the onset of inflammatory changes including induction of MMPs, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), and PAI-1 expression.[196,209,210] At menstruation and with progesterone withdrawal, Cox-2 is dramatically elevated via NF-κB with induction of prostaglandins and lipoxygenases (LOX).[211] One lipoxygenase, LOX15, is expressed with progesterone withdrawal. Since LOX15 is responsible for production of the anti-inflammatory eicosanoid lipoxin A4 (LXA4), expression may help curtail inflammatory responses.[212] Surprisingly, LXA4 was also recently shown to be a potent estrogen receptor agonist[213]

and could function to facilitate repair of the endometrium, particularly during menstruation, when circulating estrogen levels are low, but ERα (at least in the zona basalis) remains elevated.[185]

The duration of menses in ovulatory cycles is variable, generally lasting 4 to 6 days and usually similar from cycle to cycle in any individual ovulatory woman. The duration of flow is considered to be abnormal if it is less than 2 days or more than 7 days. The amount of blood lost in a normal menses ranges from 25 to 60 mL, being greater when coagulation and platelet disorders are present. Loss of more than 60 mL per month is associated with iron deficiency anemia.

Menstruation and the subsequent regeneration of the functionalis layer are notable for the lack of development of synechiae. The majority of cases of uterine synechiae causing Asherman's syndrome occur following pregnancy-related curettage, especially when curettage is performed during the first 4 weeks of the post-partum period when the uterus is particularly vulnerable.[214] Asherman's syndrome is usually associated with infection as well.

Stem Cells and Telomerase in Endometrial Renewal

Menstruation and cyclic repair of the endometrial lining requires both stem cells and an adaptation involving telomerase that imbues the endometrium with near immortality, compared to other tissues in the body. In 2004, the endometrium of women who had undergone bone marrow transplantation was examined and found to contain endometrial epithelial and stroma cells that had clearly been derived from the donor's bone marrow.[215] This was a remarkable finding for two reasons. First, this experiment suggested the possibility that bone marrow derived stem cells traffic and engraft to the endometrium and secondly, that these undifferentiated bone marrow stem cells undergo both mesenchymal and epithelial transformation adopting a phenotype indistinguishable from their host organ. This original observation was later confirmed in the mouse, using male bone marrow donors and female recipients, proving that these were not endometrial stem cells that reside in the bone marrow, but rather bone marrow derived pluripotent stem cells.

Endometrial derived pluripotent cells, whether native to the endometrium or derived from the bone marrow, appear to have unusual properties that may revolutionize how we think about stem cell research. These cells have been manipulated in the mouse model to differentiate into neuronal cell types with the potential to produce dopamine, and pancreatic beta cells that produce insulin.[216,217] Mesenchymal stem-like cells which express cluster of differentiation (CD)40b and CD146 have also been identified by Gargett and colleagues that can be differentiated into adipocytes, osteocytes, chondrocytes, myocytes and endothelium.[218] Based on these early reports, the ready access of stem cells of endometrial origin could obviate the need to perform bone marrow biopsy offering the promise of self-renewing source of autologous stem cells for grafting of a wide variety of cell types with applications for human diseases including Parkinson's or Type I diabetes, as well as treatment for Asherman's syndrome with the possibility of endometrial renewal.[219-221]

Early methods for identifying endometrial stem cells began with the study of retention of cells labelled with 5-bromo-2-deoxyuridine (BrdU).[222,223] These rare, highly proliferative and undifferentiated quiescent populations of cells maintain properties of clonicity, colony forming unit activity, and ability to reconstitute their tissue of origin.[224] The ability to identify the side population (SP) cells have been used to show that in the endometrium, these SP cells are maintained at a constant level (~1%) throughout the lifetime of a woman's reproductive life.[224,225] It was recently suggested that bone marrow derived cells do not contribute to this population of SP cells in the endometrium, suggesting they are a resident to the endometrium and therefore may be an endogenous source of self-renewing stem cells in the endometrium.[226]

The endometrium, unlike other tissues in the body, has the remarkable characteristic of longevity. Despite up to 450 cycles of menstruation and regeneration, the endometrium of the typical woman continues to renew itself with remarkable fidelity. Age is related to telomere length, which in other tissues determines the lifespan of a cell.[227] Endometrium expresses the enzyme telomerase, at levels similar to certain cancers.[228] Levels of telomerase are cycle dependent, with higher levels in the proliferative phase reaching their nadir during the midsecretory phase.[229,230] Interestingly, this enzyme is found in the epithelial but not the stromal compartment.[231] Based on recent reports, telomerase may be indirectly regulated by estrogen through WNT pathways involving beta-catenin expression.[232] In women and baboons with endometriosis associated with progesterone resistance, telomerase levels are increased, suggesting a possible link to this disease and the pathophysiology related to its chronicity and tendency to recur.[233]

Vascular Remodeling and Angiogenesis

Angiogenesis, the formation of new blood vessels from preexisting vessels, rarely occurs in the normal adult except in the female reproductive tract and ovary. Here, the cyclic processes of endometrial shedding and regeneration and corpus luteum formation entail remarkable changes in vessel growth and remodeling. The angiogenic process involves multiple steps and is tightly regulated by activators and inhibitors.[234-236] There are four phases of the endometrial cycle when important events relating to angiogenesis occur: (1) at menstruation, when there is repair of ruptured blood vessels; (2) during the proliferative phase, when there is rapid growth of endometrial tissue; (3) during the secretory phase, with the development of the spiral arterioles that feed a subepithelial capillary plexus; (4) in the premenstrual phase, when there is evidence for vascular regression. If angiogenic remodeling is abnormal, endometrial dysfunction can result, including menorrhagia.

Angiogenesis during the proliferative phase is by vessel elongation.[237] In the secretory phase, intussusception appears to account for the increase in vessel branching; this proliferation of endothelial cells inside of vessels ultimately produces a wide lumen that can be divided by trans-capillary pillars, or alternatively lead to capillary fusion or splitting. Although most prominent in the late menstrual and early and late proliferative phase, endothelial cell proliferation is continuous during the menstrual cycle. Thus, vessel growth

continues during the secretory phase despite the fact that the surrounding endometrial tissue has ceased to grow, resulting in the coiling of spiral arterioles.

Endometrial angiogenesis and vessel remodeling are directed by a network of signalling molecules and receptors that include members of the vascular endothelial growth factor family, fibroblast growth factors, angiopoeitins, angiogenin, and the ephrins and their cognate receptors—which can also exist as secreted ligand-binding domains that function as inhibitors as a result of alternative splicing.[234-236, 238] Although the temporal and spatial patterns of expression of several other angiogenic factors and their receptors have been defined in the endometrium, the specific roles of each of these factors in the endometrial angiogenesis–vessel remodeling cycle remain to be elucidated.

Of the members of the vascular endothelial growth factor family that includes VEGF-A, VEGF-B, VEGF-C, and VEGF-D, VEGF-A is most important for endometrial angiogenesis.[237-239] VEGF-A acts on two different receptors: VEGF receptor-2 (VEGFR2), which may play the dominant role in signaling endothelial cell proliferation; and another tyrosine kinase receptor, VEGFR1 (also known as FLT-1), which may play the dominant role in mediating VEGF effects on vascular permeability.[240-243] Both receptors are present on endothelial cells. VEGFR2, also known as kinase domain receptor (KDR), has been detected in stromal and epithelial cells of the premenstrual endometrium; this presence suggests actions on non-vascular compartments.

The premenstrual phase is characterized by a dramatic up-regulation of VEGFR2 receptor in stromal cells of the superficial layers of the endometrium in response to progesterone withdrawal. The action of VEGF on VEGFR2 may participate in the increased expression of MMP-1 in the stromal in the premenstrual phase.

VEGF-A expression is detectable in glandular epithelial and stromal cells in the proliferative phase, stimulated by estrogen through the actions of hypoxia induced factor -1 α (HIF-1α).[244,245] HIF-1α is also induced by prostaglandin E2, which is maximally produced at the time of menstruation.[246] VEGF released from neutrophils in intimate contact with the endothelial cells is thought to stimulate endometrial vessel growth. It is also present in uterine NK cells. During the secretory phase, VEGF-A can be identified in surface epithelial cells, which presumably secrete it into the uterine cavity. The release of VEGF from subepithelial NK cells has been suggested to play a role in directing the development of the subepithelial capillary network in the secretory phase.

VEGF-A has four common isoforms, It has four common splice variants (VEGF121, VEGF165, VEGF189, and VEGF 206). After ovulation, there is a remarkable shift in VEGF-A isoforms expressed in the uterus with the appearance of VEGF-A189 in the perivascular stromal cells; the VEGF-A189 isoform can be processed by proteolytic cleavage by plasminogen activator.[100] VEGF-A189 increases vascular permeability acting on VEGFR1 while its processed form binds to the VEGFR2 receptor, which mediates the mitogenic action on endothelial cells.

The highest VEGF-A levels are found in the menstrual phase, probably a response to pro-inflammatory cytokines. The surge might also be attributed to focal hypoxia, which is a potent stimulus to VEGF-A gene transcription. Expression of VEGFR1 and VEGFR2 is also greatest in the menstrual phase. The elevated levels of VEGF and cognate receptor expression at this time are presumed to be important for vessel repair and the preparation for angiogenesis in the proliferative phase. Notably, the functional activity of VEGFR2 (as assessed by receptor phosphorylation), a signature indicating ligand activation, is relatively low in the early menstrual phase when levels of soluble VEGFR1 (sFLT-1), which sequesters VEGF, are highest. VEGFR2 receptor phosphorylation increases substantially in the late menstrual phase, remaining modestly elevated in the early and late proliferative phase when sFLT-1 levels decline.[247]

The fibroblast growth factor (FGF) family of proteins may also participate in endometrial angiogenesis through interactions with the VEGF system. FGFs up-regulate VEGFR2 and VEGF-A expression, and, in a feed-forward loop, VEGF-A promotes the release of FGFs from the extracellular matrix. Basic FGF is a potent stimulus for αvβ3 integrin expression. This integrin is present at the site of active angiogenesis and fundamental to endothelial invasion and vessel elongation during angiogenesis.[248]

The angiopoietins (Ang) regulate vessel stability. Ang-1, expressed in vascular smooth muscle cells, binds to a cognate receptor, Tie-2, on endothelial cells, resulting in vessel stabilization. Ang-2 is a physiological antagonist of Ang-1. It also binds to the same Tie-2 receptor. Vessels atrophy when Ang-2 acts in the absence of VEGF-A, whereas angiogenesis is promoted in the presence of VEGF-A. *In situ* hybridization studies indicate that Ang-1 expression is most abundant in the glands and stroma of the early and midproliferative phase and reduced in the late proliferative phase.[249] Ang-2 expression is detected in the glands and stroma throughout the cycle, with highest expression in the early proliferative and mid to late secretory phases. In endometrium from women with menorrhagia, Ang-1 expression is consistently down-regulated; as a result, the ratio of Ang-1 to Ang-2 is reduced, which contributes to vessel instability.[249]

Angiogenin is a heparin-binding molecule that is expressed by endometrial epithelial and stromal cells at their greatest levels in the mid to late secretory phases and in the decidua of early pregnancy. Angiogenin is thought to contribute to the proliferation of vascular smooth muscle cells around the spiral arterioles. Like VEGF-A, expression of angiogenin is stimulated by hypoxia. It is also increased by progesterone.

Ephrins, a family of molecules and their cognate tyrosine kinase receptors, are believed to guide endothelial cells to specific targets. Ephrins have been detected in endometrial endothelial and stromal cells, but the functional roles of these molecules and their receptors in the uterus remains to be clarified.

The physiological consequences of angiogenesis are reflected in changes in endometrial blood flow. By measuring the clearance of radioactive xenon gas, highest endometrial perfusion was reported between day 10 and 12, and day 21 and 26 of the cycle.[86] Microvascular perfusion has been assessed by laser Doppler fluximetry with transvaginal placement of a fiber optic probe into the uterine cavity.[250] With use of this technique, endometrial perfusion was found to be highest during the proliferative phase and the early secretory phase, not too dissimilar from the finding based on xenon clearance. Uterine blood flow is greatest in the fundus, and higher flow rates are associated with better

outcomes in assisted reproduction. Notably, diminished uterine blood flow has not been found in the perimenstrual period, but these methods cannot easily identify localized areas of vasoconstriction.

Extracellular Matrix Remodeling

The biochemical basis for the dramatic structural changes in the endometrium in the perimenstrual period includes the action of specific matrix-degrading proteases, the MMPs.[195,200-203] Studies on human endometrial explants in culture demonstrated that degradation of the extracellular matrix occurs in the absence of progesterone and estrogen, which suppress expression MMPs. Moreover, this degradative process can be blocked by MMP inhibitors, but not by inhibitors of lysosomal cysteine proteinases—directly implicating MMPs in the catabolism of the endometrial extracellular matrix.

Enzymes of the fibrinolytic system, urokinase and tissue plasminogen activator, are increased in the endometrium as progesterone is withdrawn in the perimenstrual period. Moreover, PAI-l expression is reduced, allowing the plasminogen activators to activate plasmin and proteolytically cleave and activate the latent MMP proenzymes.[251]

MMPs represent a large family of proteinases that play a major role in remodeling of the extracellular matrix (Fig. 10.9). *In situ* hybridization and immunocytochemistry have been used to map the expression of MMPs and the endogenous inhibitors, tissue inhibitors of the matrix metalloproteinases (TIMPs), in the primate endometrium. Cell-specific and menstrual cycle-specific patterns were revealed, with the most profound changes occurring during the perimenstrual period.[201,252] After ovulation, the expression of interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), and stromelysin-2 (MMP-10) in the endometrial stroma is essentially restricted to the perimenstrual and menstrual phase.

Other MMPs are detected during the proliferative and secretory phases but are significantly increased in expression perimenstrually. These include the type IV collagen-degrading enzymes, MMP-2 and MMP-9. The membrane-bound MMP, MMP-14 (which activates MMP-2), is detected during menstruation in stromal inflammatory cells and epithelial cells. TIMP-1, which is detectable in the endometrium throughout the cycle, is increased in the stroma, epithelium, and arterioles at menstruation.

The importance of progesterone withdrawal in regulating endometrial MMPs and the different temporal patterns of expression have been well-documented in *in vivo* and *in vitro* systems.[91] In a primate model in which hormone levels were manipulated by steroid implants, progesterone withdrawal resulted in up-regulation of MMP-1, -2, -3, -7, -10, -11, and -14. It is important to note that the expression of MMPs in the endometrium is heterogeneous.[195,252]

At the start of menstruation, MMP-1 is found in patches of stromal cells in the superficial zone; these patches are colocalized with areas of reduced stromal and epithelial expression of estrogen and progesterone receptors and focal disruption of the extracellular argyrophilic fibrillar network—reflecting the degradative activity of MMP-1. As the process of menstruation proceeds, MMP-1 expression spreads to include the entire functionalis. MMP-2 and MMP-3 expression is also limited to the stromal cells in the functionalis. During menses, MMP-1, -2, -3, and -9 localize primarily in and around arteriolar walls. The heterogeneity of MMP expression suggests that MMP gene transcription is under the control of local rather than systemic (steroidal) factors. In other words, steroids are indirectly influencing MMP expression.

Progesterone, particularly in the presence of estradiol, can suppress expression of certain MMPs (i.e., MMP-1, -2, -7, -9, and -11) in endometrial explant culture.[203] This action is most likely explained by changes in autocrine/paracrine signals—particularly pro-inflammatory cytokines or members of the transforming growth factor family which, respectively, are potent inducers and suppressors of MMP gene transcription. In culture systems, IL-lα has been implicated as the mediator of MMP-1, MMP-3, and MMP-7 expression in response to withdrawal of progesterone. Neutralizing antibodies to TGF-β prevent the action of progesterone in blocking MMP-3 and MMP-7 expression.

Endometrial bleeding factor (EBAF) is the orthologue of the murine gene named Lefty and another likely candidate for a progesterone-regulated cytokine controlling MMP expression.[253-255] Lefty was originally identified in human endometrium as a gene up-regulated in the late

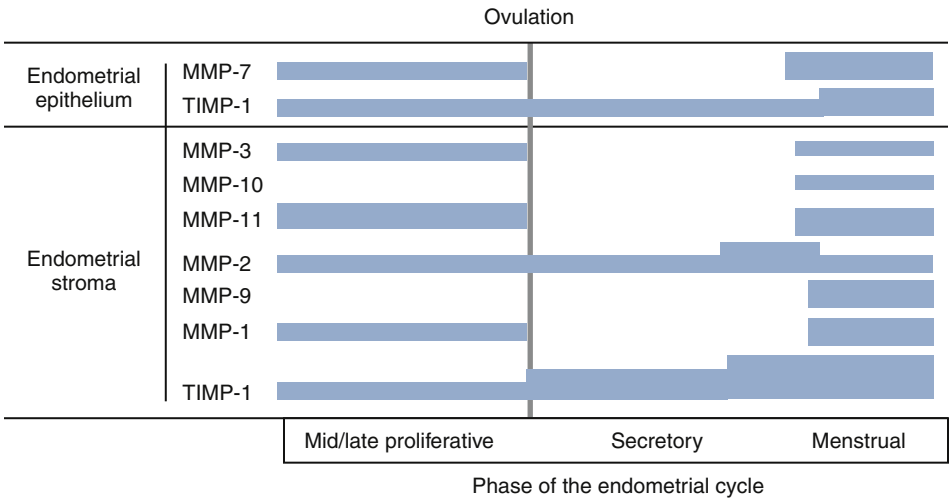


FIGURE 10.9 Schematic of matrix metalloproteinase (MMP) expression in the human endometrium during the menstrual cycle. *(Modified from Rodgers WH, Matrisian LM, Ciudice LC, el al. Patterns of matrix metalloproteinase expression in cycling endometrium imply differential functions and regulation by steroid hormones.* J Clin Invest *94:946–953, 1994.)*

secretory and menstrual phases of the normal cycle, being absent in the proliferative, early, and midsecretory endometrium. EBAF expression, which is predominantly found in the endometrial stroma and to a much lesser extent in the glandular epithelium, is suppressed by progesterone. Interestingly, endometrium from women with a history of abnormal bleeding and endometriosis expressed Lefty at unusual times including the proliferative, early, and midsecretory phases.[256] Such expression patterns may help explain the abnormal bleeding before menses, often associated with the presence of endometriosis.[257]

Unlike other members of the TGF-β family that promote the formation and stability of the extracellular matrix, EBAF down-regulates the elaboration of collagen in association with reduced expression of connective tissue growth factor while up-regulating expression of collagenolytic and elastinolytic enzymes.[258] These actions of EBAF are the result of antagonism of the SMAD (a portmanteau of two other protein names, Drosophila MAD, mothers against decapentaplegic, and C. elegans SMA, for small body size) signaling pathway that is activated by the other TGF-β growth factors. Thus, the decline in progesterone and estradiol in the late luteal phase initiates alterations in the endometrium that include up-regulation of proinflammatory cytokines (some of which may be contributed by immune cells that accumulate in the endometrium) and a natural TGF-β antagonist. The collective result is focal and then widespread expression of matrix degrading enzymes that result in the remodeling of stroma and blood vessels in the functionalis. EBAB/Lefty also has been shown to inhibit the expression of key decidual proteins, IGFBP-1 and PRL, through regulation of transcription factors FOXO1 and ETS1, thus causing infertility when prematurely expressed.[259]

Lysosomal involvement in the process of menstruation has been proposed because of three observations: (1) an increase in the abundance of lysosomes in the endometrium during the late secretory phase, (2) the cytochemical demonstration of acid phosphatase in the perimenstrual endometrium, and (3) the high specific activity of certain lysosomal hydrolases in endometrial tissue in the menstrual phase.[260] However, inhibitors of these enzymes, leupeptin and E-64, do not prevent the progesterone withdrawal—induced breakdown of extracellular matrix in endometrial explants as do the inhibitors of MMP activity. These observations suggest that lysosomal proteinases are not major contributors to the remodeling of the perimenstrual endometrium.

Vasoactive Substances

The *endothelins* are a family of potent vasoconstrictors produced by endothelial cells that act on two types of receptors present on vascular smooth muscle. Endothelin-1, produced by endometrial epithelial or stromal cells, may act on spiral artery smooth muscle cells to promote vasoconstriction. Enkephalinase, a membrane-bound metalloendopeptidase, degrades endothelin-1 and other vasoactive peptides, and is present in highest levels in the midsecretory endometrium.[116] Expression of the gene encoding enkephalinase is up-regulated by progesterone. The decline in progesterone levels at the end of the luteal phase results in a subsequent fall in enkephalinase, which prolongs the biological life of endothelin-1. Vasopressin may also function as a vasoconstrictor in the endometrium during the menstrual phase of the cycle.[261]

The production of prostaglandins, particularly $PGF_{2\alpha}$ and other eicosanoids in the endometrium, is enhanced by lysosomal phospholipases that liberate the arachidonic acid that accumulates in endometrium during the secretory phase; in turn, arachidonic acid is metabolized into prostanoids (see Chapter 6).[262] The premenstrual decrease in progesterone is also followed by an induction of the prostaglandin synthase, Cox-2, and decline in 15- hydroxyprostaglandin dehydrogenase activity which inactivates $PGF_{2\alpha}$. This induction of the prostaglandin synthase and the decline in 15-hydroxyprostaglandin dehydrogenase leads to increased production and bioavailability of $PGF_{2\alpha}$, which triggers myometrial contractions that compress the endometrial vasculature and promote hemostasis (Fig. 10.10).[263]

Hemostatic and Fibrinolytic Mechanisms

The relative activities of the hemostatic and fibrinolytic systems in the endometrium are shifted in the perimenstrual period such that clotting activity is reduced and fibrinolytic activity is increased. Consequently, menstrual fluid does not normally clot, even on prolonged storage. Decidualized stromal cells express tissue factor (TF), the primary trigger of thrombin formation and hemostasis, under the influence of progesterone.[192] TF is expressed in the endometrial stromal cells along with another hemostatic factor, plasminogen activator inhibitor-1 (PAI-1) under the influence of progestins, which suppresses MMP production.[264] Tissue factor production by the decidualized stromal cells declines with withdrawal of progesterone, along with the increase in MMP production.

The endometrial fibinolytic system includes urokinase-type plasminogen activator and tissue-type plasminogen activator, which cleave plasminogen to yield the fibrinolytic enzyme, plasmin.[192,265,266] This system is hormone regulated and can be modulated by drugs. Progesterone reduces expression of urokinase and increases that of PAI-1 in cultured endometrial cells. Removal of progesterone or the addition of the anti-progestin, RU486, reverses these

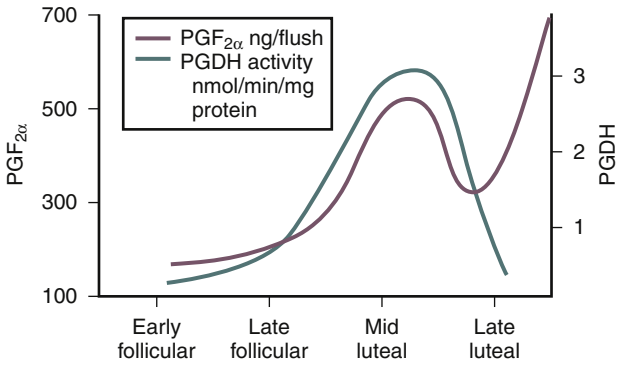


FIGURE 10.10 Changes in $PCF_{2\alpha}$ levels and endometrial 15-hydroxy prostaglandin dehydrogenase (PGDH) activity. *(From Casey ML, Hemsell DL, MacDonald PC, et al. NAD+ dependent 15-hydroxy prostaglandin dehydrogenase activity in human endometrium. Prostaglandins 19:115, 1980; Demers LM, Halbert DR, Jones DF, Fontana J. Prostaglandin F levels in endometrial jet wash specimens during the normal human menstrual cycle 10:1065, 1975.)*

responses.[266] Tranexamic acid, an antifibrinolytic agent, is a synthetic derivative of the amino acid lysine, that reversibly blocks lysine binding sites on plasminogen, and inhibits plasminogen activation to plasmin. This drug, administered orally or intravenously, can be used to control dysfunctional menstrual bleeding or bleeding associated with fibroids.

The Uterus in the Cycle of Conception and Pregnancy

In the event of oocyte fertilization, hCG from the implanting embryo rescues the corpus luteum from apoptosis and regression, maintaining serum progesterone secretion and endometrial integrity. This integrated mechanism demonstrating the importance of synchrony, creates a window of endometrial and ovarian receptivity that is essential for the success of the pregnancy.[267] Women who are delayed in establishing embryo implantation, beyond the normal window of implantation (7 to 10 days after ovulation), are at increased risk for miscarriage.

The examination of hysterectomy specimens revealed that the first consistent structural changes in the endometrium of early pregnancy are recrudescence or accentuation of glandular secretory activity, edema, and the predecidual reaction.[268] The increased prominence of the vasculature is considered to be a manifestation of increased blood flow, which together with VEGF production (a vascular permeability factor) may account for the associated edema. Endometrial biopsies in a cycle of conception suggest that stromal edema and vascular congestion are the earliest persistent morphological features of the endometrium of pregnancy.[269]

Within the first weeks of gestation, the endometrium undergoes characteristic changes in which epithelial cells of the endometrial glands become distended with a clear cytoplasm (Fig. 10.11). Many of the epithelial cells develop enlarged and hyper-chromatic nuclei. The enlarged nuclei are polyploid. Parallel channels of endoplasmic reticulum and large mitochondria are abundant in the epithelial cells, and the Golgi complexes have numerous stacked saccules. These changes are commonly referred to as the *Arias-Stella*

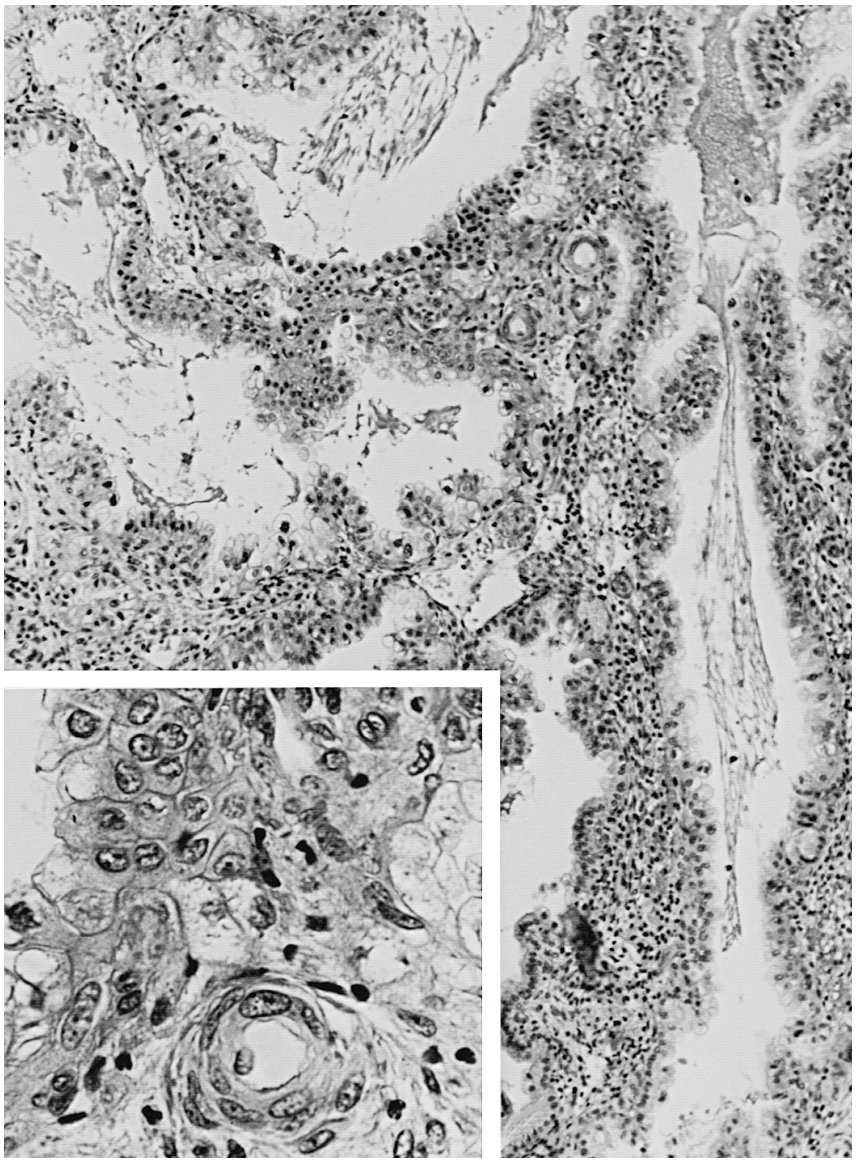


FIGURE 10.11 Arias-Stella reaction. Hypersecretory glands with enlarged hyperchromatic nuclei are present. The glands have scalloped borders. *Inset:* High-power view.

reaction.[270,271] The ultrastructural characteristics of the endometrium are consistent with a hypersecretory state, providing the earliest nutrition and maternal communication with the nascent embryo. As blood flow to the placenta requires vascular remodelling that occurs later, this uterine milk from the endometrial glands provides direct and essential sustenance for the early fetus.[272]

The Arias-Stella reaction has an irregular distribution in the uterus and is present in about 50% of the uteri of women with ectopic pregnancies. It could reflect changes in steroid hormone levels as well as the direct action of hCG on the endometrium, a subject discussed later in this chapter. The endometrium exhibits significant changes in cellular composition that are reflected in the marked alterations in the synthesis and secretion of endometrial proteins. As gestation advances, endometrial glands atrophy and are scarce at term.

The decidua develops with continued exposure of the uterus to progesterone, secreted first by the corpus luteum and later by the placenta. Based on *in vitro* studies, other factors (including hCG and relaxin) may act synergistically with progesterone to promote decidualization. Because exogenous cyclic adenosine monophosphate (cAMP) in the presence of progesterone represents a sufficient *in vitro* stimulus to induce stromal cell differentiation into decidual cells, relaxin and hCG likely exert their effects through this second messenger. Extrapolating from studies on the mouse, including findings on knock-out animals that have been found to be defective in decidualization, it is possible that amphiregulin, HOXA10, HOXA11, IL-11, LIF, Cox-2, and prostaglandins all have a role in the decidual response in humans.[273-275]

The decidualized stroma represents a tissue that is both permissive and simultaneously a restrictive barrier of trophoblast invasion and placentation; its remodeling is crucial to the morphogenesis of the placenta and the establishment of the uteroplacental circulation. Moreover, the decidualized stroma represents the arena where the fetal semiallograft is exposed to maternal immunologically competent cells. While creating a hospitable environment for trophoblast invasion, the decidua also sets limits on this process to prevent excessive penetration and tissue destruction beyond its bounds.

The plump, polygonal decidual cells are arranged in a cobblestone configuration. The ultrastructural features of the decidual cells—including prominent Golgi complexes, dilated rough endoplasmic reticulum, and dense membrane-bound secretory granules—are characteristic of secretory cells. The histologically distinct cell borders around decidual cells reflect the accumulation of a pericellular matrix (Fig. 10.12).[189,190] Abundant decidual cell prolyl hydroxylase, an enzyme involved in collagen synthesis, indicates the important role of these cells in extracellular matrix production. There is also an abundance of amorphous components, including high-molecular-weight proteins with voluminous saccharide moieties (e.g., heparin sulfate proteoglycan). Fibrillar collagens are partially broken down and reorganized. Type V collagen epitopes are unmasked; collagen type VI, "stiff" short collagen fibers that bridge other fibrillar collagens, disappears from most of the stroma and persists only in association with vessels and the basement membrane of the glands.

The deposition of a basement membrane-type matrix containing laminin and type II collagens around the decidual

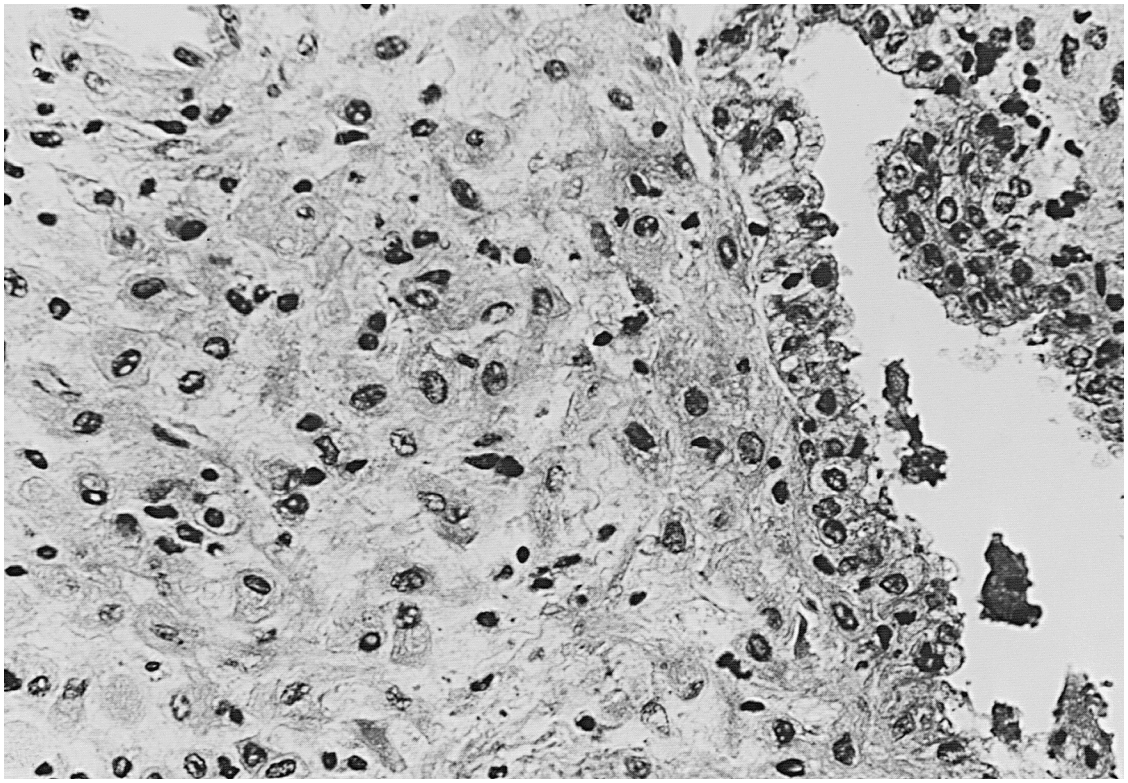


FIGURE 10.12 Decidua of pregnancy. Decidualized stromal cells are plump and have distinct cell borders. Glands are atrophic.

cells contributes to the formation of a "looser stroma" that serves as a substrate for the invading trophoblast cells. For example, entactin, a component of this basement membrane-like matrix, promotes trophoblast cell adhesion and migration. The decidual matrix is also a rich source of cytokines, protease inhibitors, protease precursors, and other factors that modulate cell behavior. These are derived, at least in part, from the decidual cells whose secretory products also include IGFBP-1 and TGF-β, which may restrain the invasion of trophoblast cells.[276,277] Forkhead box 0A1 (FOXO1A) has been shown to regulate many of the genes induced during decidualization including IGFPB-1 and prolactin.[278] Ghrelin is an endogenous ligand for the growth hormone (GH) secretagogue receptor and has also been shown to be involved in decidualization of human endometrium as well.[279,280] Ghrelin in combination with sex steroids estradiol and progesterone and cAMP increase both prolactin and IGFBP-1, while ghrelin alone antagonizes the actions of cAMP.[281] Reduced Ghrelin has been associated with infertility.[282]

The Endometrium in Advancing Age

Based on donor oocyte pregnancies achieved in post-menopausal women whose reproductive tract was programmed with exogenous steroid hormones, uterine aging in the absence of acquired pathology evidently does not preclude successful implantation and carriage of a pregnancy to term. The relentless ability of the endometrium to restore itself may hint at its longevity. A rich source of the enzyme telomerase,[230] the endometrium may have the ability to delay the normal aging process. However, there are structural changes that take place in the uterus with advancing age, pregnancy, and the cessation of ovarian function. Whether or not these changes affect the "reproductive potential" of the uterus or contribute to the increased incidence of pregnancy complications associated with advanced maternal age (e.g., increased incidence of stillbirth and intrapartum complications) are not known.

The basal endometrium interdigitates with the myometrium with advancing age, resulting in a degree of superficial adenomyosis that is a normal finding in the uterus in the fifth decade of life. The infiltrating endometrium does not undergo normal cyclic changes. This may be a consequence of a uterus hosting a past pregnancy.[283]

After menopause, in the absence of hormone replacement, endometrial atrophy is apparent and mitotic activity ceases. Epithelial cells shrink in size, and the stroma becomes fibrotic. A compact eosinophilic material is found in the lumina of the endometrial glands, occasionally engorging them and giving rise to the histological pattern referred to as *cystic atrophy*.

The Uterine Cervix

The cervix is a remarkable specialized structure at the base of the uterus that functions as a biological "gate", controlling the access of sperm and microorganisms into the uterine cavity.[284] During pregnancy, it helps retain the fetus and extra-embryonic tissues and fluids in the uterus, safely until the time of parturition. The endocervix is lined by layer of tall columnar ciliated and non-ciliated secretory cells. A rich extracellular matrix consisting of fibrillar collagens and elastin with embedded fibroblasts and a small percentage of smooth muscle cells (approximately 10%) lies beneath the endocervical epithelium. There are no true glands in the endocervix, but rather an intricate system of crypts or grooves. These endocervical cells stop at a sharply demarcated junction with the stratified squamous epithelium that covers the portio vaginalis at the external os.

The secretory cells of the endocervix elaborate mucus with an average production of 20 to 60 mg daily in a woman of reproductive age. At midcycle, this production rate increases tenfold to twentyfold. The mucus is a complex mixture of water, electrolytes, and mucins, which are large glycoproteins that are heavily laden with O-linked oligosaccharides. Human cervical mucus is approximately 92% to 94% water, but the water content rises to 98% at the time of ovulation. Inorganic salts make up approximately 1% of the mucus by weight.

Mucins represent 45% of the protein content of cervical mucus.[285,286] The mucin core proteins typically contain tandem repeat domains enriched in serine, threonine, and proline residues. These molecules have a role in protecting the upper reproductive tract from bacterial colonization by trapping microorganisms; they also protect the epithelial cell surface, and, as a result of their hydration, form a gel that keeps the cervical canal open.

The cervix expresses a number of mucin genes, including *MUCl*, *M17C4*, *M17C5AC*, *M17C5B*, *MUC6*, and *MUC8*. The periovulatory mucins form a hydrogel, a large mesh that facilitates penetration by motile sperm. The abundance of the mRNA encoding the major cervical gel-forming mucin, MUC5B, is greatest right before ovulation; the amount of MUC5B protein per unit protein in the cervical mucus reaches a peak at ovulation. Cervical expression of MUC5B mRNAs declines markedly after ovulation.

The composition of mucus determines its rheological properties, including consistency, flow elasticity, Spinnbarkeit (the capacity of liquids to be drawn into threads), thixotropy and tack, as well as its ability to form a fern-ing pattern when dried as a result of crystallization of salts (sodium chloride and potassium chloride) in the presence of protein. In the preovulatory phase the mucus is profuse, thin, clear acellular with an alkaline pH. Semi-quantitative scoring schemes that assess quantity, rheological properties like Spinnbarkeit, ferning, and the appearance of the cervix and cervical os, have been used to assess the estrogen status of women. Progestin-only contraceptives, including long-acting injectables and progestins delivered in implants, result in a viscous cervical mucus that impedes sperm penetration. These effects on cervical mucus and sperm penetration can be seen 3 days after insertion of a levonorgestrel-releasing implant.[287]

The Myometrium

The myometrium is organized into strata: an external hood-like layer covering the fundus, a dense network of fibers beneath it, and an innermost layer surrounding the internal os and tubal ostia. Magnetic resonance imaging (MRI) of the uterus of women of reproductive age reveals two distinct myometrial zones: the junctional zone underlying the endometrium (which has low signal intensity) and the outer myometrium (which has relatively high signal intensity).[288]

The junctional zone corresponds to the hypoechogenic layer of the myometrium, the subendometrial halo observed in ultrasound images. The lower T2-weighted signal intensity of the junctional zone is due to the lower water content (mostly a consequence of contractile activity of this zone that decreases its blood volume), less extracellular matrix, and more tightly packed smooth muscle cells. This zonal anatomy is apparently dependent upon sex hormones because it is not evident prior to menarche or in postmenopausal women who are not receiving hormone replacement therapy. MRI images reveal that the junctional zone is altered in early pregnancy, evidently as a consequence of embryo-derived factors.

The functions of the junctional zone of the myometrium appear to be linked to events in the menstrual cycle and the establishment of early pregnancy; those of the outer myometrium are related to pregnancy and parturition. The junctional zone has several unique features that distinguish it biochemically and functionally from the outer myometrium. Although steroid hormone receptors are expressed throughout the myometrium, the junctional zone exhibits cyclic changes in expression of estrogen and progesterone receptors that parallel those of the endometrium. This differs from the "outer" myometrium, which does not show marked cyclic changes in receptor expression.

During the menstrual cycle, subendometrial peristalsis has been observed by ultrasonography and ultra-fast MRI.[289-292] The frequency, intensity, and direction of the subendometrial contractions vary during the menstrual cycle. They average two to three cycles per minute and flow from cervix to fundus at midcycle; diminish in the luteal phase; and increase again during menstruation, but in a fundal-to-cervical direction. Changes in steroid hormone levels, expression of hormone receptors, and prostanoids govern the peristaltic activity. Some authors have suggested that the contractile activity of the junctional zone alters uterine shape from an elongated configuration in the follicular phase to a piriform configuration in the luteal phase, changes that make the uterine cavity more spherical after ovulation.[293]

In some pathological states, including endometriosis or adenomyosis, dystonic uterine contractions have been observed.[294-296] Whether or not these aberrant contraction patterns contribute to infertility and other symptoms of endometriosis remains to be determined. It is tempting to speculate that disruptions in the normal fundal-to-cervical contractions during menstruation might worsen the sensation of uterine cramping associated with endometriosis, and perhaps even contribute to the retrograde menstruation thought to underlie the pathogenesis of this disorder.

The myometrial compartment changes dramatically during pregnancy, primarily as a result of muscle hypertrophy, the elaboration of extracellular matrix, and an increase in lymphatics and blood vessels. The wet weight of the human uterus increases tenfold during pregnancy, and its carrying capacity increases from 300 mL to 4.5 L, largely owing to hypertrophy and hyperplasia of the myometrium. The myometrial hyperplasia is steroid hormone-dependent and probably mediated by growth factors, particularly the IGFs.[102] The primate myometrium expresses all components of the IGF signaling system, including IGF-1, IGF-2, and the type 1 growth factor receptor, as well as IGF-binding proteins (IGFBPs 2, 3, 4, and 5). Progesterone enhances expression of IGF-1 mRNA stimulated by estrogen. The stimulation of IGF-1 expression is accompanied by an increased number of Ki67– positive myometrial cells, indicating myometrial cell proliferation.

Immunology of the Reproductive Tract

The uterus is an immunologically privileged organ: it can accommodate the fetus and extra-embryonic tissues as a semi-allograft, yet it also is endowed with mucosal immunity, defenses against ascending foreign organisms, and a system to efficiently clear the endometrial detritus that results from menstruation (see Chapter 14). The human reproductive tract has components of both the innate and adaptive branches of the immune system. Uterine epithelial cells express members of the Toll-like receptor family (TLR 2-6, 9, and 10) which detect pathogen products and trigger a cellular response to these "foreign" molecules including peptidoglycans from Gram positive bacteria (TLR2), lipopolysaccharide from Gram negative bacteria (TLR4), and unmethylated CpG islands found in bacterial DNA (TLR9). The endometrium also produces host defense molecules, defensins, as well as cytokines and chemokines. Uterine lymphoid and myeloid cells play roles in tissue defense, immune modulation, angiogenesis, and tissue remodeling.[188,297-300] These cells are present in the fallopian tubes, uterus, and cervix, with the fallopian tubes and uterus containing a higher proportion of leukocytes than the cervix and vagina.[301]

Both innate and adaptive immune responses by the endometrium are regulated by steroid hormones. Progesterone has potent immunomodulatory effects[302]; it induces a local Th2-type cytokine response in the uterus, which includes an increase in IL-4, IL-5, and IL-15 and down-regulation of the IL-13 receptor α2, which is a negative regulator of the anti-inflammatory cytokine, IL-13 and powerful inhibitor of the Th2 response.[303] The Th2 response is believed to counter pro-inflammatory processes in the endometrium that could lead to rejection of the embryo. Steroid hormone-directed alterations in endometrial chemokine production influence the trafficking of blood leukocytes in the reproductive tract. Further, actions of progesterone are important in the overall immune suppressive phenotype adopted by the receptive endometrium.[304] Such actions of progesterone are dependent on the PR isoform (A and B) involved.[305]

During the secretory phase there is a profound recruitment of leukocytes into the endometrium starting in the perivascular locations around spiral arterioles and glandular epithelium.[306-309] Progesterone-induced alteration in endometrial cytokine/chemokine production contributes to this recruitment.[310] Cytokines IL-1, IL-11, IL-15, LIF, TGF-β regulate trafficking of leukocytes to the endometrium.[311] IL-15 recruits NK cells into the endometrium[312] as evidenced by the IL-15 null mice that lack uNK cells. Locally acting prostaglandins including PGE along with VEGF modulate vascular permeability.[309,313] Cyclooxygenase-2 (Cox-2), a rate limiting enzyme that regulates the biosynthesis of PGE2, is critical to implantation in animal models,[314,315] with PGE2 required for initiation and maintenance of decidualization.[316,317] Blockade of Cox-2 prevents decidualization in mice[315] indicating that Cox-2 plays a critical role in endometrial function at the time of implantation.[318,319] Reduced Cox-2 is associated with implantation

failure[320] and unexplained recurrent pregnancy loss,[321] and increased Cox-2 expression is found in endometriosis,[322,323] and may be associated with other immune changes seen in this disease, including increased Th1 or Th17 responses.[324] The development of an immunotolerant maternal immune response is essential for the acquisition of endometrial receptivity and the success of pregnancy.[325-327] Factors that support a more suppressed immune environment, including the recruitment of T regulatory cells (Tregs) and a shunting away from a pro-inflammatory, Th1/Th17 responses are likely critical to establishment of normal pregnancy and lack of these immune changes may underlie infertility and pregnancy loss associated with various inflammatory conditions such as endometriosis (discussed below).

Leukocytes and Lymphocytes

There are striking menstrual cycle–dependent changes in the immune cell population of the endometrium. Neutrophils, the most abundant leukocytes of the immune system, are rare in the normal endometrium until the peri-menstrual phase, when they accumulate and account for 6% to 15% of the total cell number. Eosinophils are also rarely found in the normal endometrium until the perimenstrual phase, when they also accumulate—usually in aggregates—and show evidence of activation, as revealed by the extracellular location of eosinophil cationic proteins. Macrophages are present in the endometrium throughout the cycle, increasing in number from the proliferative to the menstrual phases. Immediately prior to menstruation, the number of macrophages in the functional layer of the endometrium is equivalent to that of neutrophils.

Mast cells expressing tryptase (a characteristic of mucosal mast cells) and chymase (a characteristic of connective tissue mast cells) are found in the human endometrium. In the functionalis, mast cells are positive only for tryptase while those in the basalis express both enzymes. Degranulation of these cells may initiate activation of matrix metalloproteinases as a result of tryptase and chymase action on proMMP-1 and proMMP-3.

The endometrial lymphoid system has a distinctive composition and activity. There is good reason to believe that endometrial T cells are activated *in situ*, as judged by their expression of antigens that are characteristic of the activated state; these antigens include the major histocompatibility complex (MHC) class II molecules (also known as HLA) includes HLA-DR, HLA-DP, and HLA-DQ, and very late antigen 1. CD3+ T cells represent only 1% to 2% of the lymphomyeloid cells detectable in the human endometrium. They are present throughout the cycle in aggregates in the basalis, as well as singly throughout the stroma and in intraepithelial sites. The number of these cells increases prior to menstruation. The ratio of $CD4^+$ T helper lymphocytes to $CD8^+$ cytotoxic T cells in the endometrium is inverted compared with peripheral blood. The latter are cytolytic during the proliferative phase, but this activity diminishes in the secretory phase. This decline might reflect the influence of the tissue environment programmed by steroid hormone progesterone, or possibly an alteration in the leukocyte population through deletion or recruitment of $CD8^+$ cells with a different phenotype. Few B cells and plasma cells are present in the human endometrium.

Leukocytes make up between 15% to 30% of the cellular mass of the endometrium.[328] They communicate with the endometrial epithelium and trophoblast through complex networks of cytokines[329] and likely are involved in further leukocyte recruitment to the endometrium.[311] Reductions in this cytokine milieu is associated with pregnancy failure.[330]

In the human, progesterone is essential for establishment of pregnancy,[331] directing the coordinated biochemical modifications in the endometrium that regulate the controlled invasion of the fetal allograft, including the establishment of immunological tolerance.[332-334] The actions of progesterone on immunity are mediated in part through retinoic acid (RA), TGF-β,[335-337] and peroxisome proliferator-activated receptor γ (PPAR- γ), which exert a profound influence on CD4+ leukocyte differentiation. The actions of P (and RA and PPAR- γ) are disrupted in certain gynecological disorders including endometriosis, leading to alterations in the endometrial phenotype and contributing to infertility and early pregnancy loss.[109,338] Normal eutopic endometrium contains leukocytes in both stromal and intraepithelial locations.[306,308] Recent evidence suggests that maternal immune responses are critical for the acquisition of endometrial receptivity,[325-327] and that immune cells can directly regulate endometrial epithelial receptivity and associated gene expression.[339] Macrophages have been shown to regulate uterine epithelial gene expression in the mouse as well as human,[329,340] suggesting a mechanism for observed differences in eutopic endometrial gene expression in endometriosis. Conversely, inflammatory cytokines in peritoneal fluid or peripheral circulation of women with endometriosis could alter the differentiation pathways of monocytes toward a more hostile phenotype.[341]

T-Regulatory and Th-17 Cells and Endometrial Receptivity

As discussed in Chapter 14, the immune system is divided into "innate" immunity including monocytes, macrophages, dendritic cells (DC), neutrophils, basophils and mast cells, and natural killer cells from the lymphoid lineage. The adaptive immune system includes the T and B cells, which require innate immunity participation for establishment of an immunological memory.[342] Bone marrow (BM)-derived cells are known to traffic to endometrium, via steroid-regulated chemokine production.[215,343] Under the right circumstances, DC and Treg cells increase at the implantation site, along with uNK cells that interact with the invading placental cells to both direct and limit trophoblast invasion.[344] BM-derived decidual cells ultimately determine pregnancy outcomes.[338,345-353] Monocyte-derived leukocytes and uterine natural killer (uNK) cells comprise up to 20% to 40% of all endometrial cells in the secretory phase and are critical to the establishment of endometrial receptivity,[307,344,354] while the loss of function of these cells may account for infertility and recurrent pregnancy loss seen in women with endometriosis.[326,334,338,355-357]

Adaptation of the innate immune system is required for pregnancy and survival of the semi-allograft.[334,338] This immunotolerance is mediated in part by T-regs.[355,358] Treg cells as suppressors of inflammatory immune responses, keeps the immune system under control.[359] In the mouse

model, Treg cells proliferate and are activated in the uterus in response to paternal antigens and non-specific hormonally regulated factors.[338,360] In contrast, the subtype of inflammatory T cell called Th17 cells, is associated with multiple autoimmune and inflammatory conditions including colitis, multiple sclerosis, and rheumatoid arthritis.[361] IL-9 induces Th17 cell differentiation[362] attenuation of IL-9 decreases Th17 cells and reduces IL-6 producing macrophages,[363] contributing to Th17 differentiation. A balance in T-cell differentiation may ultimately define whether the endometrium becomes receptive to the fertilized oocyte and whether a pregnancy will be successful.

Uterine Natural Killer Cells

The uterine NK cell, also known as the granular lymphocyte, is a unique member of the lymphoid lineage found in the endometrium.[297] These are round cells that characteristically have bi-lobed or indented nuclei and a pale cytoplasm containing acidophilic granules. They are a specialized subset of NK cells based on their expression of cell surface antigens ($CD3^-$, $CD16^+$, and $NCAM/CD56^{bright}$). This pattern is different from blood NK cells, which have a phenotype of $CD56^{dim}$ and $CD16^+$.

Uterine NK cells are among the most abundant lymphomyeloid cells in the perimenstrual endometrium. Very few are present in the proliferative phase endometrium; they accumulate during the secretory phase, at which time they comprise 15% to 25% of the cells in the endometrial stroma. It has been postulated that the dynamic changes in uterine NK cell number during the cycle are determined by changes in endometrial prolactin production, which is then controlled by ovarian steroid hormones. This notion is based on the known roles of prolactin as an immunomodulator and the correlation between endometrial prolactin levels and NK cells.[364,365]

The NK cells persist in the decidua during the first trimester, when they represent 70% of the decidual leukocyte population. Their close association with stromal cells has led to the suggestion that they have an important role in initiating and maintaining decidualization. In a non-fertile cycle, the NK cells that amass during the secretory phase undergo programmed cell death.

When activated by interleukin (IL)–2 *in vitro*, the NK cells become competent to kill malignant cells (and some normal ones) through the release of cytotoxic proteins like perforin. From the late proliferative phase on, the NK cells express cytotoxic activity and are thought to play a role in protecting the endometrium against infection. The cells have also been proposed to modulate trophoblast invasion during implantation and placentation because of their abundance in the decidua during the first trimester.

Although *in vitro* studies document the killing activity of cytokine-activated NK cells, there is little evidence for destruction of trophoblast cells *in vivo*. Thus, any restraining activity that NK cells exert on invading trophoblast cells appears to be through a non-cytotoxic mechanism that may involve secreted cytokines, including colony-stimulating factor-1 (CSF-1), IL-1, LIF, and INF-γ. The apparent *in vitro* and *in vivo* resistance of trophoblast cells to death may be explained by the fact that trophoblast cells express a non-classical and nonpolymorphic major histocompatibility complex (MHC) class I antigen, HLA-G; NK killing activity is also suppressed by endometrial stromal cells.

In the transgenic mouse line TgE26, which lacks NK cells, abnormal implantation sites are found. Fetal demise follows, in association with changes in uterine arterioles suggestive of arteriosclerosis and hypertension.[365] These histopathological changes are reminiscent of pre-eclampsia in humans. In contrast, mice lacking IL-15, and consequently, circulating and decidual NK cells, are fertile but show a thickening in uterine vessels. These findings collectively implicate NK cells in vessel remodeling, a role consistent with their expression of angiogenic factors.

Factors Regulating Uterine Immune Cell Dynamics

Changes in lymphomyeloid cell populations in the endometrium, particularly their accumulation in the premenstrual phase, appear to be the result of recruitment from the peripheral circulation and intra-endometrial proliferation. The accumulation of migratory cells is directed by chemoattractant cytokines, chemokines, and the expression of intercellular adhesion molecules that attach leukocytes to the endothelium in preparation for extravasation and trafficking through the endometrium.[366] Expression of these molecules changes during the menstrual cycle, under the influence of steroid hormones as well as extrinsic inflammation, such as that seen in endometriosis.[367]

The expression of several chemokines has been documented in the human endometrium, including fractalkine (CX3CL1), Regulated on Activation, Normal T Expressed and Secreted (RANTES) (CCL5), IL-8, MCP-1 (CCL2), MCP-2 (CCL8), and eotaxin (CCL11). These chemokines bind to receptors on leukocytes, promoting the appearance of molecules that mediate adhesion to the endothelium, extravasation and chemotaxis along the concentration gradient of the chemokine, thus targeting specific leukocyte types to specific endometrial compartments. A repertoire of cell adhesion molecules expressed in the endometrium, including intercellular adhesion molecule-1 (ICAM-1), ICAM-2, VCAM-1, E-selectin, and PECAM, specifies the recruitment and location of leukocyte and platelet accumulation. ICAM-1 is present in the functionalis in the menstrual phase; ICAM- 2 is restricted to the vascular endothelium and does not appear to change during the cycle, vascular cell adhesion molecule-1 (VCAM-1) and E-selectin appear in the upper layer of the functionalis in the secretory phase, and the platelet endothelial cell adhesion molecule platelet endothelial adhesion molecule (PECAM) is abundant in the stroma during the menstrual phase.

Evidence for leukocyte proliferation in the endometrium includes the expression of Ki67 and incorporation of BrdU (both proliferation markers) by NK cells with a marked increase in the secretory phase. The uterine NK cell proliferation may be driven by progesterone-regulated stromal factors as $CD56^+$ cells proliferate *in vitro* in the presence of progesterone-treated endometrial tissue.

LIF is associated with a shift in Th1 to Th2 shift that is required for normal pregnancy and is defective in recurrent abortions in mice.[368] Maternal T cells increased LIF expression in response to paternal antigens and trophoblast, and reduced response was associated with poor reproductive

outcomes.[369] Progesterone is an anti-inflammatory immunomodulator in pregnancy and through induction of LIF and other cytokines that support trophoblast invasion.[304]

LIF and another biomarker of endometrial receptivity, αvβ3 integrin, are coexpressed in mouse and human endometrium[370,371] and like αvβ3 integrin, disturbances in LIF expression are associated with multiple reproductive pathologies.[275,372,373] While recent reports have linked these two important biomarkers,[374,375] their coregulation has only recently been demonstrated.[376]

Aberrant expression of both αvβ3 integrin and LIF is associated with decreased pregnancy success in *in vitro* fertilization (IVF).[377-379] Injection of peritoneal fluid from women with endometriosis reduced both LIF and αvβ3 integrin expression in the mouse uterus,[370] suggesting an inflammatory factor is responsible. Both LIF and αvβ3 integrin are positively regulated by paracrine actions of P (HB-EGF) and negatively regulated by inflammatory immune cells in the eutopic endometrium associated with endometriosis[370,380] and inflammatory tubal disease.[381] LIF has anti-inflammatory actions regulating cytokine action,[382] protecting against septic shock in the mouse model,[383,384] while αvβ3 integrin has been shown to interact with decay accelerating factor (DAF) and osteopontin (OPN),[109,385] that together block complement activation as part of innate immunity.[386] T cells from women with recurrent pregnancy loss secrete less LIF than normal women and defective LIF is associated with decreased Th2 cells and recurrent pregnancy loss.[368,387]

The Complement System

The human fallopian tube, endometrium, and cervical mucosa express components of the complement system.[388-391] The activation of the third (C3) and fourth (C4) component of complement induces chemotaxis of inflammatory cells, enhances phagocytosis, and mediates cell lysis. This system of natural immunity must be tightly regulated so that it can target foreign organisms and cells while avoiding untoward tissue damage. This is especially important during early pregnancy when the process of implantation might be disrupted. Complement activation is regulated by decay-accelerating factor (DAF, also known as CD55), which inactivates the C3 convertase enzymes that activate C3; and by membrane cofactor protein (MCP, also known as CD46), which serves as a cofactor for factor I-mediated degradation of activated C3 and C4.

A third protein, present only in rodents and named complement regulator decay accelerating factor (Crry), has DAF and MCP-like activities. Crry controls deposition of activated C3 and C4 on the surface of autologous cells. Fetal demise due to complement deposition and placental inflammation was observed in mice lacking Crry, reflecting unchecked activation of the complement system.[392] Complement activation appears to be an essential event in pregnancy loss associated with anti-phospholipid syndrome and can be prevented by administration of heparin.[393-395] These observations suggest that control of the complement system is essential for fetomaternal tolerance against attack by the innate immune system.

In the endometrium, complement component C3, factor B, and DAF are present in the glandular epithelium, and their expression is up-regulated in the secretory phase. MCP is expressed in the glandular epithelium throughout the menstrual cycle. Complement receptor type 1 is present in the stroma during the secretory phase; complement receptor type 2 is not detectable, and complement receptor type 3 is associated with infiltrating leukocytes in the luteal phase. The cyclic changes in C3, factor B, and DAF expression suggest progesterone regulation. In model human endometrial epithelial cell culture systems, however, steroid hormones did not alter decay accelerating factor expression, but heparin-binding epidermal-like growth factor (HB-EGF) did increase it.[151] Thus, the steroid hormone effects on expression of some of the complement system proteins may be indirect, perhaps acting through the stromal compartment.

Members of the integrin family are now thought to be involved in the regulation of C3, as part of the protective proteins that prevent the cascade of complement activation. DAF or Factor H may interact with these cell surface receptors (including the αvβ3 integrin and perhaps CD44) through binding to osteopontin (OPN) to limit C3 activation by digesting bound C3b. The αvβ3 integrin, OPN, and DAF all appear synchronously at the time of implantation in human endometrium and may serve as a primary safeguard against complement activation at the time of embryonic attachment, thus avoiding destruction of the early pregnancy by the host's defenses.

Anti-Microbial Peptides

Epithelial cells of the female reproductive tract elaborate antimicrobial peptides that presumably guard against ascending infection.[396,397] These peptides coat the epithelial surface and enter into the cervical mucus. Among the antimicrobial peptides expressed other than complement are lactoferrin, lysozyme, secretory leukocyte protease inhibitor (SLP1), and defensins. The α-defensins are elaborated by leukocytes and epithelial cells, and the β-defensins are elaborated by epithelial cells. Defensin-5, and α-defensin, are expressed in the upper half of the stratified squamous epithelium of the vagina and ectocervix, and in columnar epithelial cells of the fallopian tube and endometrium. β-defensin-1 and β-defensin-2 and secretory leukocyte protease inhibitor are produced by endometrial glandular epithelial cells. β-defensin-2 expression is increased by pro-inflammatory cytokines, and defensin-5 is also evidently up-regulated by inflammation. Defensin-5 and secretory leukocyte protease inhibitor levels in the endometrium also fluctuate during the endometrial cycle, being highest in the secretory phase.

Uterine Receptivity and Embryo Implantation

The timing of conception is exquisitely synchronized to ovarian events. With ovulation, progesterone from the nascent corpus luteum transforms the proliferative phase endometrium into a secretory structure and readies it for the newly fertilized ovum (Fig. 10.13A and B) (See Chapter 7).[114,388,398] In the appropriate endocrine milieu, the growing embryo will interact with the surface epithelium and invade into the underlying stroma, initiating gestation (Fig. 10.13C). Implantation can be divided into distinct

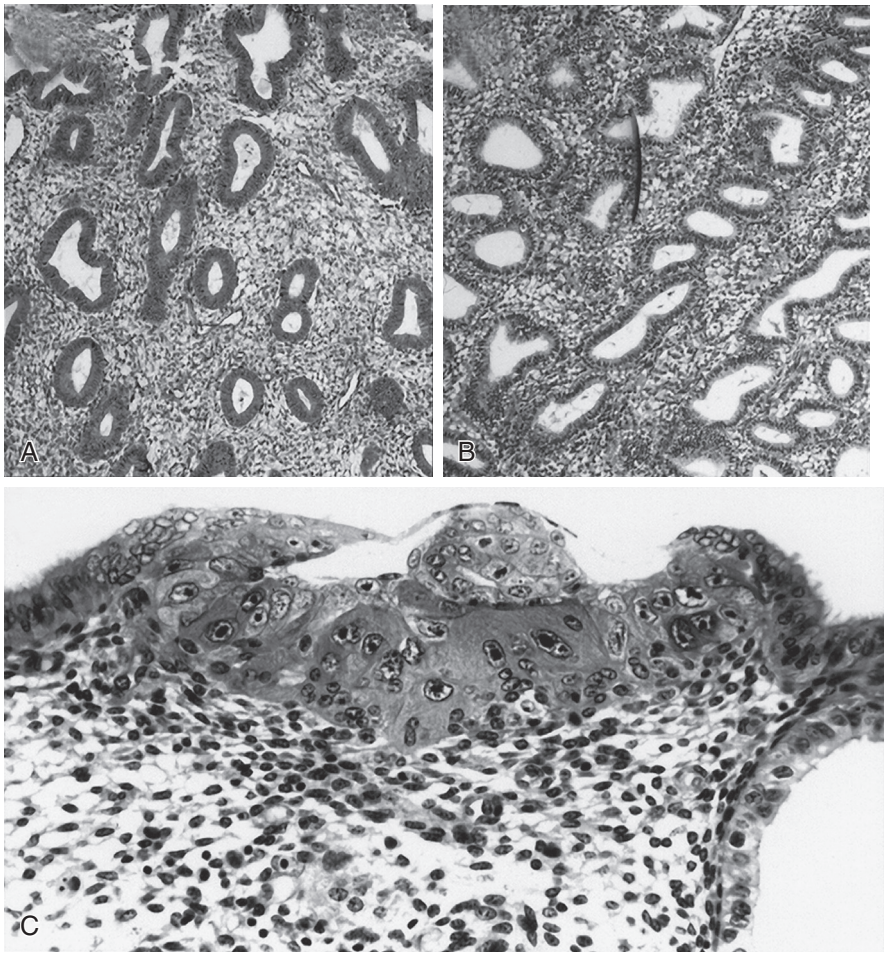


FIGURE 10.13 Proliferative (**A,** pre-receptive) and midsecretory (**B,** receptive) endometrium is regulated by ovarian steroids, resulting in successful implantation of the human embryo **(C).** *(Generously provided by Dr. Allen Enders, from the Carnegie Collection of early human implantation sites. Used with permission from Human Reproduction.)*

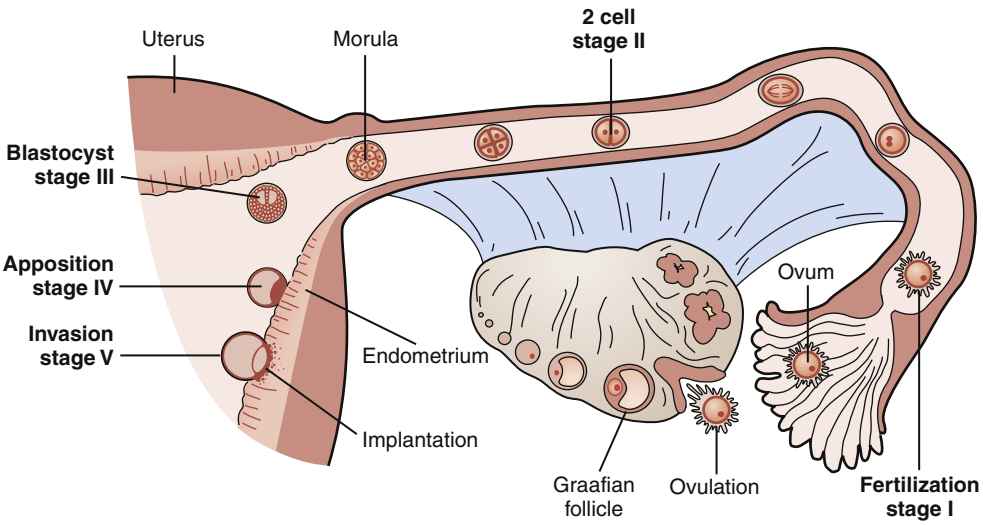


FIGURE 10.14 Stages of implantation beginning with ovulation, fertilization, then transport to the waiting endometrium. Implantation depends on synchronous development between the endometrium and the embryo.

and separate stages, timed precisely to the steroid-mediated changes ongoing in the endometrium (Fig. 10.14).[332] Following ovulation, the ovum enters the fallopian tube where fertilization occurs. The early cell divisions ensue and the embryo enters the uterine cavity at the morula stage, approximately 2 to 3 days after being fertilized. Implantation begins several days later, around the sixth to seventh day after fertilization. The initial attachment reaction (apposition) may be the rate-limiting step, and failure to adhere may preclude the subsequent stages of implantation.

Studies in experimental and domestic animals have demonstrated the synchronous development of the embryo and

endometrium that is required for normal implantation and development to occur.[399-403] In laboratory animals, there is a discrete "window" of time for implantation, which in some species lasts for only a matter of hours.[403] The concept of a "window" for implantation has also been demonstratred in the human female,[267,401,404,405] with a molecular basis that continues to be elucidated.[332,406-410]

Uterine receptivity is defined as the period of endometrial maturation during which the trophectoderm of the blastocyst can attach to the endometrial epithelial cells and subsequently proceed to invade the endometrial stroma and vasculature. The transition from the non-receptive to the receptive state is determined by the expression of membrane-bound, soluble, and secretory factors that support trophoblast attachment and subsequent migration. Factors expressed during this temporal window have been exploited as biomarkers of the receptive state.

The timing of implantation has been examined with increasing scrutiny over the past 50 years. In the 1950s, luteal phase hysterectomy samples in pregnant subjects suggested that embryos did not attach until day 20 of a 28-day cycle.[268] Using fertilized donor oocytes transferred into hormonally prepared recipients, Bergh and Navot later suggested that implantation occurred between cycle days 20 and 24.[411] More recently, by examining the time of pregnancy in 221 normal cycling women who were attempting pregnancy, Wilcox and coworkers showed implantation normally occurred between 7 and 10 days after ovulation (days 21 to 24).[267] In these studies, delayed implantation was associated with a higher miscarriage rate, possibly due to a shift in the time of implantation and the concomitant loss of synchrony between endometrium and embryo (Fig. 10.15). This explanation may provide insights into some cases of otherwise unexplained infertility and recurrent pregnancy loss.

Implantation can be viewed as a highly complex and orchestrated interaction between the maternal endometrium and the newly formed embryo.[332,412] As depicted in Figure 10.16, multiple soluble and membrane-bound factors have been elucidated that facilitate embryo growth, differentiation, attachment, invasion, and avoidance of immunological rejection. Maternal factors appear to simultaneously permit intrusion while limiting the degree of embryonic invasion into maternal tissue. Many of the embryonic signals or receptors have complementary ligands or coreceptors on the maternal surface. Mimicking of maternal antigens by the invading embryo is a strategy that is also used by the embryo to penetrate the endometrium, without triggering host defenses.[413] Some of the proteins involved in this process based on our current understanding of their roles are depicted in Figure 10.16.

Secreted Proteins of the Endometrium

The endometrium produces a large number of secreted proteins, collectively referred to as the endometrial secretome that serve autocrine, paracrine, and juxtacrine roles for the developing endometrium and embryo.[403,412] The uterine glandular epithelium also secretes glycogen which is thought to serve as a histiotrophic nutrition source for the embryo prior to 10 weeks gestation.[272] In addition to macromolecules, the endometrium also is likely to selectively secrete many small molecules, since it is well endowed with many members of the adenosine triphosphate–binding cassette transporter protein family, which are involved in the

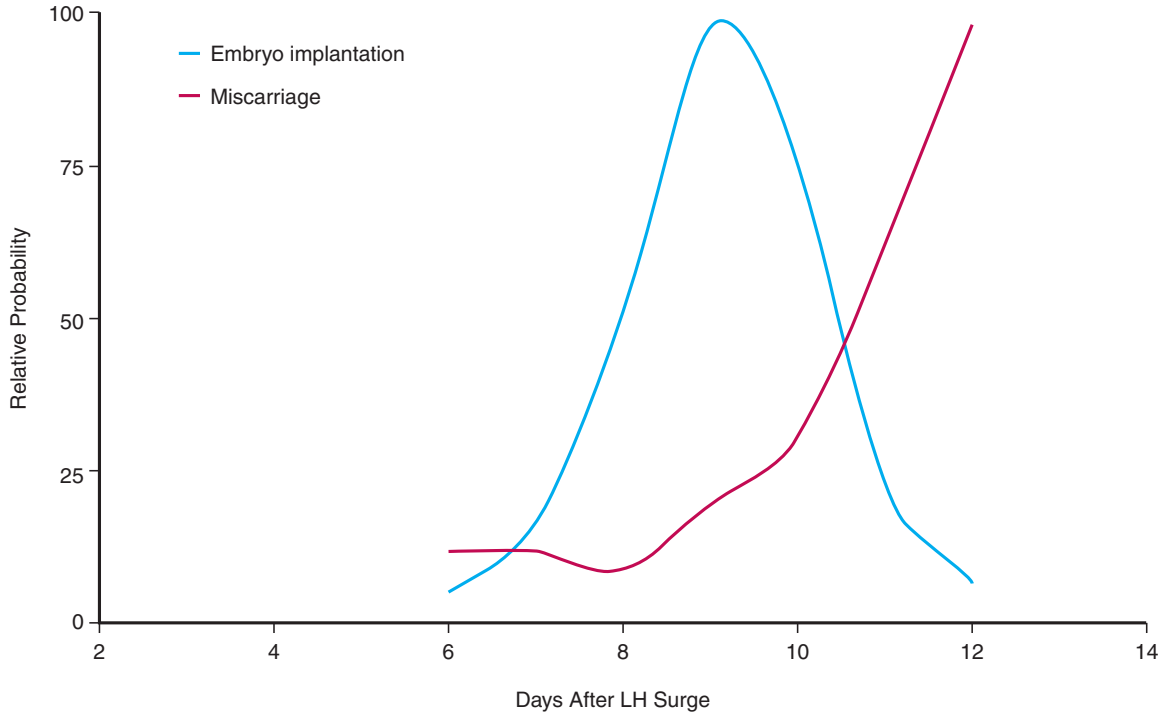


FIGURE 10.15 The timing of implantation in a population of fertile women. Most successful pregnancies implant between 7 to 10 days following a urinary luteinizing hormone (LH) surge *(blue line),* but the probability of miscarriage increases when implantation occurs outside of this 4 day window *(red line). (Adapted from Wilcox A, Barid DD, Winberg CR. Time of implantation of the conceptus and loss of pregnancy.* N Engl J Med *240:1796–1799, 1999).*

regulated secretion of a variety of small molecules, including drugs, lipids, and conjugated molecules.[414]

Endometrial secretions are found in the lumen of the uterine cavity and some are found in the general circulation (Fig. 10.16). A remarkable increase in secretory activity is associated with the luteal phase and early pregnancy, primarily in the glandular epithelial cells and later the decidua. Most of these secreted proteins are thought to be expressed because of the action, either directly or indirectly, of progesterone and many are implicated in regulation of embryo implantation and/or early embryo survival.[398,415,416] Therefore, an understanding of the mechanisms of progesterone action allows significant insight into endometrial function.

Glycodelin

Glycodelin, one of the most abundant products of the secretory phase endometrial glands and decidua, is known by several names: progesterone-associated endometrial protein, α-uterine protein, pregnancy-associated endometrial α2 globulin, endometrial protein 15, chorionic α2 macroglobulin, and placental protein 14 (an erroneous designation because glycodelin is an endometrial/decidual protein).[416,417] The mature form of glycodelin contains 162 amino acid residues and is 17.5% carbohydrate by weight. It has extensive structural homology with the β-lactoglobulins and, to a lesser extent, with retinol-binding proteins.

Glycodelin exists as isoforms depending on its glycosylation pattern. Glycodelin A is the major progesterone-regulated protein secreted into the uterine lumen. However, its functions with respect to the endometrium, implantation, and pregnancy are still largely unknown. Glycodelin A is a potent inhibitor of sperm-egg binding, and researchers have postulated that it may play a role as an immunomodulator because of its ability to suppress NK cells.[418,419] The absence of glycodelin A during the time of fertilization and its appearance during the time of implantation and placentation are consistent with these proposed functions.[420]

Glycodelin levels in uterine flushings are tightly correlated with the histological date of the endometrium (Fig. 10.17). It is not detectable in significant amounts in uterine flushings during the follicular and early luteal phase. Six days after ovulation, however, the levels rise rapidly to reach a concentration 100 times higher than plasma levels. In peripheral serum, glycodelin appears 5 days after ovulation, reaching peak concentrations in non-fertile cycles at about the time of menstruation; levels reach a nadir during the midfollicular phase of the subsequent cycle. In a cycle of conception, glycodelin levels increase rapidly after implantation, reaching a maximum at 8 to 10 weeks of gestation and subsequently declining in a pattern that mimics the changes in hCG.

The discordant pattern of glycodelin and progesterone in serum may reflect the slower turnover of the protein. Levels of glycodelin in serum fail to rise in women using combination-type oral contraceptives and in some patients with luteal-phase defects.[421] There is good—but not perfect—correlation between the progestational activity of steroids and their ability to stimulate endometrial glycodelin synthesis. Relaxin has also been implicated as a stimulus for glycodelin expression.[420,422] Specific histone deacetylase inhibitors have been shown to potentiate the action of progesterone on both endometrial epithelium and stroma.[423] The histone deacetylase inhibitor trichostatin (TSA) has

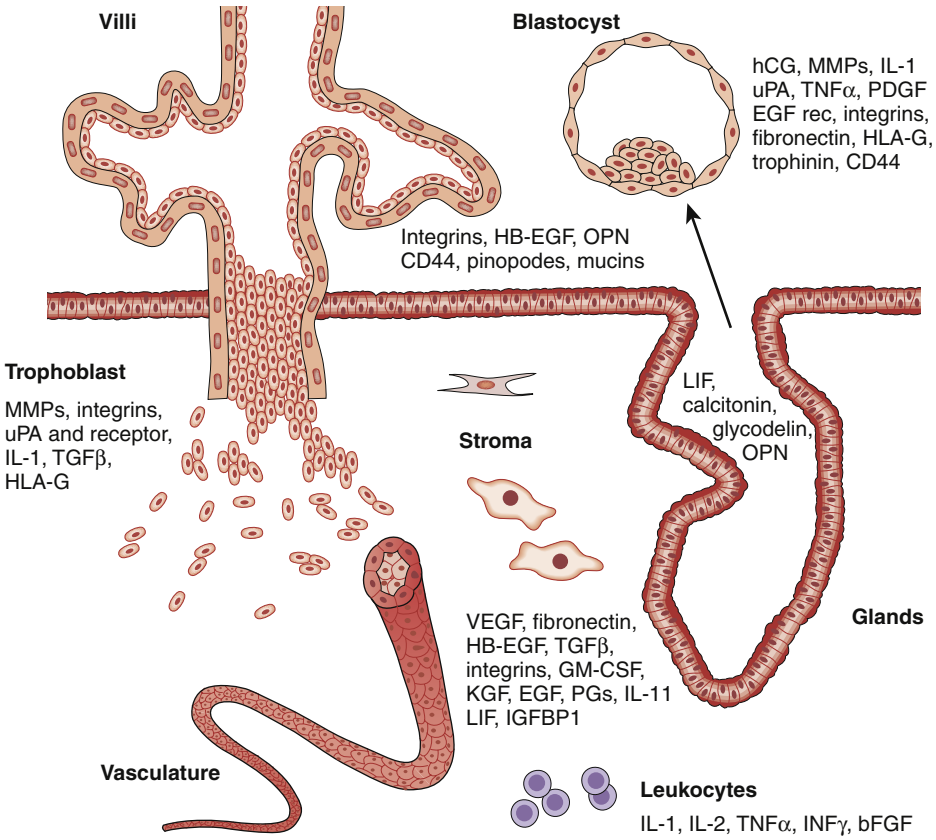


FIGURE 10.16 Schematic showing some of the factors and their cells of origin during normal implantation in women.

been shown to induce glycodelin in Ishikawa cells. Using an *in vitro* model of implantation, Uchida and coworkers showed that induction of glycodelin by a histone deacetylase inhibitor enhanced placental cell (JAR) spheroid attachment to Ishikawa cells.[424] This and other studies suggest a role for glycodelin in both the differentiation and function of the receptive endometrium.

Glycodelin has been shown to be decreased in the endometrium of women with infertility, including those with luteal phase defect[421] and polycystic ovary syndrome (PCOS).[425] In women exposed to the levonorgestrel containing IUD or the Yuzpe emergency contraception regimen, glycodelin expression was unaffected or increased.[426,427]

Insulin-Like Growth Factor Binding Protein-1

IGFBP-1, also known as *placental protein 12* or PP12, is a major secretory product of decidual cells.[428] It is one of several proteins that bind IGF-1 and IGF-2, affecting the ability of these growth factors to interact with the IGF receptors. Consequently, the binding proteins can have significant roles in modulating IGF effects. The protein undergoes post-translational modification by phosphorylation, which increases its affinity for IGF-1 and therefore its ability to neutralize IGF-1 action.

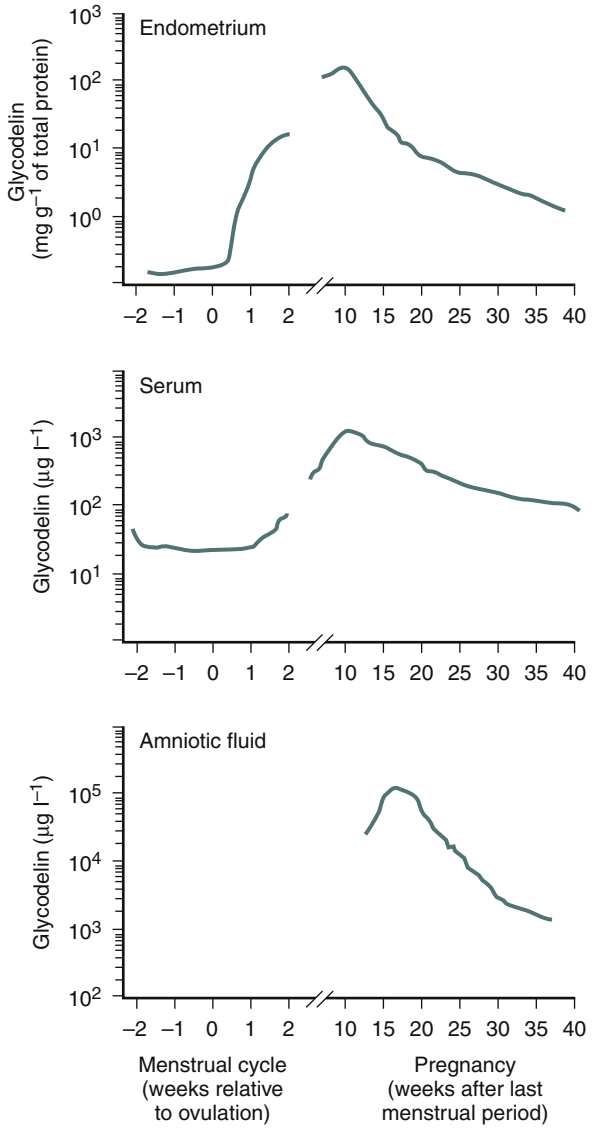


FIGURE 10.17 Glycodelin levels in endometrium, serum, and amniotic fluid. *(Reprinted with permission from Seppala M, Koistinen H, Koistinen R. Glycodelins.* Trends Endocrinol Metab *72:111–117, 2001.)*

IGFBP-1 derived from the decidua has been proposed to control the invasion and proliferation of trophoblast cells during implantation and placentation by sequestering IGFs. In a transgenic mouse model, over expression of IGFBP-1 in the decidua resulted in abnormal placental morphology due to defects in trophoblast invasion and differentiation.[429] Because IGFBP-1 contains the Arg-Gly-Asp (RGD) motif recognized by cell surface integrins that bind fibronectin, its actions may be more complex than simple IGF sequestration when it is presented to cells that express the RGD-binding integrins.[430]

Stromal cell IGFBP-1 mRNA levels are regulated by progesterone as well as by IGFs, insulin, relaxin and other growth factors as well as hypoxia.[431-433] Both IGF and insulin decrease decidual IGFBP-1 release while relaxin increases its release in a dose-dependent manner. HOXA10 may have a mild impact on IGFBP-1 expression, but in the presence of the forkhead–winged-helix transcription factor, FOX01, IGFBP-1 is markedly stimulated.[434] FOXO1, prolactin, and IGFBP1 are all induced during decidualization, and likely are involved in the progesterone-mediated stromal differentiation and prevention of apoptosis in preparation for nidation.

Intrauterine microdialysis studies revealed that IGFBP-1 is released into the uterine lumen in the late secretory phase (10 days after ovulation or later)[435] and increased secretion in response to hCG.[435] IGFBP-1 is increased between midsecretory to 6 weeks of pregnancy, as demonstrated by DNA microarray analysis on endometrium from ectopic pregnancy.[436] IGFBP-1 levels then decline in the second trimester only to rise again in late pregnancy, also accumulating in amniotic fluid. Pregnancy termination with the anti-progestin RU486 causes a marked decline in IGFBP-1 levels before a decrease in hCG levels, confirming the progesterone dependence of IGFBP-1 production by decidual cells.

Defects in IGFBP-1 have been noted in certain conditions associated with infertility and pregnancy loss, including polycystic ovary syndrome (PCOS) and endometriosis.[425,437] Such observations may reflect an altered endocrine milieu, higher insulin levels, or a relative progesterone resistance in these conditions (described later in the chapter). *In vitro*, insulin inhibits the normal process of endometrial stromal differentiation (decidualization). In addition, hyperinsulinemia down-regulates hepatic IGFBP-1, resulting in elevated free IGF-I in the circulation. Thus, the elevated androgens and estrogen seen in PCOS along with decreased progesterone in the absence of ovulation, likely contribute to endometrial dysfunction, infertility, increased miscarriage rate, endometrial hyperplasia, and endometrial cancer common in women with PCOS.

Osteopontin

Osteopontin (OPN) is a member of the SIBLING family of proteins.[438,439] Each of the proteins in this large family contain the three amino acid sequence arg-gly-asp (RGD) and have binding sites specific for two major cell surface receptors, αvβ3 and CD44.[438] Osteopontin is a 70-kDa

glycosylated phosphoprotein secreted by the glandular epithelium and is expressed during the midsecretory phase, localized to the luminal endometrial epithelium.[440] Osteopontin is regulated by progesterone.[440,441] The secretion of osteopontin and subsequent binding to the luminal surface suggests direct interaction is occurring between integrins and this molecule and a role as a "sandwich" ligand that serves as a bridge between surface receptors on the endometrial and embryonal surfaces. An additional role for osteopontin and these receptors is the prevention of complement fixation as part of a protective mechanism involving the innate immune system.[442]

Prolactin

Prolactin is produced by both the endometrium and the myometrium.[443,444] Serum levels of prolactin do not change across the menstrual cycle but rise markedly during the first trimester of pregnancy in parallel with the decidual response, reaching peak concentrations at 15 to 20 weeks of gestation. Prolactin, like IGFBP-1, is a downstream biomarker of decidualization of human endometrial stromal cells. Regulators of decidualization include ovarian steroids, cAMP or forskolin, IL-11. Recent studies suggest upstream mediators of decidualization may regulate prolactin expression, including various proteins expressed during the secretory phase including gherelin, IGFBP related protein-1, leptin, and oncostatin-M.[280,445-448] The proliferation of endometrial cells is inhibited during deciudalization perhaps through stromal factors, such as IL-6 and oncostatin-M.[445,449] Prolactins role in the decidualization process remains unclear. Prolactin receptor (PRL-R) is expressed on the endometrial stroma, and while PRL-R knockout mice had defects in decidualization, the phenotype including reduced expression of LIF, amphiregulin, HB-EGF, Cox-1, Cox-2, PPARδ, HOXA10, cyclin-D3, VEGF, and its receptors, Flk-1 and neuropilin-1, could all be rescued by exogenous progesterone. This suggests that ovarian PRL-R stimulation by prolactin inducing progesterone secretion is the critical event rather than any direct effect of prolactin acting directly on the decidualizing stroma.[450] A role for uterine prolactin controlling the NK cells has recently been suggested.[451] Prolactin produced by the decidua during pregnancy accumulates in the amniotic fluid, where it has been postulated to have effects on osmoregulation and fetal lung development.

Global Gene Expression Patterns During the Window of Implantation

The dramatic changes in growth, secretions, cell growth, and then cell death have been studied at the molecular level using DNA microarray. Demonstration of the key patterns of gene expression between the pre-receptive (early secretory) phase and receptive (midsecretory) phase were first defined in 2002; 370 genes (3.1%) displayed decreases ranging from 2 to greater than 100-fold while 323 genes (2.7%) displayed increases ranging from 2 to greater than 45-fold.[452] A survey of the gene expression patterns has recently been completed in normal fertile women.[453] The patterns of up- and down-regulated genes appear to define well the four stages of endometrial development and suggest that the functional characteristics of each phase is defined by the proteins that are expressed during each phase. Cell cycle proteins are the predominant feature of proliferating endometrium while secretory profile of gene expression characterizes the early secretory phase. In preparation for implantation, patterns of gene expression reflect changes in extracellular matrix, cell-cell communication and adhesion, negative effectors of proliferation, synthesis of amino acids, cell ion homeostatis, and modifications to the innate immune system. Finally, there are dramatic changes in gene expression toward cell death, enzymatic digestive processes and endocytoisis/phagocytosis, hemostasis, and anti-microbial responses in response to the shedding of the endometrium during menstruation.

The molecular phenotype of the human endometrium in the midsecretory phase has been characterized using DNA microarray analysis of RNA extracted from endometrial biopsies obtained from fertile women, using chips that contain the entire human genome.[452-455] A relatively small percentage of genes (less than 6%) represented on the chips were significantly increased or reduced in expression (at least two-fold) during the receptive phase. Microarray analyses have confirmed the up-regulation of transcripts previously known to be increased during the window of implantation (including osteopontin, DAF, glycodelin, and IGFBP-1) and the down-regulation of others (including MMP-7 and cyclin B). However, several previously unappreciated genes changed in expression, most notably members of the WNT signaling pathway (Fig. 10.6A and B). Such changes included the increased expression of the Wnt pathway inhibitor Dickkopf-1 (DKK1) and a striking decline in the soluble form of FrpHE (SFRP4), another Wnt pathway inhibitor.

The microarray analysis also revealed significant alterations in expression of genes encoding water and ion transporters, including up-regulation of claudin-4, *Clostridium perfringens* enterotoxin receptor. Expression of genes encoding proteins responsible for prostaglandin synthesis and action were also found to be altered, including up-regulation of phospholipase A2 and prostaglandin E2 receptor (EP1). The functional significance of these genes, as well as the alterations in others, remains to be determined. Moreover, the interpretation of the findings is complicated by the fact that alterations in specific endometrial cellular compartments could not be ascertained for the majority of the genes of interest. The data sets do, however, provide a first approximation of the molecular signature of the human endometrium during the putative time of implantation in a small group of fertile women. This information could be used to develop a diagnostic "chip" profile for establishing normal uterine function.

Now that the changes in the endometrial transcriptome across the menstrual cycle have been roughly defined, investigators can view each segment of the menstrual cycle, divided by histological stage, as a unique fingerprint detailing the role of steroid hormones and the availability of their cognate receptors in the endometrium.[453] Genes that are expressed during the proliferative phase in response to estrogen play roles in cell adhesion, cell to cell signaling, cell cycle regulation, and cell division. After ovulation in the early secretory phase in response to both ovarian estrogen and progesterone, gene expression shifts globally to include metabolic enzymes, transporter proteins, inhibitors of WNT signalling with a marked up-regulation of lipid metabolism,

phospholipase activity and prostaglandin metabolism. There is increased EGF signalling noted during this stage. The midsecretory is distinct from the other stages due to the down-regulation of ERα and PR in the epithelial compartment, reflected in the patterns of gene expression. Devoid of estrogen action and progesterone acting primarily in the stroma, there is a shift toward cell adhesion, communication and motility, reflecting an increase in intracellular signalling, anti-apoptosis and immune responses, including DAF, defensin, complement component 4, and glycodelin along with leukocyte differentiation and immunotolerance.

During the late secretory phase, in lieu of pregnancy, progesterone withdrawal leads to preparation for eventual menstruation with increased expression of matrix metalloproteinases, EBAF (Lefty; an anti-TGF-β cytokine) and signalling through the IL-2 receptor. Evidence of leukocyte infiltration with gene expression patterns reflecting leukocyte integrins. Genes encoding proteins for the humoral immune response are further up-regulated at this time, while the most down-regulated genes denote a closing of the window of receptivity, with loss of LIF, DKK-1, IGF-1, and complement proteins.

Gene Expression Differences in Women with Endometriosis and Infertility

One of the best studied gynecological conditions affecting women is endometriosis. Global comparisons of the endometrium from women with or without endometriosis has been reported, providing insights into the critical elements for implantation.[114,183] Comparing midsecretory endometrium from each group, the overall consensus is that of progesterone insensitivity in eutopic endometrium of women with endometriosis. In women with mild disease, changes in gene expression were modest, with an increase in the expression of 91 genes (0.7%) and a decrease in 115 genes (0.9%). Genes such as collagen α2 type I and bile salt export pumps are examples of genes that were over expressed. Another finding, down-regulation of an enzyme necessary for L-selectin ligands (N-acetylglucosamine-6-O-sulfotransferase) may be significant because L-selectins have been postulated to be receptors on the embryo necessary for adhesion to uterine epithelium. Glycodelin, a major progesterone-induced secretory product (see previous discussion in this chapter), was also reduced. These differences may provide a profile for endometrial dysfunction in this and other diagnoses that have been associated with implantation defects. Interestingly, when mild endometriosis was compared to moderate/severe endometriosis, there was a shift in progesterone resistance toward the early secretory phase, suggesting that stage of endometriosis matters when considering the effects on the eutopic endometrium.[114] Multiple studies have also examined the difference between eutopic and ectopic endometrium in women with endometriosis.[456-460]

MicroRNAs (miRNAs) are small non-protein coding ribonucleic acid sequences that regulate specific post-transcriptional mRNA targeting them for degradation. Genes coding for miRNA represent 1% to 4% of the genes in higher organisms and are highly processed sequences that are derived from larger RNA transcripts. These small precursor miRNAs of 70- to 90-nucleotides are transported to the cytoplasm where they undergo cleavage by the RNAse III enzyme, Dicer, to generate a double stranded miRNA duplex, eventually forming a single stranded miRNA that binds to complementary sequences on target mRNA and preventing their transcription.[461-463] This extraordinarily complex regulatory mechanism of transcription has now been studied in the human endometrium.[117,464-466] In endometriosis, complex networks of genes appear to be altered on the basis of specific changes in miRNAs,[116,117,466-469] providing a new dimension to the study of endometrial receptivity.[470]

The Role of Growth Factors, Cytokines and Gondadotropins in Establishing a Receptive State

Growth factors and cytokines play an essential role in the dramatic morphological changes that occur in the endometrium during the menstrual cycle and pregnancy. Among the growth factors whose expression has been demonstrated in the human endometrium and decidua are EGF and EGF-like molecules, including TGF-α and HB-EGF; acidic and basic fibroblast growth factor (FGF); IGF-1 and IGF-2; IL-1, IL-11 and IL-6; LIF; macrophage colony-stimulating factor (M-CSF, also known as CSF-1); granulocyte-macrophage colony-stimulating factor (GM-CSF); members of the TGF-β superfamily; platelet-derived growth factor (PDGF); tumor necrosis factor-α (TNF-α); and endothelins 1, 2, and 3. Many of these factors have been proposed to play a crucial role in endometrial function and during pregnancy.[332,428,471-486] The endometrium and decidua and embryo/trophoblast have also been shown to express receptors for many of these factors, including the EGF/TGF-α, IGF-1 and IGF-2, IL-1, CSF-1, GM-CSF, PDGF, and VEGF. With such a wide array of growth factors, it has become difficult to determine with clarity the role of each factor in endometrial growth and differentiation, or their importance in processes involving maternal embryonic interaction and placental development.[487]

Several growth factors have been shown to exert regulatory effects on the expression of extracellular matrix proteins, their cellular receptors (integrins, selectins, cadherins), and enzymes (MMPs) that also influence cell growth, differentiation, and remodeling.[488-490]

Leukemia Inhibitory Factor (LIF) and IL-11

IL-6, LIF, an IL-11 are glycoproteins that belong to the same family of cytokines whose receptors utilize gp130 as a common signalling molecule.[275,491,492] LIF named for its capacity to inhibit the proliferation of a mouse leukemic cell line, is expressed constitutively in the ampullary region of the fallopian tube and in a cyclic fashion in both epithelial and stromal cells of the endometrium, with epithelial expression being greater.[372,493-499] The functional LIF receptor, a complex consisting of LIF receptor β (which binds LIF) and gp130 (which mediates signal transduction) is present throughout the menstrual cycle in the luminal epithelium. LIF receptors are expressed by all trophoblast types, particularly the villous syncytiotrophoblast and cytotrophoblasts and to a lesser extent the extravillous trophoblast cells.

Sentinel observations in the mouse have clearly demonstrated that LIF of endometrial origin is crucial for the process of implantation, particularly the decidualization response.[494] LIF-deficient female mice failed to become pregnant or respond to a uterine decidualizing stimulus. However, transfer of their embryos to pseudopregnant, wild-type females resulted in viable pregnancies—as did infusion of LIF into the uteri of LIF-deficient females. The primary action of LIF appears to be on the uterus. However, in mice subsequent placentation is disrupted, perhaps because LIF's role in modulating trophoblast differentiation and expression of MMPs cannot be executed. Implantation of LIF receptor-deficient human embryos occurs but these offspring suffer from Stuve-Wiedemann/Schwartz-Jampel type 2 syndrome.[497]

LIF mRNA and protein are present in human endometrium, being most abundant in the glandular and luminal epithelium and peaking in the secretory phase of the cycle.[500-502] The cycle-dependent expression of LIF in human endometrium may be a function of other growth factors rather than a direct effect of steroid hormones. If implantation occurs, LIF expression by the endometrial glands is down-regulated while there is a concomitant increase in LIF expression by endometrial NK and T cells. LIF has been found to enhance human blastocyst formation and to modulate trophoblast differentiation *in vitro*.[493] LIF levels in uterine flushings rise 7 days after ovulation, reaching a maximum 5 days later. LIF levels in uterine flushings and secretion of LIF from cultured endometrium obtained from patients with repeated implantation failures or unexplained infertility is decreased,[372,503] and defects in LIF have been implicated in some cases of recurrent pregnancy loss.[493]

Leptin has been suggested as a regulator or LIF, through its receptor, OB-R.[504] LIF stimulates hCG in trophoblast.[498] LIF has been implicated in activation of Signal transducer and activator of transcription-3 (STAT-3); mice homozygous for STAT activation site on gp130 have a defect nearly identical to LIF deficient mice.[505] Suppressor of cytokine signaling protein-3 (SOCS-3) is stimulated by LIF and blocks phosphorylation of gp130 and STATs. In LIF null mice, stromal Cox-2 and epithelial HB-EGF is absent at the site of implantation.[506,507] Two other EGF family members, amphiregulin and epiregulin are also reduced in LIF knockout mice. IL-1 has been shown to induce Cox-2.[508,509] Prostacylin acting through the PPAR γ is essential for decidualizaton.[314,510] Cox-2 null mice have multiple defects in ovulation, fertilization, and implantation.[315] Together, LIF stimulation of luminal epithelium along with blastocyst may trigger IL-1 that triggers decidual changes.

Mutations in the coding sequence of one copy of the LIF gene were identified in a small number of infertile women (3 of 74 infertile nulligravid subjects), and a presumed polymorphism was found in one of 75 fertile controls and none of 131 non-obstetrical patients.[511] One of the mutations in the infertile group was in the 5′-regulatory region of the LIF gene; the two others were in the coding sequence in a domain thought to be important for LIF binding to its receptor. Unfortunately, the authors did not determine whether LIF levels or bioactivity in uterine flushings or endometrial biopsy material were correlated with genotype. Collectively, however, these observations are consistent with an important role for LIF in human implantation, trophoblast differentiation, or placentation. IL-11 is another member of the IL-6 family that is implicated in the decidual response.[492,512,513] As with the LIF knockout mice, female mice lacking the IL-11 receptor alpha chain are infertile because of defective decidualization.[512]

IL-11 is present in the human endometrium and it advances progesterone-induced decidualization of cultured endometrial stromal cells. Both relaxin and prostaglandin E2 increase IL-11 expression. An inhibitor of IL-11 (W147AIL-11) reduces prolactin secretion by ESC in response to RLX and PGE2 suggesting IL-11 is the critical factor in this signalling cascade.[514] Like LIF, IL-11 is an activator of the JAK/STAT signalling pathway through STAT3, which stimulates suppressor of cytokine signalling -3 (SOCS3), a negative feedback mechanism for receptor activity. Ovarian steroids and cAMP differentially stimulate STAT3 and SOCS3, respectively, *in vitro*, while IL-11 activates both by phosphorylation.[515] Treatment with an anti-progestin increases SOCS3, attenuating IL-11 induced STAT3, suggesting multiple regulatory components including progesterone. Recent investigations in women suggest that IL-11 and phosphorylated STAT3 are significantly lower in the infertile endometrium compared to controls, while IL-11 receptor and LIF were not different.[516]

EGF Family of Growth Factors

The EGF family of growth factors appears to play a major role in uterine development and physiology.[332,428] The EGF family of ligands is produced by a family of genes, epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AMP), betacellulin (BTC), epiregulin (EPR), tumor growth factor α (TGF-α), and neuregulins (NRG). This EGF family of ligands has the ability to bind and activate one or more dimers of four structurally homologous ErbB receptor subunits via a conserved 60 amino acid "EGF-like" binding domain. The receptor subunits for the EGF ligand family, ErbB1, ErbB2, ErbB3, and ErbB4, are structurally homologous and specificity of ligand binding is determined by differences in extracellular domain sequences (Fig. 10.18). The ErbB proteins function as hetero- or homo-dimers and possibly also as higher order multimers.[517] ErbB2 lacks ligand-binding activity and ErbB3 lacks functional tyrosine kinase activity. Thus, ErbB2 and ErbB3 likely function only as heterodimers, with another ErbB protein supplying the missing function in a dimer.

Interacting with their receptors, EGF family members act as autocrine and juxtacrine factors, and some exist as membrane bound forms that are released by proteolytic cleavage to function in a paracrine or endocrine manner. The proteolytic cleavage is accomplished by cell-surface metalloproteinases, similar to those involved in L-selectin ligand and MUC-1 cleavage, suggesting an important role for metalloproteinase action at the maternal-fetal interface.[518-520]

EGF and TGF-α stimulate the proliferation of endometrial stromal cells.[428] The synthesis of fibronectin and vitronectin by epithelial cells is enhanced by EGF. EGF also stimulates stromal cell differentiation and enhances the synthesis of laminin and fibronectin. These growth factors also enhance the morphological and functional differentiation of decidual cells *in vitro*. EGF has not been identified in

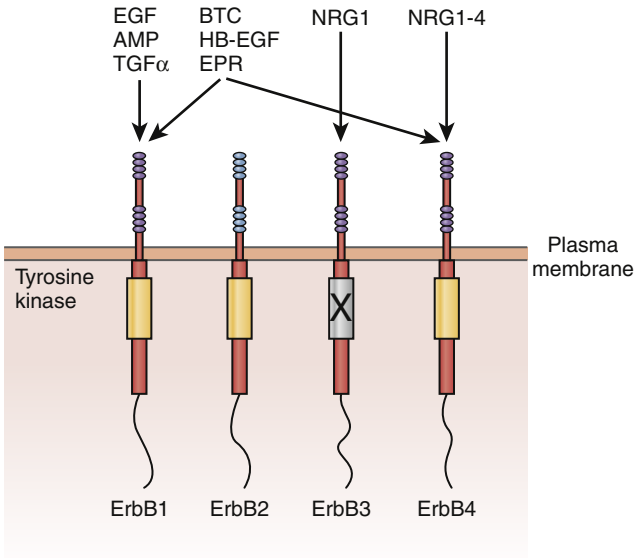


FIGURE 10.18 The EGF family of growth factors and receptors. EGF and its family members associate with specific receptors that signal through intracellular tyrosine kinase action. The 4 receptors can dimerize in predictable ways.

the circulation of cycling or menopausal women, but it has been detected in the serum of pregnant women with peak concentrations occurring in early pregnancy. Binding studies indicate that EGF-R peaks at the time of ovulation (or shortly thereafter) and declines during the secretory phase to a nadir immediately before menses. The binding sites are present on both stromal and epithelial cells, as well as in decidua of pregnancy. Studies using immunohistochemistry suggest that decidualization is associated with an increase in EGF-R. Abnormal EGF and EGF-R activity has been reported in cases of intrauterine growth retardation. It has been hypothesized that EGF and related molecules play a role in the induction of trophoblast differentiation.

Multiple EGF family members appear to directly participate in the process of implantation.[274] HBEGF exists as a membrane-anchored precursor (HB-EGF™) that gives rise to a soluble processed form. These proteins can bind to two different EGF receptors, HER1/ErbB1 and HER4/ErbB4. HB-EGF mRNA is found in the stroma during the proliferative phase, but in the midsecretory phase it is also detected in luminal and glandular epithelium.[521,522] This expression pattern appears to be driven by a combination of progesterone and estrogen.

HB-EGF is postulated to play roles both in adhesion via the membrane-anchored precursor (which binds to HER4/ErbB4 on the apical surface of the trophectoderm) and in stimulating embryo growth. In the rodent, HB-EGF is one of the first cytokines found around the implanting embryo.[523,524] In the human endometrium, mRNA levels of HB-EGF increase during the secretory phase, reaching a peak at the time of implantation.[521] HB-EGF™ can mediate attachment of human embryos in an *in vitro* assay of implantation.[525] HB-EGF also may promote embryonic development. When added to serum-free medium, it increases the number of embryos reaching the blastocyst stage and stimulates hatching. HB-EGF may also participate in paracrine actions within the endometrium. Stromal derived HB-EGF promotes expression of LIF, HOXA10, αvβ3 integrin, and DAF by endometrial cells *in vitro*. Other members of the EGF family likely play important roles during early pregnancy as well.[372]

The TGF-β Family

TGF-β family, multifunctional proteins that regulate cell growth and differentiation, includes five dimeric polypeptides encoded by related genes.[258,486,526-529] These growth factor members bind to three cell-surface proteins designated type I, II, and III receptors. The type I and type II receptors are thought to mediate the actions of TGF-β through the SMAD signaling pathway. All three isoforms of TGF-β can be found in human endometrium, including TGF-β1, TGF-β2, and TGF-β3. Null mutant mice bearing TGFβ mutations for each of the three genes produce a distinct phenotype suggesting a separate role for each member of this growth factor family.[530] All three and the type II receptor were identified in all cell types of the endometrium.[531] TGF-β1 and TGF-β3 are found on both epithelial and stromal cells, while TGF-β2 is found primarily on the stroma and increases during the secretory phase.[529,532] TGF-β3 increases at menstruation and remains high throughout the proliferative phase, while TGF-β1 is maximal at menstruation. Ovarian steroids strongly repress TGF-β2 and TGF-β3 in stroma but only TGF-β2 is repressed in glandular epithelium. cAMP prevents this inhibition of TGF-β2 by progesterone, while MAP kinase (MAPK) inhibitors differentially stimulated TGF-β2 and TGF-β3. These opposite effects provide insight into the discreet and highly temporal and spatial expression of the TGF cytokines during the menstrual cycle.

TGF-β has profound effects on the extracellular matrix, increasing the synthesis of collagen while decreasing its degradation.[195,533] In the secretory phase, TGF-β is a potent inhibitor of MMP activity in both stroma (MMP-3) and epithelium (MMP-7) while simultaneously promoting TIMP expression. This cytokine is induced by progesterone, acting in concert with retinoic acid to maintain these pro-fibrogenic activities. In the event of pregnancy, progesterone acting through TGF-β will maintain the integrity of the endometrium throughout gestation. In lieu of pregnancy, the fall in progesterone releases the MMP inhibitory factors, resulting in programmatic breakdown of the extracellular matrix and subsequent menstruation.

Other members of the TGF-β family are expressed in the endometrium.[486] EBAF (or Lefty) may participate in menstruation process, acting as a potent inhibitor to TGF-β.[253] Antagonism of TGFβ1 by over expression of EBAF (Lefty; a TGFβ antagonist) reduces the number of implantation sites in rodent models,[534] consistent with human data showing high levels of lefty in infertile women.[256]

Activins are highly abundant in the endometrium. Activin β (βA and βB) is found in the glandular epithelium with peak expression during the secretory phase. Activin A promotes decidualization *in vivo* while follistatin opposes the actions of activin.[535] Activin A enhances production of LIF and enhances MMP-2 during decidualization. MMP and LIF activity are both crucial for decidualization in rat and primate.

TGF-β1 and TGF-β2 have been reported at the interface between embryo and endometrium and likely plays multiple

roles during embryo attachment and invasion.[528,536] In pregnancy, TGF-β is most abundant in the decidua in the first trimester of pregnancy, where it is thought to restrain trophoblast invasion by promoting trophoblast differentiation away from the invasive phenotype. TGF-β is sequestered in the extracellular matrix where it can be activated by embryo-derived proteases. TGF-β up-regulates the expression of cellular fibronectin by trophoblast cells and induces integrins on the trophoblast that interact with the ECM during invasion promoting MMP activity.[537,538] TGF-β induces TIMPs and PAI-1 activity to counterbalance the invasiveness of the trophoblast. TGF-β as a potent immunosuppressant may prevent the maternal immune rejection of the fetal allograft. Its actions include suppression of chemotaxis and macrophage and T-cell activity. ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin repeats) has been thought to play a critical role in ECM remodelling during implantation. IL-1 increases, while the anti-inflammatory effects of TGF-β1 decreases ADAMTS expression.[539] These opposing actions illustrate the balance between factors favoring embryonic invasion and maternal efforts to control this invasion and reflects the essential role of TGF-β in this process.

Other Growth Factors and Cytokines Participate in Endometrial Growth, Differentiation, and Immune Function

Platelet derived growth factor (PDGF) is produced by endometrial stromal cells and also released in the endometrium by activated platelets.[540] PDGF is a potent mitogen that acts on endometrial receptors that are most abundant in the proliferative phase of the cycle.

FGFs encompass a family of growth factors that can stimulate growth of endometrial cells and smooth muscle cells.[541,542] Acidic and basic FGFs bind to proteoglycans. Because these proteins do not contain secretory signal sequences, they may be most important in the endometrium during menstruation when they could be released from dying cells. Basic FGF is angiogenic but also stimulates stromal cell proliferation in the presence of progesterone. FGF-7, also known as keratinocyte growth factor, stimulates epithelial cell proliferation. FGF-7 mRNA is expressed at highest level in the late secretory phase endometrium in the stroma; its receptor is most abundant in the glandular epithelium in the late proliferative phase. These findings suggest that FGF-7 is progesterone dependent, whereas its receptor is estrogen sensitive. FGF-8 is a uterine growth factor isolated from bovine uterus, and FGF-9 was recently described in the late proliferative phase of human endometrium.[542]

The IGF system of growth factors encompass not only IGF-1 and IGF-2 hormones, but also two distinct receptors and multiple different binding proteins that modulate IGF activity.[429,430,543-547] IGF-1 and IGF-2 are mitogenic growth factors that share structural similarities to insulin and are present in the mouse uterus and throughout the menstrual cycle in women.[548] Both hormones are present in the stroma, though the receptors are present in both epithelial and stromal cells. IGF-1 is more abundant in the proliferative phase and may function in epithelial proliferation, while IGF-2 is expressed in a more robust fashion in secretory phase and is thought to be a mitogen for the stroma of pregnancy.

IGF-1 binds to both the type 1 IGF receptor (IGF-1 receptor), structurally similar to the insulin receptor, and the type 2 receptor (IGF-2 receptor) with high affinity. IGF-2 binds the latter with higher affinity.[549]

IGF-1 mRNA is localized by *in situ* hybridization primarily in the syncytial trophoblast. IGF-2 mRNA has been demonstrated in mesenchymal fibroblasts of the villous core and also expressed in first-trimester and term cytotrophoblasts. The expression of IGFs in the placenta seems to be under the regulatory control of hormones (insulin, human placental lactogen, and estrogens) as well as growth factors, including PDGF. Type IGF-1 receptors have been detected in the placenta during the earliest periods of gestation, and it has been hypothesized that IGFs promote trophoblast proliferation.

TNF-α is a pleiotropic factor that exerts inflammatory, mitogenic, mitostatic, angiogenic, and immunomodulatory effects in a variety of tissues.[550] It is a membrane-bound 14-kDa polypeptide that is derived by proteolytic cleavage from the 26-kDa precursor. Expression of TNF-α mRNA and protein has been demonstrated in human endometrium, decidua, and trophoblasts; its receptors have also been found in all these tissues. TNF-α in the human endometrium is subject to regulation by steroid hormones—namely, estrogens and progesterone.

TNF-α and its receptors (TNF-R) are expressed by trophoblasts during both early and late gestation.[551] There is differential expression of the two genes encoding TNF-R, thus allowing some regulation of TNF-α activity. In cultured human chorionic cells, TNF-α affects cellular fibronectin secretion. Scientists have hypothesized that, along with other endometrial and trophoblast factors, TNF-α controls trophoblast adhesion and invasion and alters the integrin expression pattern in endometrial stroma.[552,553]

Colony-stimulating factors are a family of cytokines that were initially identified by their ability to stimulate hematopoietic stem cells to form colonies in semisolid culture media. CSF-1 is a glycosylated disulfide-linked homodimer. CSF-1 and its receptor are expressed in the endometrium, decidua, and placenta.[428,554] CSF-1 levels in the endometrial glands are higher in the secretory phase compared to the proliferative phase and the decidua expresses higher levels of CSF-1 mRNA and protein than the proliferative phase endometrium. The CSF-1 receptor (CSF1R encoded by FMS) is highly expressed by extravillous trophoblasts in the cell columns that anchor the placenta to the uterus.[555] The endometrial cells, both of local and of bone marrow origin, in proximity to the anchoring trophoblasts appear to be the major source of CSF-1 found at the placental-uterine interface. Lower serum levels of CSF-1 have been reported in women with recurrent spontaneous abortions. Although CSF-1 has been proposed to regulate trophoblast proliferation and differentiation, this role is not confirmed by the phenotype of the *op/op* (osteopetrotic) mouse that lacks CSF-1. This mouse has reduced fertility and smaller litter sizes, evidently due to a defect in ovulation, not implantation or placentation.

Human Chorionic Gonadotropin

In vitro studies demonstrate that the addition of follicle-stimulating hormone, luteinizing hormone (LH), hCG, thyroid-stimulating hormone, and free β subunit have an effect on human reproductive tract tissues, including the

stimulation of prolactin production, enhancement of decidualization, and myometrial relaxation.[556,557] The presence of LH/hCG receptors in the fallopian tube, myometrium, and endometrium has also been reported. However, the transcripts detected are smaller than those encoding the gonadal LH/hCG receptors; the proteins are also smaller, detected as 50 to 60 kDa molecules as compared with the observed receptor molecular weights in gonadal tissue (83 to 95 kDa). Thus, the extragonadal LH/hCG receptors appear to be truncated, evidently lacking extracellular domains, but still retaining the capacity to signal after binding LH and hCG. The primary signal transduction cascade initiated by the truncated endometrial LH/hCG receptors may not involve the classical cAMP-protein kinase A system as the primary pathway, but rather the mitogen-activated protein kinase pathway or the activation of prostaglandin synthesis.

Although extragonadal LH/hCG receptors have been proposed to play various roles in the reproductive tract, they are probably most important in the context of pregnancy where high levels of hCG are present. Thus, hCG could be an important embryonic signal in the bidirectional dialogue between conceptus and uterus. The best evidence that extragonadal LH-hCG receptors have an important role in primate reproduction is derived from the study of the baboon, where administration of recombinant hCG causes alterations in both epithelial and stromal cells. Preliminary studies in women indicate that hCG can also affect the human endometrium *in vivo*,[558] including prevention of endometrial apoptosis.[559]

In the baboon, the response to intra-uterine administration of hCG by osmotic minipump over a 4-day period starting on day 6 after ovulation includes the formation of an epithelial plaque, hypertrophy of the surface epithelium, and rounding off of the glands characteristic of pregnancy in this species.[557] Glycodelin expression in the endometrial glandular epithelium is enhanced, and α-smooth muscle cell actin is expressed in the stroma. *In vitro*, hCG inhibits stromal cell apoptosis and stimulates decidual changes—reflected by increased IGFBP-1 expression as well as increased expression of Cox-2. As previously noted, *in vitro* studies have shown that the glycoprotein α subunit stimulates production of prolactin, another uterine secretory product. Activation of myometrial LH/hCG receptors *in vitro* causes myometrial relaxation, which could facilitate nidation *in vivo*.[558] The response to hCG was blunted in animals with endometriosis.[560] Using intrauterine microdialysis in the human, it was shown that administration of hCG significantly reduces IGFBP1 and M-CSF expression in human after post-ovulatory day 10, while LIF, VEGF, and MMP-9 were all dramatically increased,[435,561] suggesting that hCG is an important modulating factor during early pregnancy. *In vitro* studies, using hCG coated beads, have demonstrated an induction of trophinin on the endometrial epithelium when IL-1 is present, suggesting a mechanism for enhanced embryo-endometrial interactions at the maternal-fetal interface.[562] LIF and its receptor are up-regulated in response to hCG treatment *in vitro*.[563] As an endocrine factor, hCG likely has independent effects on a wide variety of endometrial genes, independent of direct trophoblast interactions.[436]

Prostanoids and Other Lipids

The role of prostaglandins in the implantation process has long been suspected because of their effects on the vascular system and association with inflammatory processes. The fact that mice deficient in Cox-2 display abnormalities in the implantation process, particularly the early decidual response, is consistent with this notion.[558] Evidence suggestive of a role of prostanoids in human implantation includes the presence of Cox-1 and Cox-2 in the human endometrium (mainly the glandular epithelium) during the presumptive implantation period[564]; an examination of prenatal use of non-steroidal anti-inflammatory drugs (NSAIDs), drugs that inhibit Cox enzymes, indicated an increased risk of miscarriage in users of aspirin and other NSAIDs.[565] One of the key prostanoids involved in implantation is thought to be prostacyclin, a ligand for the peroxisome proliferators-activated receptor delta (PPARδ), a nuclear receptor family member expressed in subluminal stromal cells in the rodent uterus, whose role in implantation has been suggested.[510,566-568]

Another lipid implicated in implantation is the arachidonate derivative known as arichidonylethanolamide or anadamide, a ligand for the cannabinoid receptors.[569-572] Anandamide, referred to as an endocannabinoid, binds to cannabinoid receptors CB1-R and CB2-R, which are expressed in the preimplantation embryo and in the reproductive tract (CB1-R). The embryo is enriched in CB1-R, and in the blastocyst the expression of this receptor is most abundant in the trophectoderm. Uterine anandamide levels in the mouse are reduced at the time of implantation and are highest at inter-implantation sites. Endocannabinoids at low levels accelerate trophoblast differentiation but at high levels inhibit trophoblast differentiation and arrest embryo development. Tetrahydrocannabinol and synthetic agonists of the cannabinoid receptors have similar effects. Thus, it has been postulated that endocannabinoids play an important role in controlling the synchrony of embryo development for implantation in rodents. Anandamide is present in human reproductive tract fluids,[571] and high anandamide levels are associated with *in vitro* fertilization failures.[572]

Early Implantation Events in the Human

Prior to interaction with the surface epithelium, the blastocyst must hatch from the confines of the zona pellucida. Gradual zona thinning, as well as complete hatching of embryos, can be observed *in vitro*. The existence of ectopic implantation suggests that the endometrium is not obligatory for this process to be successfully completed. Nevertheless, there may be more subtle regulation of hatching within the endometrial cavity. Although degradation of the zona pellucida is a process controlled by the embryo, inhibitors or agents that induce "zona hardening" may affect the timing of the process.

Work on subhuman primates demonstrates that mononuclear cytotrophoblasts of the trophectoderm of the blastocyst have fused into syncytia before the attachment of these cells to the endometrial epithelium.[573] Careful histological descriptions of very early human implantation sites (such as those studied by Hertig and colleagues)[268] indicate that the syncytial trophoblast layer of the human embryo comprises the invading front during the first few days of implantation. Thus, the consensus appears to be that it is a syncytial trophoblast cell that initially interacts with and adheres to the endometrial epithelium; only after the human embryo is completely embedded in the endometrium do the cytotrophoblast cell columns start to stream out of the trophoblastic

shell and further invade the uterus.[574,575] This process starts approximately 1 week after the initiation of implantation and continues well into the second trimester of pregnancy.

The early human implantation sites that have been examined histologically demonstrate that by day 12 after ovulation, the embryo is almost completely covered by endometrium. The endometrial stroma around the implantation site displays the predecidual reaction and is edematous. By the classic histological criteria of Noyes and associates,[576] the endometrium of the implantation site is not that different from the non-pregnant midsecretory phase endometrium. The glands adjacent to the embryo are themselves deflected by invading trophoblasts but maintain the tortuosity and secretion-filled appearance typical for this stage of the menstrual cycle. *In vitro* observations using human embryos or human trophoblasts have attempted to characterize some morphological features of the early events of trophoblast-endometrial interactions.[577-581]

There is growing consensus that initial apposition and attachment is an evanescent and rate limiting step in the initiation of implantation. A receptor-mediated paradigm for embryo attachment and invasion has long been postulated.[582] Numerous endometrial and trophoblast cell-adhesion molecules and associated moieties have been suggested as candidates to serve as attachment receptors.[525,583-586] Historically, surface modification of the glycocalyx was a subject of much interest,[587-589] but more recently the molecules that populate the luminal surface have come under scrutiny. The actual number of receptors and ligands on this surface appear to be somewhat restricted compared with the other cells in the endometrium. As shown in Figure 10.19, a limited number of adhesion-attachment components have been suggested. MUC-1 (not shown) is a large glycoprotein that is downregulated on the endometrial surface in most species at the time of implantation,[590] but expressed throughout the menstrual cycle in humans.[586] Nevertheless, debate continues regarding this large glycoprotein as a possible attachment receptor for the human embryo. Other smaller molecules are present and may serve the purpose of initial attachment, including trophinin, integrins, CD44, L-selectins, and HB-EGF™.[109,591]

The cascade of events leading to successful implantation likely requires many critical proteins with different functional contributions. A novel cell surface protein called *trophinin* has been suggested as a homologous pairing partner between trophoblast and endometrium during implantation.[584] The αvβ3 integrin and its ligand osteopontin, are expressed at the time of implantation on the luminal surface of receptive endometrium. Secreted osteopontin binds to this integrin through an RGD sequence.[440,592] Since osteopontin can also bind to the CD44 hyaluronate receptor through non-RGD binding sites, it has been suggested that these serve in a "sandwich"-pairing mechanism at the point of interface.[593,594] In the human, surface epithelial cells on both the embryo and endometrium express the αvβ3 integrin *and* CD44.[595] There is also ample evidence that RGD binding is vital to the process of implantation,[596,597] peptides containing this sequence reduce implantation efficiency in animal models, including the rabbit and mouse.[598,599] Thus, integrin-mediated adhesion may somehow play a role in successful implantation. More recently it has been suggested that osteopontin, αvβ3 integrin, and CD44 binding serves to suppress the innate immune system through decay-accelerating factor (DAF)-mediated interference with complement subunit C3,[438,442] a role that could be critical for the protection of the embryo at the time of initial attachment and invasion.

The transmembrane form of HB-EGF and its receptor ErbB-4 are expressed on the surface of endometrium and on the outer cells of the embryo, respectively. Evidence in both rodents and human suggests that these molecules could serve as an attachment receptor-ligand pair during implantation.[523,525] Soluble HB-EGF interferes with this process, perhaps through competitive inhibition. Transmembrane HB-EGF could also play a paracrine role especially if cleaved from its transmembrane location when the embryo enters the uterine cavity.

Perhaps the most promising mechanism involves that previously elucidated for leukocyte-endothelial interaction. L-selectin and an oligosaccharide ligand are expressed on the blastocyst and endometrial surface in the human, respectively.[585] This type of adhesion reaction between embryo and endometrium at the time of implantation is quite appealing and likely involves integrin mechanisms for embryo invasion, similar to leukocyte intercalation at sites of inflammation.

Evidence from humans illustrate a temporal and spatial regulation of L-selectin ligand expression on the luminal and glandular epithelium.[600] Likewise, each mechanism could be disrupted in infertile women, leading to a failure

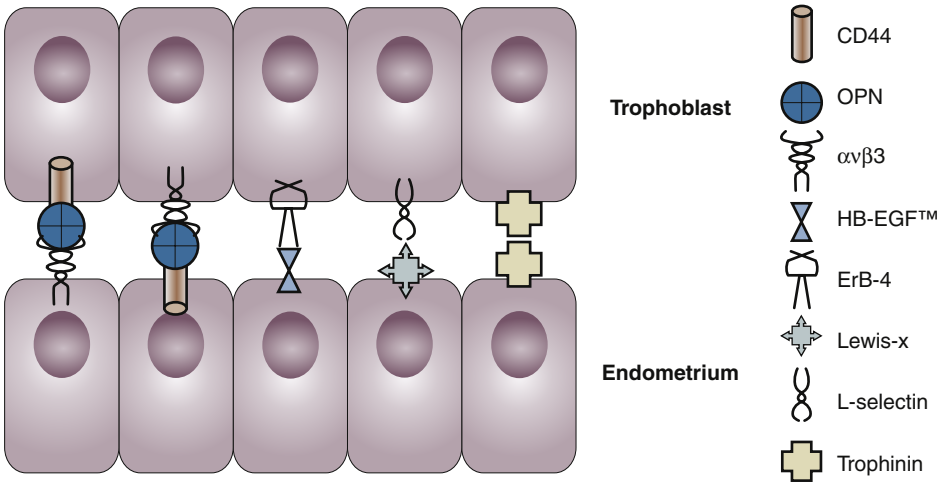


FIGURE 10.19 Luminal surface proteins that may interact with complementary proteins on the embryonic surface. Experimental evidence exists for each of these possible attachment reactions. It is likely that there is redundancy in these systems and that each may serve specific functions, such as signaling or attachment during the initial stages of implantation.

of implantation.[601] Methods to diagnose and correct such defects may provide new hope for those with otherwise unexplained infertility or recurrent pregnancy loss.

Structural alterations accompany the biochemical changes noted on the surface epithelium. Scanning electron microscopy reveals that the human endometrial epithelium consists of secretory and ciliated cells (Fig. 10.20). The ratio of non-ciliated to ciliated cells changes during the menstrual cycle, decreasing in the late proliferative phase and increasing in the secretory phase. In general, estradiol levels correlate directly with the presence of ciliated cells, and withdrawal of estrogen is associated with loss of cilia. The ciliated cells do not undergo surface morphology changes during the menstrual cycle, whereas the secretory cells display significant cycle-dependent surface modifications.

Transient surface specializations of the secretory cells called *pinopodes*, also known as pinopods or uterodomes, have been a focus of research, because the temporal patterns of expression seemed to coincide with the time of maximal uterine receptivity.[602] These surface structures were first identified on the luminal epithelium of rodent endometrium during the limited period (approximately 12 hours) when the uterus is receptive to implantation; they were shown to be involved in pinocytosis, hence the appellation of pinopode (from the Greek "drinking foot").[603] Similar structures, although with different morphologies, were subsequently discovered in numerous species, including humans; their appearance again generally correlated with the time of implantation.

While it is certain that pinopodes are involved in pinocytosis in the rodent uterus, *in vitro* studies failed to show that they serve this function in women—hence the suggestion that the structures should be designated "uterodomes" as opposed to "pinopodes."[604,605] Mechanisms underlying the formation of pinopodes in the human endometrium have not been elucidated. They may form from the accumulation of membrane components, as a consequence of secretory activity, or from reorganization of the cell cytoskeleton. Some researchers have suggested that they serve to elevate the endometrium above the ciliated cells, providing a platform with the necessary complement of surface adhesion receptors.

The role of pinopodes in human embryo implantation (beyond the temporal correlation between their appearance and the estimated time of nidation) is supported by *in vitro* studies demonstrating that human blastocysts implant on human endometrial epithelial cells only in areas bearing pinopodes (Fig. 10.21). Other studies have demonstrated that surface biomarkers are present on the pinopodes. HB-EGF, a molecule implicated as a membrane bound ligand and juxtacrine-paracrine factor important in signaling to the embryo, is on the surface of pinopodes at the expected time of implantation.[371] The αvβ3 integrin and its ligand are also both present on these apical protrusions during the window of implantation (Figs. 10.20 and 10.22).[606] MUC-1 and osteopontin appear to be on different cell types of the luminal surface, based on electron immunohistochemistry; MUC-1 is present solely on the ciliated cells while osteopontin is present on the secretory or pinopode-bearing cells.

The formation of pinopodes appears to be dependent on progesterone, while estrogen causes them to regress. The earlier appearance of pinopodes in controlled ovarian stimulation cycles is correlated with the preovulatory rise in plasma progesterone. Administration of a low dose of the progesterone receptor antagonist mifepristone on days

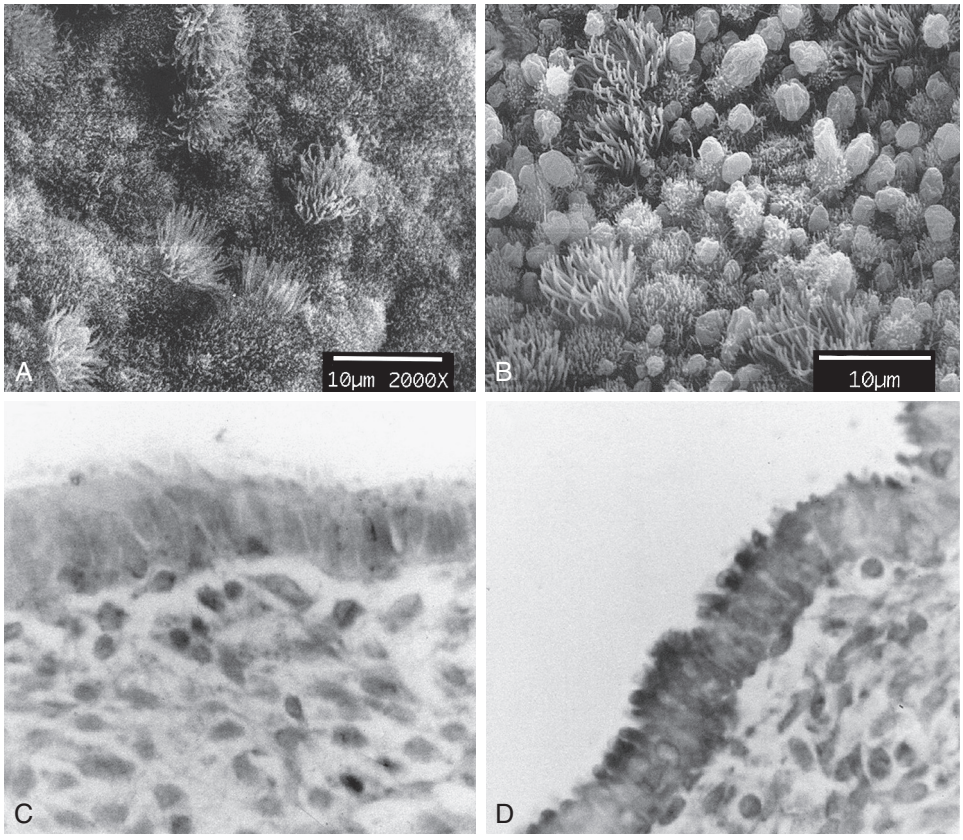


FIGURE 10.20 **A,** The surface epithelium of human endometrium is composed of ciliated and secretory cell types. **B,** During the time of maximal receptivity, the surface of these cells transform into a sac-like protrusion seen in many species, now called pinopodes. During the prereceptive phase, surface receptors such as the αvβ3 integrin are not present **(C),** but are expressed during the window of implantation on these pinopode structures **(D).** *(Used with permission of Human Reproduction.)*

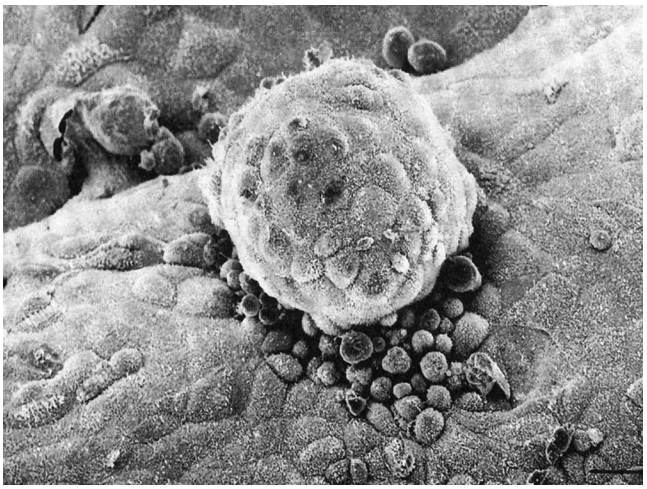


FIGURE 10.21 *In vitro* culture of human embryos on cultured endometrial epithelium appears to show that embryos prefer to attach to areas with pinopode expression. *(Reproduced with permission from Bentin-Ley U. Relevance of endometrial pinopodes for human blastocyst implantation.* Hum Reprod *15[Suppl 6]:67–73, 2000.)*

14 and 15 of a cycle delays pinopode formation. Studies in the mouse suggest that *HOXA*10, a progesterone-regulated gene, is required for pinopode formation,[607] though pinopodes have been described in both *LIF* and *HOXA10* null mice.[608] The actual temporal distribution of pinopodes has also been brought into question in the past several years, with several studies showing little correlation with the actual window of implantation.[609-611] While likely involved in embryo-endometrial interactions, the utility of these structures as markers of uterine receptivity appears limited.

Biochemical Evaluation of the Endometrium

Biomarkers of Endometrial Receptivity

Attempts have been made to find suitable biomarkers of endometrial receptivity.[470,612,613] A list of major biomarkers of receptivity are listed in Table 10.1. These endometrial factors appear relevant on the basis of their temporal patterns of expression that correlate with the putative window of implantation on cycle days 20 to 24. While certain biomarkers, such as calcitonin or LIF, are aligned closely within

Table 10.1 Endometrial Biomarkers Used for Assessment of Endometrial Receptivity

FUNCTIONAL CATEGORY	BIOMARKER	EXPRESSION PATTERN	COMMENTS
Chemokine/cytokine	LIF	m---------o---------m	All essential for implantation
	IL1,11	m---------o---------m	
Enzymes	17HSD-II	m---------o---------m	Converts E2 to less active E1;
	Aromatase	m---------o---------m	Should not be present in normal endometrium
Extracellular matrix/ receptors/ligands	Integrins		These 3 integrins frame the window of implantation;
	α1β1	m---------o---------m	
	α4β4	m---------o---------m	MMPs and lefty (EBAF) come up at menses;
	αvβ3	m---------o---------m	
	MMPs	m---------o---------m	Epithelial glycoprotein
	EBAF (lefty)	m---------o---------m	Binds L-selection;
	MUC-1	m---------o---------m	Stromal marker
	L-selectin ligand	m---------o---------m	
	Cadherin-11	m---------o---------m	
Growth factors	TGFβ	m---------o---------m	Mediates P action;
	EGF Ligand family	m---------o---------m	Involved in both proliferation and implantation;
	Hepatocyte growth factor (HGF)	m---------o---------m	
	Heparin binding-EGF like growth factor (HB-EGF)	m---------o---------m	Involved in decidualization Stromal and epithelial expression;
	IGF-I	m---------o---------m	Proliferative phase
	IGF-II	m---------o---------m	Secretory phase
Major secreted proteins	Glycodelin	m---------o---------m	All 3 are epithelial proteins
	Calcitonin	m---------o---------m	
	Osteopontin	m---------o---------m	
Paracrine or autocrine	Indian hedgehog (IHH)	m---------o---------m	Induced by P;
	Bone morphogenic protein 2 (BMP2/TGFb1)	m---------o---------m	Induced by IHH
Steroid receptors and related cofactors	ERα	m---------o---------m	Estrogen induced and P inhibited; *PRA persists in decidua
	PR-A/PR-B	m---------o---------m	
	FKBP51, FKPB52	m---------o---------m	Each induced by P;
	MAGE-11	m---------o---------m	Expressed in midsecretory phase
Transcription factors	HOXA10	m---------o---------m	Both essential for implantation;
	HOXA11	m---------o---------m	Expressed during WOI
	CCAAT enhancer binding protein- β (C/EBPβ)	m---------o---------m	Essential for decidualization; Expressed during proliferation
	Forkhead box O1 (FOXO1)	m---------o---------m	
	Beta catenin	m---------o---------m	
Miscellaneous	CD44	m---------o---------m	During WOI;
	Prolactin	m---------o---------m	Marker of decidualization

this window, others have a pattern of expression that is inversely related to the time of maximal endometrial receptivity (ERα, PRs, and telomerase).

Some of the best-characterized biomarkers for assessment of uterine receptivity are the integrins. As shown in Figure 10.22, three epithelial integrins undergo changes in expression during the luteal phase. This pattern has now been consistently observed in multiple studies.[109,614-619] Of these, αvβ3 is the most studied. Dyssynchronous or delayed maturation of the endometrium is associated with delayed expression of the αvβ3 integrin. While patterns of integrin expression suggest roles in cell adhesion and maintenance of tissue architecture, the αvβ3 integrin is present on the upper most portion of the luminal epithelium corresponding to the localization of osteopontin and pinopodes. This integrin has been used for the assessment of the endometrium in women with endometriosis and other benign conditions,including hydrosalpinges, unexplained infertility, and polycystic ovary syndrome, although not all studies agree on their usefulness.[109]

Based on the use of biomarkers like the integrins, two distinct types of deficiencies have been identified in women with infertility. This model fits well with data presented by Wilcox and colleagues that suggests delayed implantation may reduce cycle fecundability.[267] The normal uterine receptivity provides the opportunity for the embryo to attach and invade the maternal lining. In some cases, such as in luteal phase defect, this window may be shifted or delayed, resulting in pregnancy loss or failure to conceive. This might occur when progesterone levels are suboptimal or when the response to progesterone is decreased. In other cases, the histological development is normal but the biochemical changes associated with a receptive endometrium fail to develop in a timely fashion. Such a mechanism of occult defects has been suggested as the underlying implantation defect in women with endometriosis and hydrosalpinges.[109] The cause of this type of receptivity defect remains a topic of debate and an active area of research.

Clinical Evaluation of the Endometrium

Endometrial Biopsy

The endometrial biopsy is used to assess endometrial histology. While used extensively in the setting of abnormal bleeding as a diagnostic tool for endometrial cancer or hyperplasia, its primary role in the evaluation of the infertile couple has been the assessment of the luteal phase. However, as described below the clinical utility of the endometrial biopsy as a routine test is questionable. The existing literature reflects a wide range of opinions regarding the use of this test,[620-623] including when to perform the biopsy, the

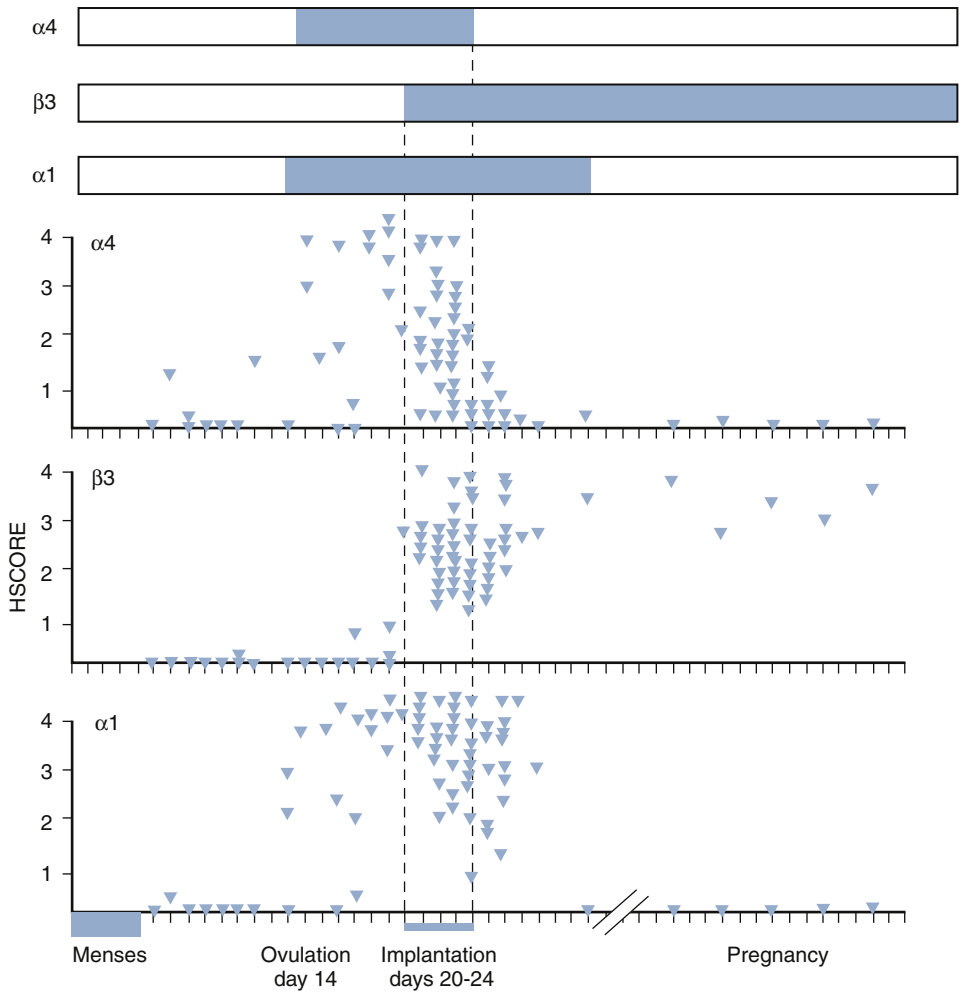


FIGURE 10.22 Scattergram showing distribution of three cycle-dependent epithelial integrin subunits in cycling endometrium. While each of the integrin subunits has a unique pattern of expression, all three are coexpressed during the time of maximal receptivity between cycle days 20 and 24. *(From Lessey BA. Two pathways of progesterone action in the human endometrium: implications for implantation and contraception.* Steroids. *68: 809-815, 2003.)*

number of days' lag time that should be required to consider a sample abnormal, and the utility of other modalities to evaluate the luteal phase.[624] True standardization of the endometrial biopsy to evaluate histological criteria has never been established despite over 50 years of use, and recent studies have questioned its value.[620,625] The chronological reference point, crucial to the interpretation of results, has shifted from retrospective to prospective determination during that time.[626] These variations in the use of the endometrial "dating" may explain, in part, the wide diversity of opinion in the literature regarding the incidence and clinical importance of luteal phase deficiency (LPD).[621]

LPD is uncommon and probably accounts for no more than 3% to 5% of infertile population judging from many studies. The high prevalence of LPD in some reports is understandable given the variability of endometrial histology between women and even in the same woman in subsequent cycles. Add to that the variation in timing of the biopsy and inter- and intraobserver variation in reading histology, the real significance of luteal phase biopsies may become of historic interest only.

The histological changes that serve as the basis for this technique were described in 1950 in the now classic paper by Noyes and associates.[576] Unfortunately, these criteria are only an approximation of the cumulative effect of progesterone on the endometrium. Cycle-to-cycle variability, high inter- and intra-observer variation, and uncertainty as to the timing of ovulation also reduces the overall reliability of these dating criteria. The original indices, as defined, were only obtained in an *infertile* population, making it somewhat difficult to know whether the same histological changes occur in normal fertile women. Indeed, recent studies in normal fertile controls do not support this technique of dating endometrium as a precise or reproducible method of endometrial assessment.[620] Currently the best method for diagnosing luteal phase defect may be the presence of a shortened luteal phase, as shown by methods such as basal body temperature charting (Fig. 10.23). While the normal luteal phase should be approximately 12 to 14 days in length, premature luteolysis could potentially compromise the establishment of pregnancy. Such charting can also demonstrate anovulatory cycles.

Of note, there may be a basis for some of the observations regarding luteal function that is worthy of further consideration. With the completion of the human genome project and the availability of high throughput DNA microarray analyses, it appears that progesterone resistance does occur, based on patterns of gene expression in women with endometriosis compared to fertile controls.[114] Whether this precedes or is a result of endometrial pathology has not been settled. And while women with endometriosis are the first group associated with this phenomenon, it also appears to be common in ovulatory women with PCOS.[48] Such changes may account for the infertility or pregnancy loss associated with these conditions. Defects in progesterone receptors or in progesterone-mediated regulators of cell growth or differentiation have all been implicated in progesterone resistance, including changes in *HOXA* genes, integrins, Mig-6, FKBP4, PR-B, or DKK-1.[18,59,90,183,627-629] These findings provide new underpinnings of understanding subtle defects in progesterone responsiveness and promises to keep attention focused on LPD, even 60 years after its original description.

Ultrasonography

Abnormalities of the structure of the uterus from congenital defects, neoplasia, or synechiae can impair fertility. Diagnostic imaging methods play an important role in the assessment of these uterine defects.[630]

Transvaginal sonography (TVS) has become widely accepted as a tool for high-resolution imaging of the female internal reproductive organs, because it is non-invasive, convenient, and safe and provides rapid diagnoses with high correlation with pathology.[631] This non-invasive, convenient, and safe technology provides rapid diagnoses with high correlation with pathology. TVS is used primarily to monitor follicular development and endometrial thickness during exogenous hormone treatments of infertile patients. The method might offer significant advantage for use as a diagnostic aid in various other endometrial conditions, such as endometrial polyps, submucus and intramural leiomyomata, endometrial hyperplasia, and carcinoma. TVS has certainly found its use in the assessment of early pregnancy.

Growth of the endometrium can easily be measured using ultrasound. Endometrial thickness and texture is commonly assessed, especially when women are sequentially monitored as part of their treatment with human menopausal gonadotropins. Endometrium in the early proliferative phase immediately following menses is typically thin; in response to estrogen, the endometrium thickens and becomes trilaminar in appearance, growing between 0.1 to 0.5 mm daily. Following ovulation, the endometrium becomes hyperechoic as secretory changes ensue. Various attempts have been made to classify these patterns on the basis of thickness and texture. Most authors suggest that a thickness of 8 mm or greater with a trilaminar appearance is adequate for implantation in an *in vitro* fertilization cycle.[632-636] Beyond a certain threshold, however, there is no correlation between implantation rates and endometrial thickness.

The cyclic endometrial changes induced by varying estrogen and progesterone levels result in predictable sonographic changes, in both blood flow and vessel density. Both endometrial thickness and echogenic pattern have been studied as potential markers of uterine receptivity and predictors of successful embryo implantation. Transvaginal pulsed Doppler ultrasound measures uterine artery blood flow (or the impedance to flow) and is expressed as the *pulsutility index* (PI). The PI varies across the menstrual cycle and may be an additional index to predict implantation after assisted reproductive techniques. Aside from endometrial thickness, morphology, blood flow, and uterine artery pulsatility have all been examined as possible markers of a receptive endometrium. Increased PI has been associated with elevated markers of pregnancy loss, including anti-cardiolipin antibodies.[637,638] Studies in pregnancy using Doppler flow ultrasound and pregnancy outcomes have been inconclusive.[639]

Sonohysterography and Three-Dimensional Ultrasound

Identification of uterine fibroids, endometriomas, and uterine septa are now fairly routine (see Chapter 27). Lesions such as polyps within the uterine cavity may be misinterpreted as thickened endometrium and therefore go

undiagnosed. Instillation of sterile saline into the uterine cavity as part of sonohysterography provides an enhanced view of the uterine cavity and may detect even small lesions. This approach is valuable in assessing the effects of selective estrogen receptor modulators like tamoxifen on the endometrium.[640] A sonohystogram in a normal uterus shows a smooth contour (Fig. 10.24A). In some cases, abnormalities can be more clearly seen with sterile saline installation (Fig. 10.24B). Three-dimensional sonohysterography can provide dramatic 3-D rendering of the uterine cavity, giving a better overall appreciation of such lesions, and better determine the location and point of attachment (Fig. 10.24C).[641] This evolving technology may further improve the sensitivity and specificity of the technique used to detect small polyps or fibroids. With the advent of three-dimensional ultrasound, differential diagnoses between a septate versus a bicornuate uterus can also be more readily determined, avoiding the need for more expensive modalities like MRI. The use of three-dimensional ultrasound for the assessment of uterine receptivity is also evolving.[642]

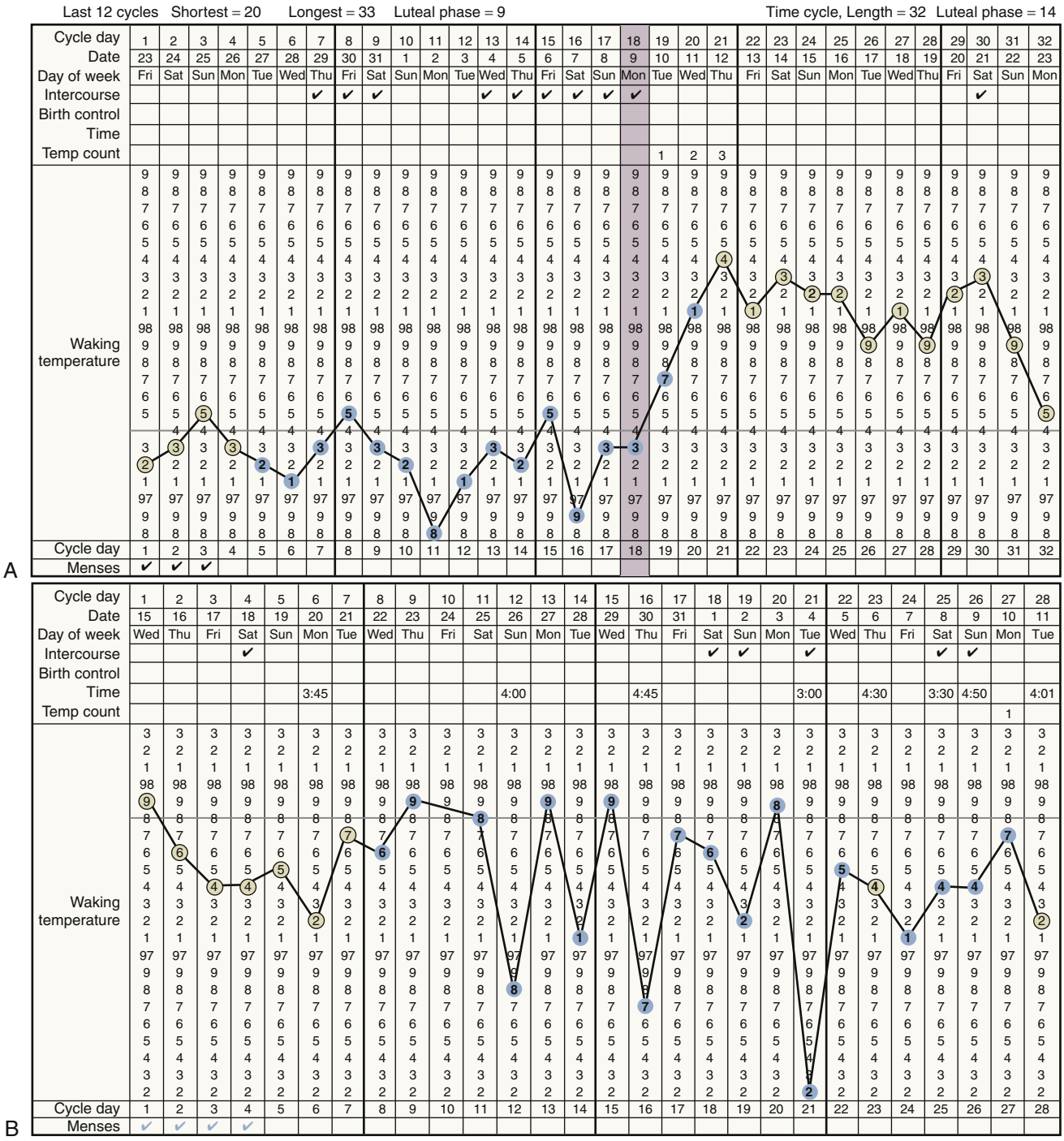


FIGURE 10.23 Basal body temperature charts can be helpful for the diagnosis of ovulatory dysfunction. Compared with normal 14-day luteal phase **(A)**, anovulatory cycles fail to show a normal thermogenic shift **(B)**.

C

Last 12 cycles Shortest = 20 Longest = 33 Luteal phase = 9 Time cycle, Length = 32 Luteal phase = 14

Cycle day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Date	23	24	25	26	27	28	29	30	31	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Day of week	Fri	Sat	Sun	Mon	Tue	Wed	Thu	Fri	Sat	Sun	Mon	Tue	Wed	Thu	Fri	Sat	Sun	Mon	Tue	Wed	Thu	Fri	Sat	Sun	Mon	Tue	Wed
Intercourse							4	4	4				4	4	4	4	4	4									
Birth control																											
Time										7:30			7:30														
Temp count																			1	2	3						
Waking temperature	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
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	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
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	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98	98
	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
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	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97	97
	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Cycle day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Menses	4	4	4																								

FIGURE 10.23, cont'd In some cases, a short luteal phase is evident **(C).**

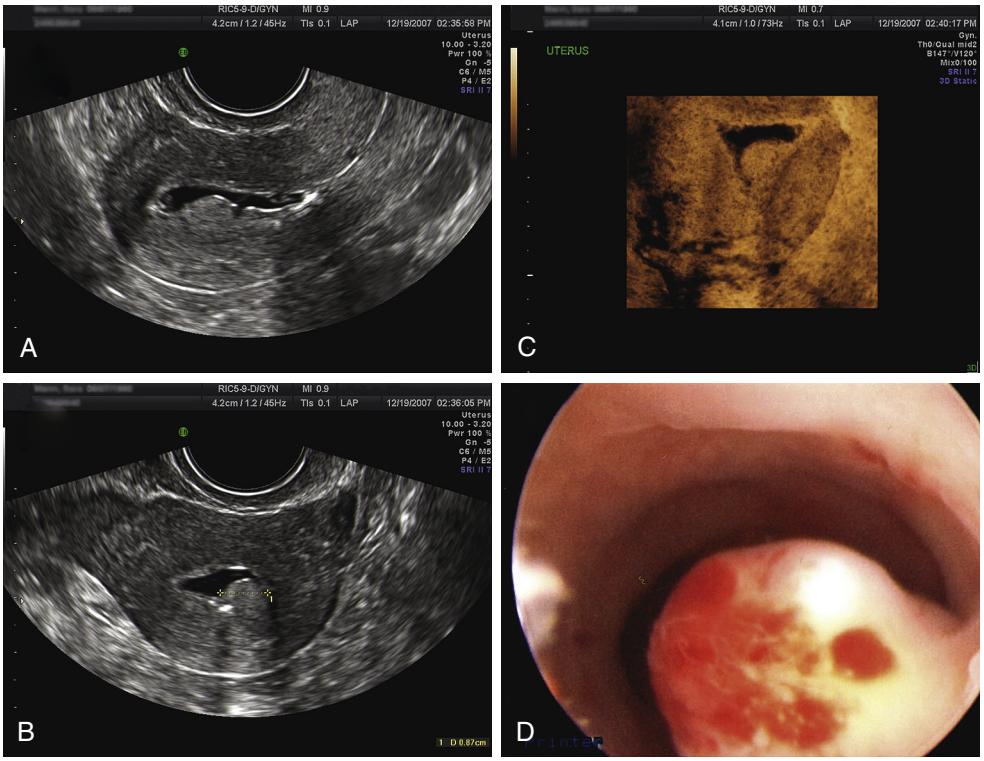


FIGURE 10.24 Sonohysterography can be used to visualize intracavitary lesions. Injection of sterile saline shows the uterine cavity to be free of polyps or fibroids **(A).** In the case of intrauterine pathology, lesions such as a polyp can be easily delineated either in 2-D **(B)** or 3-D rendering **(C).** Hysteroscopy confirms the polyp **(D)** that can then be easily resected.

Hysteroscopy

Hysteroscopy is a highly useful tool to identify and correct lesions identified by hysterosalpingography or sonohysterography (Fig. 10.24D).[643,644] Visualization of the endometrial cavity has proven useful for the inspection of the uterine cavity in women with abnormal bleeding, infertility, and recurrent pregnancy loss. Direct visualization allows the operator to resect or biopsy lesions, once identified. Resection of uterine septa in müllerian fusion defects is commonly performed. More recently, sterilization techniques through hysteroscopy have been refined to a point that challenges

other techniques and are readily available in many centers around the world.[645]

Magnetic Resonance Imaging

MRI may occasionally be useful for diagnosis of myometrial lesions, including fibroids and adenomyosis (see Chapter 27). T2-weighted images may provide evidence for endometriotic lesions outside the uterine fundus and aid in the diagnosis of congenital uterine anomalies prior to surgical correction.

Uterine Transplantation

Aside from generation of a female reproductive tract using stem cell technologies,[646] restoration of a cervix and uterus for purposes of reproduction may soon be an option for women who have lost or due to müllerian agenesis never had normal uterine anatomy.[647] Transplantation surgery of many organs including heart, lung, liver, and even the face or hands has become routine. Further, women with acquired gynecological disease such as fibroids and adenomyosis and gynecological malignancy, Asherman syndrome, or congenital absence of the uterus may one day be able to undergo a uterine transplant with reasonable chances for success. The first (and currently only) attempt at uterine transplantation in the human was performed in 2000, which ended with graft removal due to thrombosis.[648] Of greatest concern is the immune suppression required to prevent graft vs. host reaction and graft rejection. While pregnancy itself may be considered an immunotolerant state, graft rejection for other organs has been reported to be higher during pregnancy.[648] Further, it is not yet clear what the effect cyclosporine or other immunosuppressant therapies might have on the recipient, her fetus, or on the graft during pregnancy.[649]

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 11

The Breast

Robert L. Barbieri

The main biological function of the breast is to produce milk to nurture newborns. Mammals are differentiated from other animal classes by the presence of hair on the skin and the production of breast milk to nurture newborns. The word, mammal, is derived form the Latin *mamma* which means, "resembling the milk gland or breast." The breast overlies the second to the sixth ribs. The medial border of the breast is the sternum, and the lateral border is the latissimus dorsi. The superior and inferior borders are the clavicle and the costal margin and upper rectus sheath. The breast consists of glandular, adipose, and connective tissues. The left breast is often somewhat larger than the right breast.

The glandular tissue of the breast is arranged in approximately 15 to 20 lobes (Fig. 11.1). Each lobe consists of a branching structure made up of lobules and acini (also referred to as *alveoli*). The acini are lined by a single layer of milk-secreting epithelial cells. Each acinus is surrounded by an interwoven pattern of contractile myoepithelial cells. The lumens of the acini connect to collecting intralobular ducts, which empty into the main 15 to 20 lobar collecting ducts. In turn, each lobe drains into the nipple. The nipple is surrounded by a pigmented areola. The sebaceous glands located on the perimeter of the areola are referred to as the *glands of Montgomery*. The glandular tissue is embedded in fat, which accounts for most of the mass of the breast. The lobules are separated by connective tissue septa (Cooper's ligaments) that run from the subcutaneous tissue to the chest wall.

The breast is sensitive to the sex steroids estradiol, progesterone, and testosterone, and to numerous protein hormones, including prolactin and oxytocin. During puberty and pregnancy, the breast is stimulated to grow. In many mammals, estradiol is especially important in stimulating the growth of the ductal system, and progesterone is important in stimulating the growth of the acini and stromal elements. During pregnancy, estradiol, progesterone, and prolactin are important factors stimulating breast growth. Androgens tend to inhibit breast growth, an effect that can be characterized as antiestrogenic.

Development of the Breast

The development of the breast occurs in three major phases: *in utero*, at puberty, and during pregnancy. This process has been best studied in mice. During *in utero* development of the breast, the growth of epithelial elements into the underlying mesenchyme results in the development of a rudimentary ductal system. This process occurs independent of stimulation by estradiol, progesterone, prolactin, or growth hormone. Immediately after birth, the rudimentary mammary ducts are quiescent. In the mouse, the majority of mammary duct morphogenesis occurs during puberty.

The mammary gland is a tubular structure that fully develops during puberty through branching morphogenesis. The branched ductal network that forms extends into the stromal fat pad and the branching process is led by the terminal end bud which consists of epithelial progenitor cells. The differentiated mammary gland is composed of luminal epithelial cells and surrounding basal myoepithelial cells. Estradiol stimulation of estrogen receptor alpha (ERα) is critical for pubertal mammary gland development. ERα-null mice do not have mammary development.[1] Estrogen receptor beta (ERβ) is abundantly expressed in mammary glands but mice lacking ERβ have normal mammary development.[2] Forkhead box protein A1 (FOXA1) helps open up chromatin so that transcription regulatory factors, such as the estrogen receptor, can gain access and better bind to deoxyribonucleic acid (DNA) regulatory elements. FOXA1 null mice do not have normal mammary development but develop stunted ductal structures with end buds.[3] The coactivators and corepressors that modulate ER and progesterone receptor (PR) action are described in detail in Chapter 5, Steroid Hormone Action.

In the mouse, knock-out of the prolactin gene by homologous recombination causes infertility in female mice, but not in male mice. The mammary glands of prolactin knock-out mice have a normal ductal tree, but the ducts fail to develop lobules.[4] In the mouse, knock-out of the prolactin receptor gene by homologous recombination causes infertility and mammary development is normal up to puberty. At puberty ducts develop but branch less frequently and the terminal end buds do not differentiate to alveolar structures.[5]

In pubertal mice, trans-acting T-cell-specific transcription factor GATA3 (GATA3), a transcription factor involved in cell fate specification, is highly expressed in luminal cells. Conditional deletion of GATA3 results in the failure of terminal end bud formation in breast tissue.[6] In addition, mammary duct development is highly dependent on the release of amphiregulin from luminal epithelial cells by the action of the transmembrane metalloproteinase, a disintegrin and metalloproteinase domain 17 (ADAM17). In turn, amphiregulin binds to the epidermal growth factor receptor (EGFR) located on stromal cells which stimulates duct formation. In the adult mouse, tertiary side-branching of the ductal system is stimulated by progesterone.

With the onset of pregnancy, a round of breast epithelial and gland proliferation occurs. Numerous hormones play roles in this process, including estradiol, progesterone, androgens, prolactin and other lactogenic factors, thyroxine, glucocorticoid, insulin, growth hormone, transforming

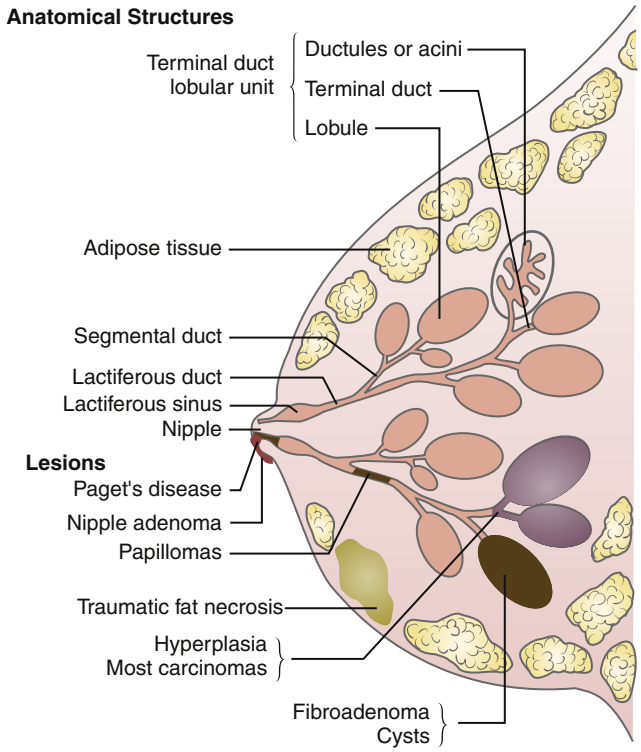


FIGURE 11.1 Anatomy of the breast and major lesions at each site within the breast. *(From Cotran RS, Kumar V, Robbins SL.* Pathologic Basis of Disease, *5th ed. Philadelphia: WB Saunders, 1994, p 1090.)*

growth factor-β, and epidermal growth factor (EGF). In the mouse, conditional knock out of ERα during pregnancy or lactation causes a loss of ductal side-branching and a decrease in the number of lobuloalveolar units.[7]

During the normal menstrual cycle, the breast undergoes cycles of growth and quiescence. In the early follicular phase, the ductal cells proliferate and continue to develop throughout the remainder of the cycle. In the luteal phase of the cycle, progesterone stimulates the proliferation of the terminal duct structure, basal epithelial cells, and stromal cells. Accompanying these proliferative changes are an increase in stromal edema and vacuolization of epithelial cells. These effects may account, in part, for the sense of breast fullness women experience during the late luteal phase. At menses, the decrease in estradiol and progesterone is associated with a decrease in cell proliferation, stromal edema, and the size of the ducts and acini.

The two most common congenital anomalies of the breast are supernumerary nipples and accessory axillary breast tissue. Supernumerary nipples result from the persistence of epidermal thickenings along the embryologic milk line. Supernumerary nipples can extend from the axilla to the suprapubic area. On occasion, breast tissue extends into the axillary fossa. Ectopic breast tissue in this region can be mistaken for an axillary mass or may cause pain after pregnancy because of the engorgement of the tissue with milk.[8-10]

Mammary Stem Cells

Mammary epithelial structures in both mice and women contain cells spanning a developmental hierarch from pluripotential stem cells, to progenitor cells committed to populate the epithelial structures to mature terminally differentiated luminal cells.[11] Mammary stem cells (MaSCs) become highly activated during pubertal development and pregnancy. MaSCs which are located in the basal epithelium do not express ERα or the progesterone receptor, but administration of estradiol, progesterone, or prolactin to female mice increases the number and clonogenicity of MaSCs.[12] This suggests the presence of paracrine cross-talk between the luminal epithelium and the basal MaSCs. Progesterone and prolactin appear to stimulate luminal epithelial cells to produce and secrete receptor activator of nuclear factor kappa-B ligand (RANKL) and Wnt4 which in turn stimulate the clonogenicity of MaSCs.[13,14]

Estradiol and Testosterone: Duelling Steroids

A genetic experiment of nature, *androgen insensitivity syndrome*, provides a clinical example of the important interplay between estrogens and androgens in the regulation of breast growth.[15] In androgen insensitivity due to mutations in the androgen receptor, genetic males (46,XY) do not have a fully functional androgen receptor. Testosterone is produced by the testis, but target tissues are not capable of responding to the high levels of circulating androgens. In this syndrome, estradiol levels are in the range of 50 pg/mL, comparable to early follicular-phase levels. Breast volume in individuals with androgen insensitivity is typically above average. This suggests that, in the complete absence of androgen inhibition, modest levels of estradiol are capable of stimulating significant breast growth. Progesterone levels are low in individuals with loss of the androgen receptor. This suggests that breast volume is not absolutely dependent on progesterone stimulation.

In the mouse, androgen receptors are present in mammary luminal epithelial cells, stromal fibroblasts, and adipocytes.[16] In female mice, administering androgen antagonists during puberty markedly increases the number of proliferating breast epithelial cells.[17] In the adult female monkey, administering androgen antagonists enhances estrogen-stimulated breast epithelial cell proliferation.[18] In cultures of human breast tissue, dihydrotestosterone (DHT) and testosterone inhibit estradiol induced stimulation of cell proliferation, cell survival, and the density of ERα containing cells.[19] The antagonist effect of androgens on estrogen stimulation of breast epithelial proliferation is the basis for the use of androgens to treat breast pain syndromes (see section titled, Cyclical Mastalgia).

Lactation

In reproduction, the main role of the breast is secretion of milk. Breastfeeding is very important for infants because it reduces the risk of infectious, immune, and atopic diseases and reduces neonatal mortality.[20] For mothers, breastfeeding protects against breast and ovarian cancer, type 2 diabetes, cardiovascular disease, and postmenopausal hip fractures. Breast milk consists of milk proteins, including casein and lactalbumin, free fatty acids, the milk sugar lactose, water, minerals, and vitamins. Lactose is a disaccharide consisting of glucose and galactose. During pregnancy, the breast is prepared for lactation by the actions of estradiol, progesterone, prolactin, and other factors—including placental lactogen. Lactose, water, and some proteins

accumulate in Golgi bodies and are then secreted into the duct system through a process of exocytosis. Many proteins and circulating hormones, including immunoglobulins and albumin, enter the duct system through a process of protein mediated transcytosis. Lipids are secreted into the duct system through a process of reverse pinocytosis. Lipids accumulate in the endoplasmic reticulum, form droplets, and then become enveloped in the apical plasma membrane leading to the emptying of the droplets into the duct system.

In pregnancy, the breast is exposed to high levels of lactogenic hormones, including prolactin, human placental lactogen, and placental growth hormone. In nonpregnant women, prolactin levels are typically below 25 ng/mL. During the third trimester of pregnancy, prolactin levels are typically fifteenfold higher, in the range of 200 to 300 ng/mL.[21] Human placental lactogen reaches concentrations in the range of 6000 ng/mL during the third trimester.

These lactogenic stimuli cause the breast to grow and prepare the alveoli for lactation. During pregnancy, the high concentration of progesterone blocks lactogenesis. After delivery, the decrease in circulating estradiol and progesterone concentrations and the continued elevated prolactin concentration result in an increase in the production of all of the components of milk.[22] The molecular mechanisms subserving these effects are discussed later in this chapter.

After the decrease in estradiol and progesterone, milk production requires 2 to 5 days to become established. In the postpartum interval, milk production can be suppressed by replacing estradiol and progesterone, or by suppressing prolactin secretion.[23] Milk production also requires the synergistic action of growth hormone, insulin, thyroxine, and cortisol.

During suckling, sensory signals originating in the nipple travel through thoracic nerves 4, 5, and 6 to the central nervous system. When the suckling-induced signals reach the paraventricular and supraoptic nuclei, they stimulate the release of oxytocin from the posterior pituitary. Noradrenergic, histaminergic, and excitatory amino acids play a role in oxytocin release.[24] Oxytocin release during suckling causes the contraction of the myoepithelial cells of the mammary acini and ducts, which induces the flow of milk. In addition to suckling, oxytocin can be released from the posterior pituitary by visual and auditory stimuli.

Prolactin maintains lactogenesis by stimulating the transcription of the casein and lactalbumin genes and other genes needed for the synthesis of free fatty acids and lactose. Suckling-induced signals also cause the hypothalamus to induce an acute release of prolactin, in part by suppressing dopamine secretion.[25] In lactating women, in the first 6 weeks postpartum, basal prolactin levels are elevated and each episode of breastfeeding initiates a pulsatile increase in prolactin which peaks at levels 8 times higher than basal concentration.[26,27] From 7 to 28 weeks postpartum, basal prolactin levels are in the normal range and the magnitude of the spike in prolactin levels is about sixfold above baseline. Use of a breast pump produced spikes in prolactin similar to those seen with suckling, but manual stimulation of the areola did not reliably produce a spike in prolactin. Having breastfeeding mothers play with their infants caused milk let-down, but did not produce a spike in prolactin levels. As the frequency and duration of suckling diminishes, the magnitude of the spike in prolactin level following suckling declines, eventually becoming extinguished.[26,27]

The Effect of Prolactin on β-Casein Gene Function

The cellular mechanisms that control milk protein production have been best elucidated for the β-casein and whey acidic protein genes. For the β-casein gene, gene function is regulated by prolactin, progesterone, estradiol, insulin, glucocorticoids, thyroxine, cell substratum proteins, and growth factors like EGF. In the murine mammary epithelial cell line HC11, expression of the β-casein milk protein gene is largely regulated at the transcriptional level.

Prolactin is the key endocrine regulator of β-casein gene transcription. The prolactin receptor is a class 1 receptor with extracellular cysteine motifs, a single transmembrane-spanning domain, and the absence of an intrinsic receptor-associated tyrosine kinase in the intracellular domain. Prolactin binding to its cognate receptor results in dimerization of the receptor and activation of the tyrosine Janus kinase 2 (JAK2) associated with the membrane-proximal, intracellular domain of the receptor (see Chapter 3, Prolactin in Human Reproduction).[28]

In turn, JAK2 phosphorylates and activates STAT5, a member of the signal transducers and activators of transcription (STAT) family. STAT5, previously known as mammary growth factor (MGF), increases transcription of the β-casein gene.[29] Various phosphatases, which are themselves regulated, de-phosphorylate STAT5 and reduce the transcription of β-casein gene transcription.[30]

The addition of prolactin to cultures of mammary epithelial cells results in casein gene transcription within 30 minutes. This effect requires the presence of both insulin and glucocorticoid. Deletion and point mutagenesis studies in mouse mammary epithelial HC11 cells transfected with the rat β-casein promoter have demonstrated that a region of approximately 340 bases immediately upstream of the transcription start site is a major site for gene regulation.[31,32] The 340 bases 5′ of the transcription start site contain the bases that confer gene inducibility by insulin, glucocorticoids, and prolactin.

Lactation and Amenorrhea

In mammals, suckling activates multiple systems that inhibit gonadotropin-releasing hormone (GnRH) secretion (see Chapters 1 and 3). The inhibitory systems activated during suckling include dopamine, serotonin, gamma-aminobutyric acid, and corticotropin-releasing factor. In turn, inhibition of GnRH secretion results in oligoovulation or anovulation. In rats, lactation reduces the number of kisspeptin containing neurons in the arcuate nucleus and median eminence, thereby suppressing GnRH secretion.[33,34] The degree to which breastfeeding suppresses GnRH secretion is modulated by the intensity of the breastfeeding, the nutritional status of the mother, and the body mass of the mother.[35-37] Lactation represents a metabolical energy burden. When nutrition is adequate and the basal body mass and composition is normal, intensive lactation is less likely to result in prolonged suppression of GnRH. When nutrition is

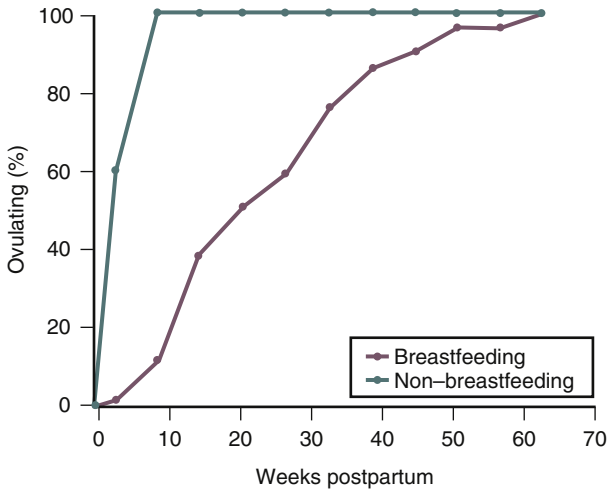


FIGURE 11.2 Cumulative proportion of ovulation by time postpartum for non–breastfeeding and breastfeeding women. *(From Campbell OMR, Gray RH. Characteristics and determinants of postpartum ovarian function in women in the United States.* Am J Obstet Gynecol *169:55, 1993.)*

inadequate to meet the energy demands of both daily living and lactation, oligoovulation or anovulation is more likely to persist for an extended time period.[38,39]

During exclusive breastfeeding, approximately 40% of women will remain amenorrheic at 6 months postpartum (Fig. 11.2).[40] In observational studies performed in the United Arab Emirates, the median duration of postpartum amenorrhea in breastfeeding women was 7 months.[41] Women who remain amenorrheic during breastfeeding may have higher prolactin levels than do women who become ovulatory while breastfeeding.[42] Among postpartum women who do not breastfeed, return of ovulation occurs between 45 and 94 days postpartum.[43] In some women who exclusively breastfeed, with at least six episodes of suckling per day, totaling more than 80 minutes in 24 hours, basal prolactin levels remain elevated up to 52 weeks postpartum and amenorrhea can persist for up to 1 year.[44]

The Effect of Steroid Contraceptives on Lactation

The available data indicate that (1) estrogen-progestin contraceptives reduce milk volume and change breast milk composition, (2) progestin-only contraceptives have no effects on milk volume and minimal effects on breast milk composition, and (3) long-term studies of the infants of breastfeeding mothers who used either estrogen-progestin contraceptives or progestin-only contraceptives show no detrimental effects of contraceptive steroid use on infant growth or neurodevelopment. Taken together, these data indicate that there is no proven effect of the use of contraceptive steroids by breastfeeding mothers on the development of infants who were breastfed. However, if a patient remains concerned about the possible impact of steroid contraceptives on breastfeeding and infant development a progestin-only contraceptive can be recommended. The progestin-only contraceptives include the progestin-only minipill, an etonogestrel releasing implant or depot medroxyprogesterone acetate.[45]

Most studies, but not all, report that estrogen-progestin contraceptives reduce breast milk volume more than progestin-only contraceptives. For example, in one study of more than 170 lactating women, the combination of 30 μg of ethinyl estradiol and 150 μg of levonorgestrel reduced daily milk volume by 31%. In contrast, a progestin-only minipill containing 75 μg of norgestrel increased daily milk volume by 10%. Similarly the use of depot medroxyprogesterone acetate increased milk volume by 10%.[46] Combination estrogen–progestin contraceptives also decrease the concentration of nitrogen, lactalbumin, lactoferrin, and lactose in breast milk. These changes in the composition of breast milk are modest and within the normal range of physiological variation observed in lactating women who are not taking contraceptive steroids.[47] In contrast to these findings, a recent, short-term randomized trial of the effects of a progestin-only minipill (norethindrone 0.35 mg daily) versus a cyclic estrogen-progestin contraceptive (35 μg ethinyl estradiol plus 1 mg of norethindrone in a 21/7 regimen) on breastfeeding reported no differences in the mother's perception of an adequate milk supply by contraceptive regimen.[48] In addition, consistent with other studies, the growth of the infants was similar with the two contraceptive methods.

The development of the newborn does not appear to be significantly affected by the changes in milk composition and volume associated with the use of combination estrogen–progestin oral contraceptives. In one study, the weight and neurological development of newborns of lactating mothers who took estrogen-progestin contraceptives were similar to those whose mothers did not.[46,49] A comparative study of the effects on lactation of a progestin-only contraceptive implant versus a copper intrauterine device (IUD) reported that infant growth was similar for both treatments.[50] Medicated and non-medicated IUDs do not appear to affect the growth of breastfed children.[51] The combination of lactation plus a progestin-only contraceptive is likely to be associated with a prolonged interval of amenorrhea compared to lactating women who use an IUD for contraception.[52]

Suppression of Lactation

Prolactin secretion is crucial to the maintenance of lactation. Suppression of prolactin secretion with dopamine agonists such as bromocriptine will suppress lactation.[53] The frequency of adverse effects associated with bromocriptine use in the puerperium is low.[54] However, there are a few case reports of severe hypertension and seizures that occurred in postpartum women who used bromocriptine.[55,56] Owing to these adverse outcomes, many authorities do not recommend bromocriptine as a first-line agent to suppress lactation. There may be a few clinical situations, such as mothers assigning their newborn for adoption, in which bromocriptine or cabergoline could be considered for the suppression of lactation. In most mothers, lactation suppression can be achieved with minimization of the mechanical stimulation of the breast and nipple, ice packs, and antiprostaglandin treatment. Milk stasis appears to decrease the production of STAT5, the main intracellular activator of milk protein production.[31,32]

Induction of Lactation

Induced lactation is defined as breastfeeding in the absence of a recent pregnancy. On occasion, adoptive mothers desire to breastfeed.[57] In countries where access to formula is limited, induced lactation in a surrogate may be important if the biological mother cannot continue to breastfeed.[58] The key hormone involved in lactation is prolactin. Medications that raise prolactin levels can be used to help induce lactation. For example, metoclopramide, 10 mg three times daily, will cause an increase in prolactin secretion.[59] When metoclopramide is coupled with nipple stimulation every few hours using an electronic breast pump, lactation can be established in the majority of women. In many cases the amount of milk produced may be insufficient to breastfeed the baby exclusively. Agents such as sulpiride (50 mg daily) and chlorpromazine can also be used to induce lactation, but these agents may be associated with drowsiness and extrapyramidal symptoms.[60,61]

Recombinant prolactin has been reported to effectively treat insufficient milk production in mothers with documented prolactin deficiency and mothers who cannot pump sufficient quantities of breast milk to feed their preterm children. At a prolactin dose of 60 μg/kg administered subcutaneously every 12 hours, prolactin levels and breast milk volume significantly increased in both groups of women.[62] The prolactin treatment also caused breast milk lactose and calcium concentrations to increase.[63]

Lactation and Hormone Responsive Reproductive Disorders

Women who are breastfeeding and are amenorrheic have circulating estradiol levels in the range of 20 to 40 pg/mL. In contrast, normally cycling women have a preovulatory estradiol concentration in the range of 250 to 300 pg/mL. Many gynecological diseases, such as endometriosis and uterine leiomyomas, are stimulated by high estradiol levels. The hypoestrogenic effect associated with amenorrhea and breastfeeding may have a beneficial effect on these estrogen-dependent disease processes and may account for the experimental and epidemiological observations that the risk of estrogen-dependent disease is reduced in women who have breastfed.[64,65] In one study of the relationship between lactation and the risk of developing laparoscopically proven endometriosis, a lifetime duration of lactation of greater than 23 months resulted in an 80% decrease in the risk of being diagnosed with endometriosis.[66] The protective effect of lactation was no longer apparent 5 years after the woman's last birth.

Lactation is associated with a reduced life-time risk of developing breast cancer (Fig. 11.3)[67,68] and ovarian cancer.[69]

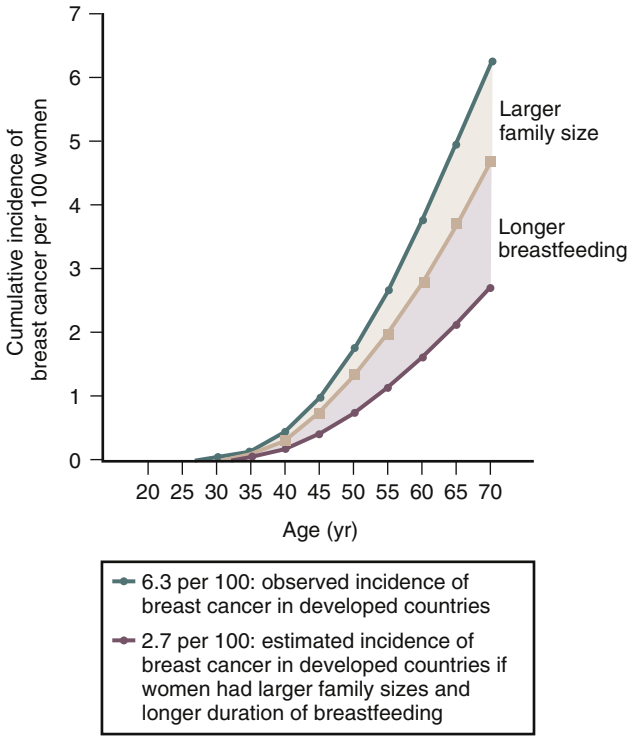


FIGURE 11.3 Estimated cumulative incidence of breast cancer in developed countries if women had family sizes and breastfeeding patterns typical for developing countries. Estimated incidence of breast cancer in developed countries if women had, on average, 6.5 births instead of 2.5 births, and if women breastfed each child, on average, for 24 months instead of a lifetime mean of 8.7 months. *(From Collaborative Group on Hormonal Risk Factors in Breast Cancer. Breast cancer and breastfeeding.* Lancet *360:187–195, 2002.)*

Lactation and the Risk of Developing Diabetes or Cardiovascular Disease

Lactation has been reported to reduce a women's risk of developing type 2 diabetes mellitus and the risk of cardiovascular disease. In the Nurses Health Study, a history of at least 1 year of lactation was associated with a 15% reduction in the risk of being diagnosed with diabetes.[70] In another prospective study of 522 women with an index pregnancy where gestational diabetes was diagnosed, women who breastfed their child, had lower fasting glucose, fasting insulin and 2-hour insulin after a glucose challenge than women who did not breastfeed their child.[71] In addition, with continued follow-up, the women who breastfed their child had a lower prevalence of diabetes and prediabetes. In another large prospective cohort, mothers who did not exclusively breastfeed their children had an increased risk of being diagnosed with diabetes compared with mothers who exclusively breastfed all their children (odds ratio [OR] 1.52, 95% confidence interval [CI] 1.11 to 2.10).[72] Breastfeeding, by increasing metabolical rate and energy expenditure, may help postpartum women achieve a lower body weight and waist circumference compared to non-breastfeeding mothers thereby improving metabolical function and reducing the risk of developing diabetes.[73]

Breastfeeding appears to reduce the risk of developing arteriosclerosis and having a cardiovascular event. In the Study of Women Across the Nation, women who reported breastfeeding all their children for at least 3 months had less carotid artery, coronary artery, and aortic calcifications than women who had never breastfed.[74] In the Women and Infants Healthy Hearts study, women who had breastfed for ≥3 months after every birth had less carotid artery narrowing as determined by high-resolution sonography than women who had never breastfed.[75] In the Nurses Health Study, women who breastfed for ≥2 years had a 37% lower risk of having a myocardial infarction than women

who had never breastfed (95% confidence interval 23% to 49%, p<0.001).[76] The mechanisms by which breastfeeding reduces the severity of atherosclerosis and coronary artery events is unknown, but in animal models, lactation reduces circulating triglyceride and increases high-density lipoprotein cholesterol.[77,78] In humans and animals, lactation reduces blood pressure and heart rate.[79]

Lactation and Bone Metabolism

During pregnancy, calcium is mobilized from bone, in part, to provide a stable source of calcium for fetal development. Pregnancy is associated with a 1% to 2% decrease in bone mineral density as determines by dual x-ray absorptiometry. Following birth, women who breastfeed lose additional bone mineral from the lumbar spine. Women who bottle feed their infants typically regain the bone they lost during pregnancy. After discontinuing breastfeeding, bone density returns to prepregnancy values.[80] Although breastfeeding may be associated with a reduction in bone density while breastfeeding, long-term follow-up studies report that a history of breastfeeding may slightly reduce the risk of hip fracture in postmenopausal women.[81,82]

Lactation and the Transmission of Human Immunodeficiency Virus

Human immunodeficiency virus (HIV) can be transmitted from mother to child by breast milk.[83,84] In developed countries where women have access to clean water and the rate of infant death from diarrheal-related illnesses is low, the consensus recommendation is for HIV-infected women to not breastfeed their children. However, in countries with a high rate of infant death from diarrheal-related illnesses due to lack of access to clean water, breastfeeding is preferred to formula feeding.[85]

If access to clean water *is* possible, formula feeding is probably better than breastfeeding. For example, in one study in Kenya, HIV-positive, postpartum women were randomized to use formula or to breastfeed their infants. The women assigned to the formula feeding group were educated about diarrheal illnesses and given access to clean water. In this study, the rate of HIV infection in the infants at follow-up 2 years later was 17% in the formula-fed group and 33% in the breastfed group. In this study, where the women had access to clean water, there was no increase in diarrheal-related illnesses in the formula-fed group.[86] Lactating HIV-positive women who are administered highly active antiretroviral drugs have a low rate of vertical transmission of HIV to their breastfeeding child.[87] The metabolical energy burden of HIV disease plus lactation is often associated with weight loss among these women.[88]

Galactorrhea

Galactorrhea is the secretion of breast milk at a time remote from nursing. In contrast to other breast secretions, the unique feature of galactorrhea is that the secretion contains milk. This can be demonstrated by drying the secretion on a glass slide and staining for the presence of fat. Galactorrhea is usually due to hyperprolactinemia or excessive sensitivity of the breast to normal circulating levels of prolactin. When galactorrhea is associated with normal ovulatory menses, the most likely cause of the galactorrhea is excessive sensitivity of the breast to normal circulating levels of prolactin. When galactorrhea is associated with amenorrhea, it is likely that the circulating prolactin level is elevated. Approximately 75% of women with both galactorrhea and amenorrhea have hyperprolactinemia. The causes of hyperprolactinemia are reviewed in detail in Chapter 3. Briefly, the most common causes of hyperprolactinemia are a prolactin-secreting pituitary tumor, the use of a dopamine antagonist (such as metoclopramide, phenothiazines, resperidone), the use of other neuroactive medications (selective serotonin reuptake inhibitors), pregnancy, renal disease, and primary hypothyroidism.

Galactorrhea of any cause can be suppressed by the use of dopamine agonist medications such as bromocriptine or cabergoline. In women with galactorrhea, normal ovulatory menses, and normal prolactin concentration, low doses of bromocriptine (1.25 to 2.5 mg) or cabergoline (0.25 mg once or twice weekly) are often effective in the suppression of galactorrhea. The presence of a unilateral nipple discharge, or the presence of blood in nipple discharge should be evaluated by referring the patient to a breast specialist.

Benign Breast Disease

Cyclical Mastalgia

Many women experience cyclic breast discomfort that begins during the late luteal phase and resolves within a few days of the onset of menses. Cyclical mastalgia is a more severe and persistent presentation of breast pain that interferes with daily activity. The hormonal basis for cyclical mastalgia is unclear, but estrogen and progesterone are likely involved. Diet interventions such as reducing caffeine intake may be effective in some women with cyclical mastalgia. Danazol, an attenuated androgen, and tamoxifen, an antiestrogen, are effective endocrine treatments for cyclical mastalgia. In women with cyclical breast pain, danazol, at doses of 100 to 400 mg daily is effective in reducing pain in about 80% of women.[89] Danazol is a teratogen, and doses less than 400 mg daily do not reliably suppress ovulation and women taking danazol should use a barrier contraceptive. Tamoxifen at a dose of 10 mg daily for 3 months has been reported to reduce breast pain in about 90% of women with cyclical mastalgia.[90] Bromocriptine, 2.5 mg twice daily, may also be moderately effective in reducing cyclical breast pain.[91,92]

Fibroadenoma of the Breast

Fibroadenomas are the most common benign tumor of the breast. They are characterized by a proliferation of both epithelial and mesenchymal cells. Stromal cells proliferate around tubular glands or compressed ducts. Women with a fibroadenoma present with a firm, usually non-tender, mobile breast mass. In 80% of cases, they present as a unifocal lesion. In 20% of cases, they present with two or more tumors in the same breast or tumors in both breasts. A core biopsy is required to make the diagnosis. The growth of fibroadenomas appears to be stimulated by estrogen and they typically regress in the postmenopause. The use

of estrogen-progestin contraceptives has been reported to reduce the risk of developing a fibroadenoma.[93]

The molecular mechanisms that cause fibroadenomas to develop are unknown. In some cases, fibroadenoma tissue appears to contain a somatic mutation in the prolactin receptor gene (I146L) that causes constitutive activation of the receptor.[94] In a study of 74 women with more than three fibroadenomas in one breast, 4 women were identified who had an A to C substitution in exon 6 of the prolactin receptor gene causing a substitution of Leu for Ile_{146} that was detected in the fibroadenoma tissue but not healthy tissue. The investigators reported that this allele resulted in constitutive activation of the prolactin receptor in three different cell models. This study suggests that prolactin receptor antagonists may be potential therapeutic agents to treat fibroadenomas.[95]

Breast Cancer

Risk factors for breast cancer include advancing age, genetic mutations, family history of breast cancer, breast density, personal reproductive history (early menarche, late menopause, parity, duration of lactation), and body mass.[96,97] The endocrine factors which modify the risk of breast cancer and the endocrine treatment of breast cancer are reviewed in Chapter 29, Hormone Responsive Cancers.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 12

The Endocrinology of Human Pregnancy and Fetal-Placental Neuroendocrine Development

Sam Mesiano

The birth of a healthy baby is dependent on an ordered sequence of biological events during pregnancy. Important among these are:

1. Successful implantation of the developing embryo
2. Adaptation of maternal physiology to accept the fetal allograft and satisfy its nutritional, metabolical, and physical demands
3. Appropriate growth and functional development of key organ and homeostatic control systems in the fetus in preparation for life outside the uterus
4. Proper timing of birth so that it occurs when the fetus is mature enough to survive outside the uterus

The hormonal interactions between the fetus, placenta, and mother that control these processes are discussed in this chapter.

The chapter begins with a brief review of the hormonal events that control endometrial growth and function during the menstrual cycle so that it becomes receptive to blastocyst implantation and the establishment of pregnancy. The processes of implantation and placental development and the mechanism by which the fetal allograft overcomes attack by the maternal immune system are also discussed.

Once pregnancy is established, the fetus, placenta, and mother initiate and maintain communication via the endocrine system. The endocrine milieu of human pregnancy is dominated by placental hormones, the major function of which is to modify maternal physiology to satisfy the nutritional, metabolical, and physical demands of the growing fetus. In this context, the physiological role of the principal placental hormones is discussed.

The fetal neuroendocrine system must become functionally competent by term so that the neonate can maintain homeostasis after birth. These systems are also critical for the fetus' future health. The fetal programming hypothesis posits that adaptation of key fetal neuroendocrine systems to the intrauterine environment affects their function during postnatal life and in some cases this adaptation can be the origins of adult diseases such as metabolical syndrome and obesity. Development of fetal neuroendocrine systems and the influence of the fetal-maternal environment is therefore discussed separately.

Finally, the endocrine interactions between the fetus, placenta, and mother that culminate in the induction of parturition are reviewed. The appropriate timing of birth, so that it occurs when the fetus is sufficiently mature to survive as a newborn, is a major determinant of postnatal survival in all viviparous species. Current understanding of the hormonal interactions that control the process and timing of human parturition is described.

Establishment of Pregnancy

The first step in the establishment of pregnancy is implantation of the blastocyst into the endometrium. The goal of the embryo is to establish a conduit through which it can access a supply of nutrients from the mother. The process of embryo apposition, adhesion, and invasion into the endometrium involves a complex bidirectional hormonal dialogue between the blastocyst and the endometrium such that embryo viability and endometrial receptivity are synchronized to allow for successful implantation and the establishment of pregnancy. As the embryo is semi-allogeneic with respect to the mother, its invasion into the endometrium represents a major breach of the maternal immune system. The hormonal dialogue between the embryo and the mother at the implantation site is critical in preventing attacked by the maternal immune system.

Endometrial Receptivity

During the menstrual cycle, the ovarian steroid hormones, estradiol and progesterone, induce structural and functional changes in the endometrium essential for implantation and the establishment of pregnancy (see Chapter 10). During the luteal phase and under the influence primarily of progesterone, the proliferative endometrium is converted to a secretory phenotype. It becomes thick (5 to 6 mm) and spongy and highly vascularized. Secretions from the glandular epithelium establish an intrauterine environment that favors embryo survival and the epithelial cells produce a variety of chemokines, growth factors, and cell adhesion

molecules (CAMs) that attract the blastocyst to specific docking sites for implantation. Increased vascularization and the development of spiral arterioles within the endometrial stroma provide an optimal substrate for invasion and placentation.

Receptivity of the endometrium to blastocyst adhesion and implantation has temporal and spatial characteristics.[1-3] In humans, implantation between days 21 and 24 of the menstrual cycle is associated with a high (~85%) success rate for continuing pregnancy, whereas implantation after day 25 has a low success rate (~11%).[4] The receptive phase of endometrial development is termed the implantation window which opens 5 to 6 days after ovulation and closes 3 to 4 days later. During the implantation window, the endometrial epithelium develops domelike structures known as pinopodes, which correlate with implantation sites.[5] The tips of the pinopodes express chemokines and CAMs that attract the embryo and appear to be the preferred site for embryo adherence. Interestingly, areas between the pinopodes produce a repellent molecule, MUC-1, that prevents embryo adhesion.[6] Analysis of the human endometrial transcriptome during the implantation window has revealed numerous genes that are up- and down-regulated in comparison with late proliferative phase endometrium,[7,8] demonstrating the remarkable complexity of molecular interactions underlying endometrial differentiation.

Implantation

After fertilization, the embryo undergoes an intrinsic developmental program. The sequence of cell divisions and differentiation is not dependent on the hormonal milieu of the fallopian tube or the uterus, as fertilization and early embryonic development occur successfully *in vitro*. By the third to fourth day after fertilization, the embryo comprises a solid ball of cells encapsulated by the translucent remnant of the zona pellucida. At about the fifth day after fertilization, a fluid filled cavity, the blastocele, forms within the embryo. At this stage the embryo is referred to as a blastocyst. The outer layer of blastocyst cells adjacent to the zona pellucida is known as the trophectoderm. These cells eventually give rise to the placenta and chorion. The fetus, amnion and mesenchymal, and vascular components of the placenta are derived from the inner cell mass, a group of cells lying under the trophectoderm at one end of the blastocele.

Although there is considerable variation in the process of implantation between eutherian mammals, the end result is the same: the blastocyst becomes fixed in position and forms a physical and functional contact with the uterus.

In humans, the blastocyst enters the uterus at around the fifth day after fertilization and floats freely for 2 to 3 days. By this stage, the blastocyst escapes the confines of the zona pellucida (i.e., it hatches) and is in an optimal condition to implant. The developing blastocyst interacts in a paracrine manner with the endometrial epithelium by producing multiple cytokines, chemokines, and CAMs that facilitate adherence.

Almost immediately after fertilization, hormonal signals from the conceptus are transmitted to the mother. During its journey through the fallopian tube the cumulus cells surrounding the embryo produces progesterone and estrogen. These steroids are thought to act locally to modulate tubal motility to facilitate transport of the conceptus toward the uterine cavity.[9] Studies of the secreted proteins (the secretome) produced by the preimplantation embryo show that the profile of secreted proteins changes every 24 hours and with the different embryonic stages.[10-12] Thus, a specifically orchestrated sequence of secretions from the embryo plays a key role in the paracrine interaction between the embryo and epithelial cells of the endometrium. Any delay in embryo development or in its transit through the oviduct disrupts the synchrony between blastocyst development and endometrial receptivity and increases the likelihood of reproductive failure. Thus, development of the "seed" and conditioning of the "soil" are synchronized. Given this delicate balance, it is not surprising that the highest rate of embryo loss occurs during the peri-implantation period.

The embryo implants in a polarized fashion. After hatching from the zona pellucida, trophoblast cells overlying the embryonic pole are the first to interact with the uterine epithelium (Fig. 12.1). Those cells dock with specific sites in the epithelial lining of the endometrium, most likely at the pinopodes.[13-16] The trophoblast cells proliferate and secrete proteases that degrade the extracellular matrix between endometrial cells forming a path for the blastocyst to enter the uterine stroma. At this stage the trophoblast cells are referred to as cytotrophoblasts and differentiate along two distinct paths: villous and extravillious. The extravillious cytotrophoblasts become highly invasive and form columns penetrating to the basal membrane beneath the decidual cells and into the myometrium. Eventually the entire embryo is embedded in the uterine stroma and anchored by villous cytotrophoblasts. During this time some villous cytotrophoblasts fuse their plasma membranes to become a single multinucleated cell known as the syncytiotrophoblast, which becomes the outer layer of the functional floating villi in direct contact with maternal blood.

As with adhesion, implantation also involves a complex paracrine dialogue between the embryo and the endometrium-decidua. Many of the molecules (produced by the endometrial cells or the blastocyst) involved in the implantation process have been identified.[3,12,17-19] These include:

- Leukemia inhibiting factor
- Interleukin–11
- Heparin-binding epidermal growth factor-like growth factor
- Prostacyclin
- Prostaglandin E_2
- Peroxisome-proliferator-activator receptor
- HOXA10 and HOXA11
- Metalloproteinase-2 and -9
- Basic and acidic fibroblast growth factor
- Bone morphogenetic proteins (BMPs) and the BMP antagonist Noggin
- Wnt and Indian Hedgehog signaling molecules

Decidualization of the Endometrium

Late in the luteal phase the endometrial stroma undergoes a series of morphological and functional changes collectively referred to as decidualization.[20,21] The process of decidualization is dependent on progesterone and occurs independently of conception and implantation. It begins around blood vessels during the midsecretory phase and

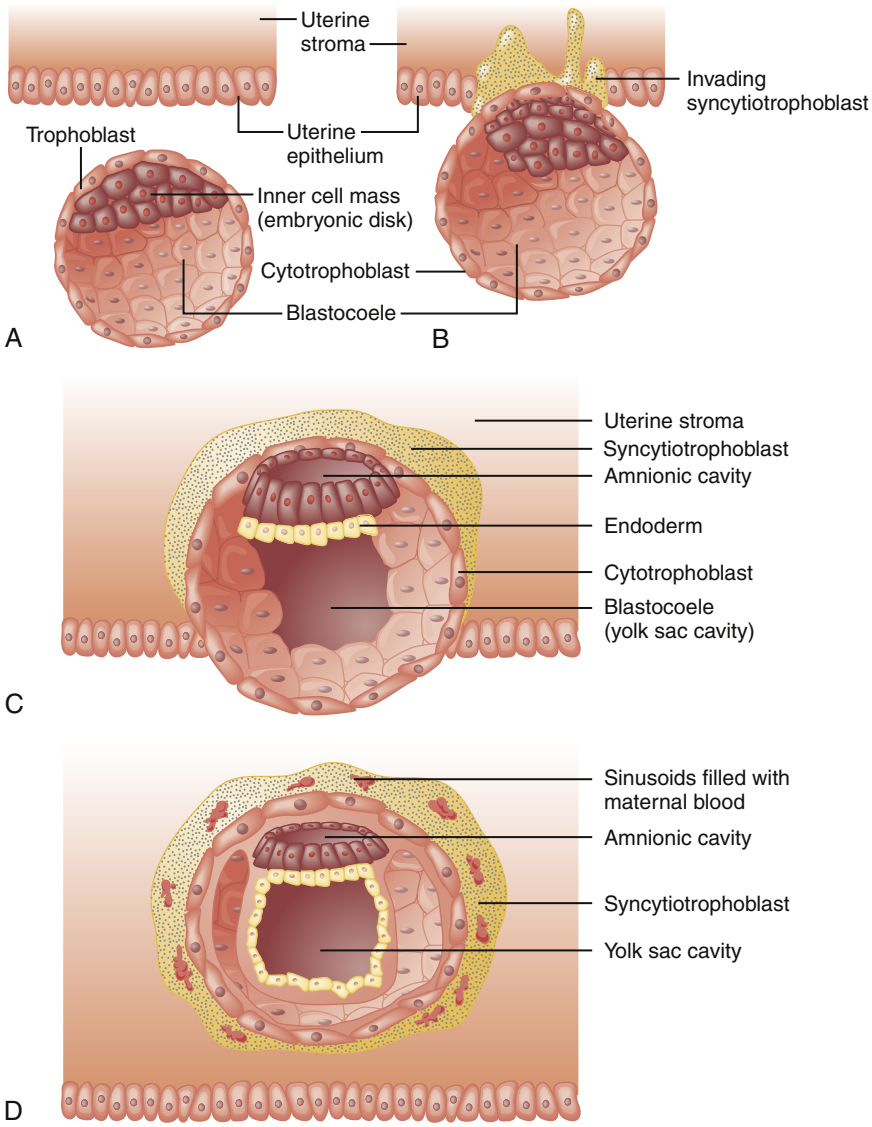


FIGURE 12.1 Implantation of the human embryo. **A,** Floating blastocyst. **B,** Attachment to the uterine epithelium and initial invasion of the syncytiotrophoblast cells. **C,** The blastocyst penetrates deeper into the uterine stroma and develops an amniotic cavity. **D,** The fully implanted embryo invades the maternal vasculature and the uterine epithelium grows over the implantation site and undergoes the decidualization. *(From Jones RE. 1997 Human Reproductive Biology by Academic Press,* San Diego, *p 189.)*

progressively encompasses the entire endometrial stroma during the latter part of the secretory phase. The fully decidualized endometrial stroma is composed of large polyhedral cells, containing high levels of glycogen and lipids that secrete a tough pericellular capsule composed of collagen, laminin, fibronectin, and heparan sulfate proteoglycan. Bone marrow-derived immune cells, primarily large granular lymphocytes (LGLs), also accumulate and extensive cell to cell contacts between the lymphocytes and the stromal cells form, especially in the vicinity of blood vessels.

The decidualized endometrium is hostile to implantation. Therefore, the blastocyst has a limited window of opportunity to establish a successful implantation before the decidual barrier is established. Attachment of the blastocyst to the uterine epithelium accelerates the decidualization process further limiting the window of opportunity for implantation. In an infertile cycle, menstruation occurs to eliminate the nonreceptive decidualized endometrium. The endometrium is renewed to a receptive state in the subsequent cycle. Thus, the term decidua is highly appropriate because this tissue is cast off at menstruation, miscarriage, and delivery.

The decidua is thought to form a critical barrier between the mother and the conceptus and represent an attempt by the mother to restrict blastocyst invasion. The large component of immune cells (mainly LGLs) within the decidual tissue may also restrict the passage of fetal antigens to the maternal compartment.[22,23] Interestingly, decidualization of the endometrium appears limited to species with interstitial implantation in which the blastocyst is completely imbedded in the endometrial stroma, further supporting the argument that it limits the invasiveness of the blastocyst during implantation.

Once pregnancy is established the decidua can be divided into three types, depending upon anatomical location: (1) The decidua basalis, which underlies the site of implantation and forms the maternal component of the placenta; (2) The decidua capsularis, which overlies the gestational sac (this portion disappears in the later stages of pregnancy); (3) The decidua vera, which lines the remainder of the uterine cavity and becomes intimately approximated to the chorion.

The decidua of pregnancy, in association with the fetal membranes, is considered to be an endocrine organ. Hormones produced by the decidua can act on the adjacent

tissue (chorion and myometrium) or communicate with the fetus via the amniotic fluid. The decidua produces prolactin, relaxin and prostaglandins that are thought to be involved in the process of parturition (discussed later in the chapter). Its close proximity to the myometrium is ideal in this regard.

Placentation

Early in the implantation process some cytotrophoblast cells aggregate and form migrating columns that penetrate into the inner third of the myometrium. The invading cytotrophoblasts target maternal blood vessels and, via interstitial and endovascular routes, completely surround and plug spiral arterioles. They then displace maternal endothelial cells and musculoelastic tissue and effectively create a low-resistance arteriolar system by increasing vessel diameter. Blood flowing from those vessels fills spaces known as lacunae, between the invading columns of cytotrophoblast cells, and leave through venous connections. Eventually, the syncytiotrophoblasts come into contact with maternal blood and form chorionic villi that are bathed in maternal blood within the lacunae.

According to Poiseuille's law of fluid dynamics, flow through a cylinder is proportional to the radius multiplied to the fourth power. On average the radii of arterioles in the pregnant human uterus are around 10-fold greater than in the nonpregnancy state. Such a change would increase flow 10,000-fold, which is ample to serve the future requirements of the growing fetus. Cytotrophoblasts within the spiral arterioles not only facilitate this large diversion of maternal blood flow to the conceptus, but also prevent maternal vasomotor control. Through this process the supply of resources to the conceptus is optimized and the capacity for maternal restriction of uterine blood flow is subverted.

Placentation across all eutherian mammals is characterized by high angiogenic activity and blood vessel growth. This is particularly the case for the site of placental attachment. The human placenta produces several known angiogenic factors, including platelet-derived endothelial cell growth factor, vascular endothelial growth factor (VEGF), angiopoietin-1 and angiopoietin-2.[24,25] In addition, two potent inhibitors of angiogenesis have been isolated from mouse placenta.[26] The specific interplay between angiogenic factors and their inhibitors in the placenta is not well understood. However, it is possible that the presence of anti-angiogenic factors within the placenta may serve to control vessel growth. This may be important for preventing maternal endothelial cells from resealing the ends of spiral arterioles that have been occupied by trophoblasts. In addition, anti-angiogenic factors may prevent the overgrowth of maternal and fetal vessels, thereby preventing maternal blood vessels from entering the fetal compartment and fetal vessels from growing out into the uterus.

The extent and depth of the placental incursion appears to be the sum of the intrinsic pro-invasive characteristics of trophoblasts and the physical and biochemical barriers mounted by the maternal tissues. Imbalances in this equation can lead to failed implantation and pathological conditions. The angiogenic events occurring during implantation and placentation likely are critical factors in the etiology of hemodynamic disorders in pregnancy. For example, in preeclampsia, cytotrophoblast invasion is restricted to the superficial decidual segments, leaving the myometrial spiral arterioles undisturbed and still responsive to maternal vasomotor influences.[27] These pathological changes result in shallow implantation and impaired blood supply to the placental unit, which then attempts to compensate by increasing maternal systemic blood pressure, leading to hypertensive disorders such as preeclampsia (Fig. 12.2).

Because cytotrophoblasts are genetically distinct from maternal tissue, implantation represents an extraordinary breach of maternal immune defenses. The mother must distinguish between the fetal allograft as being immunogenically foreign or self and/or the placental trophoblasts must protect themselves from immune destruction by activated maternal immune cells. The decidua is composed mainly of a uterus-specific subset of LGLs with natural killer (NK) cell phenotype.[23,28-30] NK cells preferentially attack targets lacking polymorphic Class I or Class II human leukocyte antigens (HLAs). Trophoblasts do not express polymorphic Class I or Class II HLAs and therefore are highly susceptible to attack by decidual NK cells.[31] However, differentiated cytotrophoblasts that lie at the maternal-fetal interface produces a unique Class I molecule known as HLA-G.[32-34] Because of its reduced polymorphism, HLA-G is less immunogenic and could prevent immunological recognition by NK cells. In addition, trophoblasts express the Fas ligand, which induces apoptosis in immune cells that carry the Fas receptor.[35] Thus, the fetal allograft may escape immune attack by presenting the unique HLA-G camouflage or by actively subverting immune recognition via the Fas ligand.

The functional unit of the human placenta is the chorionic villus, which is formed by a sprouting of syncytiotrophoblasts that project into a pool of maternal blood surrounding the blastocyst. The fingerlike chorionic villi have a central core of loose connective tissue with an extensive capillary network linking it with the fetal circulation. Surrounding the core are the outer syncytiotrophoblast and inner cytotrophoblast cells that form the functional barrier between the maternal and fetal circulations. This arrangement is referred to as hemochorial placentation since the syncytiotrophoblast layer separates the maternal and fetal circulations.

The Endocrine Placenta

Placental hormones dominate the endocrine milieu of human pregnancy.[36] This remarkable organ produces a plethora of protein (including cytokines and growth factors) and steroid hormones, which it secretes in large quantities primarily into the maternal circulation (Table 12.1). The hemochorial anatomical arrangement of the human placenta is ideal for this purpose. Cytotrophoblast and syncytiotrophoblast cells of the placenta have direct access to the maternal circulation. In contrast, the syncytiotrophoblast layer prevents most maternal hormones from entering the fetal compartment. Consequently, the fetal-placental endocrine system generally develops and functions independently of that of the mother.

Most placental hormones are counterparts to those produced in the nonpregnant adult and therefore bind to maternal hormone receptors. In this context, placental hormones may be regarded as allocrine factors, i.e., hormones produced by one organism—the fetus—to act on the receptors

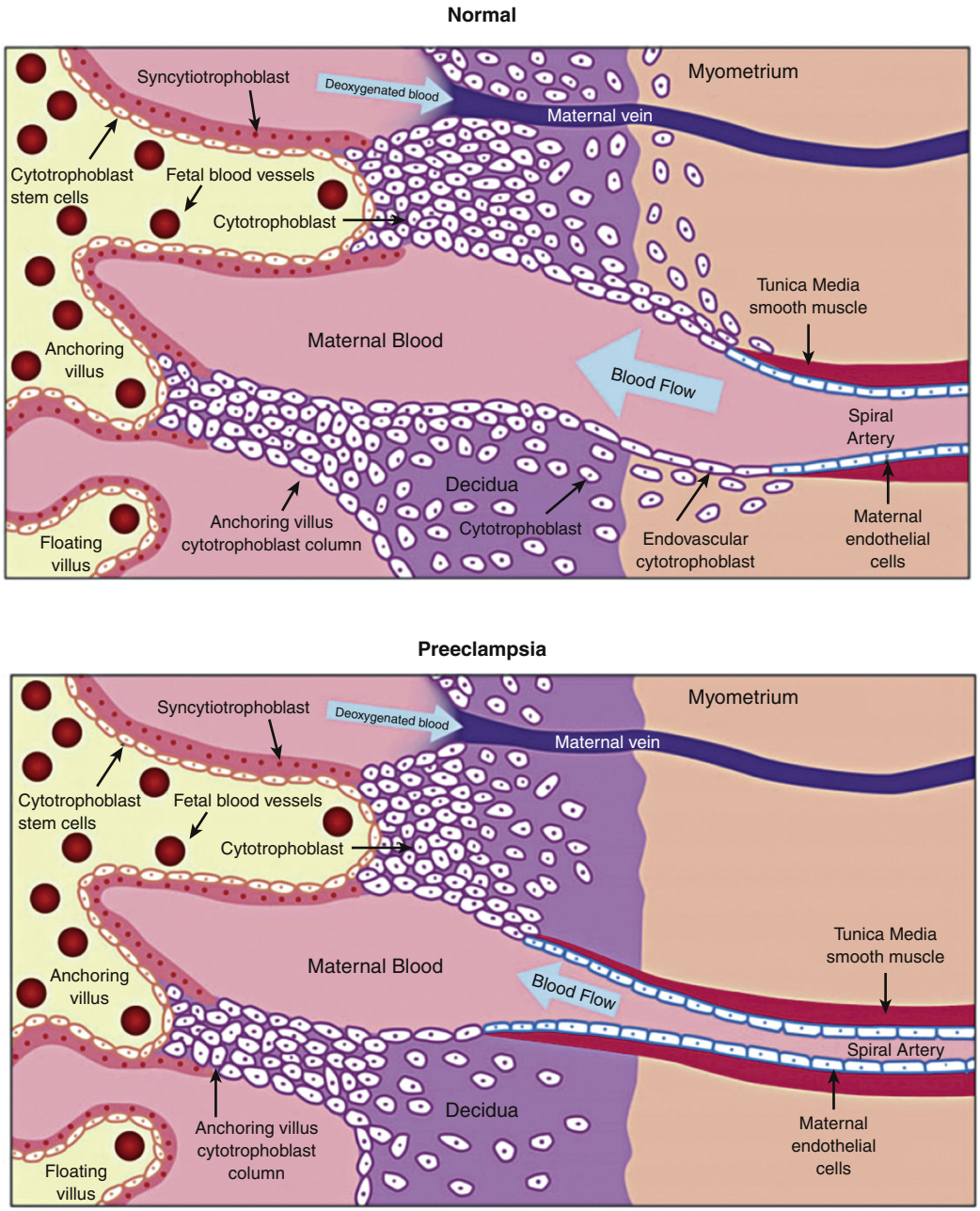


FIGURE 12.2 Invasion of cytotrophoblast cells into the spiral arteries. Shallow invasion restricts blood flow to the placenta and is thought to be a precursor for the development of preeclampsia. *(From Circulating angiogenic factors in the pathogenesis and prediction of preeclampsia. Chun Lam, Kee-Hak Lim, S. Ananth Karumanchi* Hypertension. *2005;46: 1077–1085)*

of another—the mother. Importantly, the placenta produces and responds to functional cohorts of hypothalamic and pituitary hormones and functions as a secondary neuroendocrine control center.[37] This extra brain in many instances overrides the maternal system to adjust maternal physiology usually in favor of supporting the needs of the fetus. The role of some of the principal placental hormones in the endocrine control of human pregnancy is discussed below.

Placental Gonadotropin

Gonadotropins are glycoprotein hormones produced by the anterior pituitary and the placenta that primarily affect growth and function of the gonads. Chorionic gonadotropin (CG, designated hCG in humans) is the primary embryonic endocrine signal for the establishment of pregnancy. hCG produced by the trophoblastic cells of the early embryo acts on the corpus luteum (CL) to prevent luteolysis and maintain steroidogenic function. This causes progesterone levels to be sustained which prevents sloughing of the endometrial lining. In nonconceptive cycles, the CL usually regresses at about the second week after ovulation and the subsequent decline in progesterone leads to menstruation. For pregnancy to establish, demise of the CL and the associated withdrawal of progesterone must be prevented. Thus, one of the first endocrine interactions between the conceptus

Table 12.1 Peptides, Steroid Hormones, and Monoamines Produced by the Human Placenta

Neuropeptides	Pituitary-like Hormones	Steroid Hormones	Monoamines and Adrenal-like Peptides
CRH	ACTH	Progesterone	Epinephrine
TRH	TSH	Estradiol	Norepinepherine
GnRH	GH	Estrone	Dopamine
Melatonin	PL	Estriol	Serotonin
Cholecystokinin	CG	Estetrol	Adrenomedullin
Met-enkephalin	LH	2-Methoxyestradiol	
Dynorphin	FSH	Allopregnanolone	
Neurotensin	B-Endorphin	Pregnenolone	
VIP	Prolactin	5α-Dihydroprogesterone	
Galanin	Oxytocin		
Somatostatin	Leptin		
CGRP	Activin		
Neuropeptide Y	Follistatin		
Substance P	Inhibin		
Endothelin			
ANP			
Renin			
Angiotensin			
Urocortin			

VIP, vasoactive intestinal peptide; CGRP, calcitonin gene-related peptide; ANP, atrial natriuretic peptide.
(From: Reis FM, Petraglia F. The placenta as a neuroendocrine organ. *Front Horm Res* 27:216, 2001.)

and the mother involves signaling by the early embryo that pregnancy is occurring and that the functional life span of the CL must be extended. This event is referred to as the maternal recognition of pregnancy and is mediated by hCG.

As with other glycoprotein hormones such as luteinizing hormone (LH) and follicle stimulating hormone (FSH), hCG is a heterodimer composed of α and β subunits. The α-subunits of hCG, LH, and FSH are identical, whereas the β-subunits differ, conferring specificity to each hormone. hCG is biologically and immunologically similar to pituitary LH. The α subunit of hCG is produced by cytotrophoblast cells especially as they differentiate during implantation. The β subunit of hCG and is primarily produced by syncytiotrophoblast cells but can also be detected in mature cytotrophoblast cells just before they fuse to form the syncytiotrophoblast. The syncytiotrophoblast produces both subunits and is the principal source of functional hCG.

Multiple variants of hCG have been identified in the human placenta.[38] In addition to native hCG, the human placenta produced a hyperglycosylated hCG and the free monomeric β-subunit of the hyperglycosylated hCG. hCG variants are produced by different trophoblast cells. The syncytiotrophoblast produces native hCG, extravillious invasive cytotrophoblasts produce hyperglycosylated hCG, and the free hyperglycosylated β subunit is produced by non-trophoblastic advanced malignancies.[39]

The three hCG variants have distinct physiological functions. Like LH, native hCG is a potent luteotropin, and as such, it stimulates progesterone secretion by the CL. Importantly, hCG prolongs the functional life of the CL and converts the CL of the menstrual cycle into the CL of pregnancy, insuring the production of progesterone necessary for the establishment of pregnancy. In an evolutionary context, this may provide a basis for selection as pregnancy will not ensue if the embryo is defective and cannot gain control of the CL. The capacity of the embryo to produce large amounts of hCG may represent a selective test of the embryo's endocrine competence. The maternal level of resistance to the embryo's efforts to control the CL would select embryos with more robust endocrine function.[40] The hyperglycosylated hCG and the monomeric β-subunit are thought to exert primarily extragonadal actions and are associated with malignancies.[41,42] The actions of all hCG variants are mediated through interaction with the LH receptor.

During the first 5 to 7 weeks of pregnancy progesterone is produced exclusively by the CL in response to hCG. Consequently, the ovaries are obligate organs for pregnancy maintenance during this time, and abortion rapidly ensues if they are removed. However, after weeks 6 to 7 of pregnancy the placenta begins producing large amounts of progesterone and at around the same time progesterone production by the CL decreases. This transition in the source of progesterone is referred to as the luteal-placental shift. The placenta supplies the bulk of progesterone for the remainder of pregnancy.

In normal pregnancies, hCG is detectable 9 to 11 days after the midcycle LH peak, which is around 8 days after ovulation and only 1 day after implantation.[43] Therefore, pregnancy can be detected before the first missed menstrual period. This has clinical utility when it is important to determine the presence of pregnancy at an early stage. In early pregnancy, there is an approximate doubling of circulating hCG levels every 2 to 3 days and concentrations of hCG rise to peak values by 60 to 90 days of gestation. Thereafter, hCG levels decrease to a plateau that is maintained during the remainder of the pregnancy (Fig. 12.3). Assayable LH and FSH levels in the maternal blood are virtually undetectable throughout pregnancy.

Extragonadal Actions of hCG

The actions of hCG may not be limited to maintaining progesterone production by the CL.[42] Much of the increased thyroid activity that occurs in pregnancy has been attributed to hCG,[44] which binds specifically to thyroid cell membranes and displaces thyroid-stimulating hormone (TSH).[45] hCG also influences the development and function of

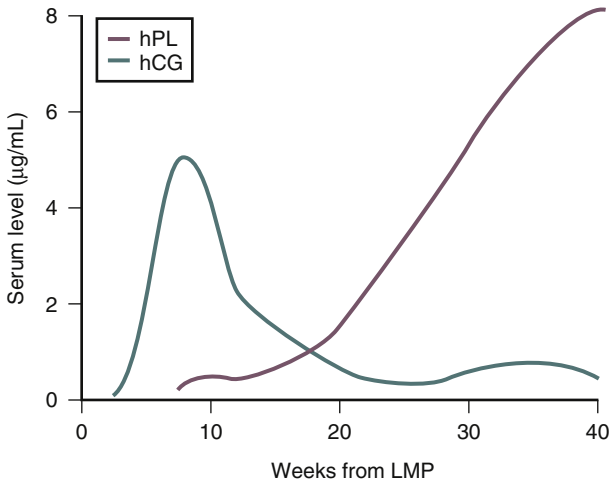


FIGURE 12.3 Schematic representation of concentrations of human chorionic gonadotropin (hCG) and placental lactogen (hPL) throughout gestation. Note differences in the magnitude of the concentrations of the two hormones in early and late gestation. LMP, last menstrual period.

the fetal adrenals and testes.[46] In addition, hCG may have actions on the maternal reproductive tract, including the decidual response,[47] relaxin production by the CL,[48] and relaxation of uterine smooth muscle.[49] hCG receptors have been detected in fetal membranes[50] and *in vitro* hCG inhibits spontaneous contractility in strips of human myometrium.[51]

Production of hCG by the invading blastocyst may contribute in a paracrine manner to the implantation process. *In vitro* and *in vivo* studies in nonhuman primates demonstrate that hCG can promote decidualization of the endometrial stroma.[52] The hormone appears to have marked effects on endometrial physiology at the implantation site before its levels are detectable in the circulation. Studies suggest that hCG produced by the blastocyst prolongs the window of implantation by inhibiting endometrial insulin-like growth factor binding protein -1 (IGFBP-1) production, augmenting angiogenesis at the implantation site by increasing VEGF expression, modulating local cytokine and chemokine expression, augmenting local protease activity and, via an autocrine effects on the trophoblasts themselves, promoting differentiation and augmenting invasive potential.[42,47]

The placenta may not be the only source of hCG. The fetal kidney and fetal pituitary gland synthesize and secrete biologically active hCG.[53] The function of extraplacental hCG in human pregnancy is unclear.

Placental trophoblasts may have an autoregulatory mechanism for hCG synthesis that involves intrinsic hormonal axes analogous to those operating in the hypothalamic-pituitary-gonadal (HPG) axis. Regulators of pituitary gonadotropin can also influence placental hCG production (at least *in vitro*). These regulators include progesterone, inhibin, activin, and gonadotropin-releasing hormone (GnRH).

Gonadotropin-Releasing Hormone

The human placenta produces GnRH, which is identical to that produced by the hypothalamus.[54] Levels of GnRH in the circulation of pregnant women are highest in the first trimester and correlate closely with hCG levels.[55] The close relation between GnRH and hCG suggests a role for GnRH in regulating hCG production.[56] GnRH stimulates the production of both the α-subunit and β-subunit of hCG in placental explants and specific GnRH-binding sites are present in the human placenta.[57] Thus, there appears to be autoregulation of hCG production within the placenta suggesting that placental GnRH is essential for the establishment and maintenance of early pregnancy.

hCG also may influence placental steroidogenesis, suggesting a complete internal regulatory system within the placenta. This concept is further strengthened by the presence of other regulators of GnRH expression including inhibins and activins, in the human placenta.

Inhibins and Activins

Inhibin is a heterodimer composed of an α-subunit and one of two β-subunits, βA or βB. Inhibins (αβA and αβB) derive their name from their ability to preferentially inhibit pituitary FSH secretion. In contrast to inhibins, the homodimers βA βA and βB βB stimulate FSH production. These compounds have been termed activins.

Inhibins are produced by the human placenta—all three subunits are expressed in the syncytiotrophoblast and the levels of expression do not change with advancing gestation.[58,59] Activin-A is also produced by the CL, decidua, and fetal membranes during human pregnancy.[60] The placenta also produces follistatin, the binding protein for activin.[61] These factors are secreted into the maternal and fetal circulations and amniotic fluid and their production varies with stage of gestation.[62]

Although the exact function of the inhibin-activin system in human pregnancy is not known, several studies indicate their involvement in the pathogenesis of gestational diseases. Levels of inhibin-A and activin-A in the maternal circulation can be indicative, albeit with relatively weak predictive value, of pathologies such as placental tumors, hypertensive disorders of pregnancy, intrauterine growth restriction, fetal hypoxia, Down syndrome, fetal demise, preterm delivery, and intrauterine growth restriction.[63]

Because inhibin and activin are synthesized in the cytotrophoblast, they may be involved in autoregulating placental hCG production by modulating local GnRH activity. *In vitro* studies have shown that inhibin decreases GnRH-stimulated hCG production by placental cell cultures whereas, inhibin antiserum increases GnRH release and causes a parallel rise in hCG secretion.[64] Further, this effect of blocking endogenous inhibin action is reduced by the addition of a GnRH antagonist, pointing to an interaction of endogenous inhibin and GnRH on hCG secretion.[65] Therefore, it is possible that inhibin exerts an autocrine or paracrine effect on hCG secretion by suppressing GnRH action. In contrast, activin augments the GnRH-induced release of hCG in cultured trophoblast cells, an effect that can be reduced by the addition of inhibin.[64] Thus, at least *in vitro*, activin and inhibin via their paracrine effect on placental GnRH production, contribute to the regulation of hCG secretion.

Placental Somatotropins

In most eutherian mammals, the placenta expresses and secretes members of the growth hormone (GH)-placental

lactogen (PL) gene family.[66] These genes are encoded by a 66 kb segment of chromosome 17q22-q24. This locus includes five closely related genes: *GH1*, *GH2*, *CSH1*, *CSH2*, and *CSHL1*, each derived from the duplication of a common ancestral gene. The *GH1* gene encodes pituitary GH and is expressed only in the pituitary. The other four are expressed exclusively in the placenta. The *GH2* gene encodes a placental GH variant, which differs from human pituitary GH by 13 amino acids. The *CSH1* and *CSH2* genes are identical and encode human placental lactogen (hPL). The *CSHL1* gene has a high degree of homology with *CSH1* and *CSH2*, however, it is considered a pseudogene due to a G to A transversion at the second intron splice site.

Human Placenta Lactogen

hPL is a single-chain polypeptide of 191 amino acids with two disulfide bridges and has a 96% homology with hGH. hPL can be detected in the placenta from around day 18 of pregnancy and in the maternal circulation by the third week of pregnancy. Low levels of hPL (7 to 10 ng/mL) are present in the maternal circulation by 20 to 40 days of gestation. Thereafter, hPL levels in the maternal circulation increase exponentially, reaching levels of 5 to 10 µg/mL at term.[67]

In contrast to the elevated levels of hPL in the maternal circulation, concentrations of hPL in the fetal circulation range from 4 to 500 ng/mL at midgestation and only 20 to 30 ng/mL at term.[68] This indicates that hPL is preferentially secreted into the maternal compartment. The concentration of hPL in maternal peripheral blood is about 300 times that in umbilical vein blood, and the concentration of hPL in the blood leaving the gravid uterus is markedly greater than that in the peripheral circulation.

hPL is detectable in the serum and urine in both normal and molar pregnancies, and it disappears rapidly from the serum and urine after delivery of the placenta—it cannot be detected after the first postpartum day. After removal of the placenta, the half-life of the disappearance of circulating hPL is 9 to 15 minutes. To maintain circulating concentrations, this would imply placental production of between 1 and 4 g of the hormone per day at term.[69] Thus, production of hPL represents one of the major metabolic and biosynthetic activities of the syncytiotrophoblast.

In normal pregnancy, hPL is first synthesized by the cytotrophoblasts of the developing placenta during the first 6 weeks of pregnancy. Thereafter, expression switches to the syncytiotrophoblasts, which eventually become the exclusive source of hPL. The extent of hPL expression by the syncytiotrophoblast does not change during the course of pregnancy, although total placental production increases substantially.[70] Therefore, the rise in hPL production is thought to be due to the increase in placental mass, as maternal hPL levels rises concordantly with the amount of syncytiotrophoblast tissue as gestation progresses (Fig. 12.4). Like hCG, hPL appears to be expressed by all types of trophoblastic tissue; it has also been found in the urine of patients harboring trophoblastic tumors and in men with choriocarcinoma of the testis.

Factors that regulate hPL production have been assessed in cultured cytotrophoblast cells. Insulin and growth hormone-releasing factor (GRF) stimulate hPL secretion, whereas somatostatin (SS) inhibits its secretion. The

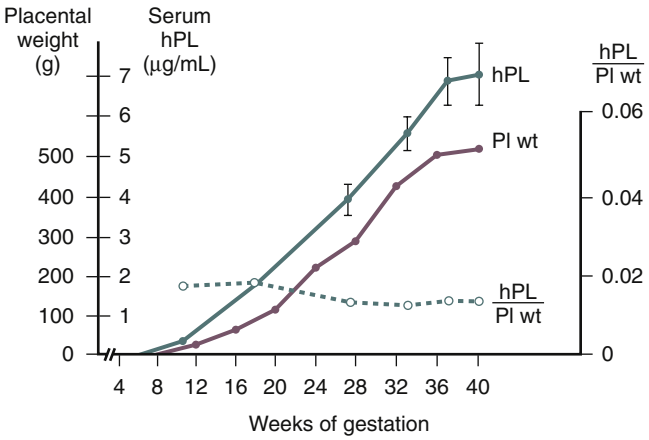


FIGURE 12.4 Levels of hPL in the maternal circulation in relation to placental weight (Pl wt). *(From Selenkow HA, Saxena BM, Dana CL, Emerson K Jr. Measurement and pathologic significance of human placental lactogen. In: Pecile A, Fenzi C [eds]. The foeto-placental Unit. Amsterdam Ecerpta Medica, 1969, pp 340–362. Elesvier Science Publishers).*

presence of hPL, hPGH, SS, and GRF in the same cell suggests that another autoregulatory loop analogous to the hypothalamic-pituitary axis, may operate within the placenta. In the third trimester, maternal hPL and GRF levels are closely correlated.[71] Interestingly, SS expression is maximal in early pregnancy and decreases during the second and third trimesters, a pattern opposite to that of hPL secretory activity.[72] Thus, locally produced GRF and SS may regulate placental hPL expression.

Several studies have demonstrated changes in maternal hPL levels in response to metabolic stress. Specifically, prolonged fasting at midgestation[73] and insulin-induced hypoglycemia raise maternal hPL concentrations.[74] However, hPL levels do not change in association with normal metabolic fluctuations during a typical 24-hour period.[75] Although extreme metabolic stress influences hPL production, hPL expression does not appear to be modulated by metabolic status within the normal range.

Human Placental Growth Hormone

Two forms of hPGH have been identified both of which are expressed in syncytiotrophoblast cells.[76] The smaller, 22 kDa form is almost identical to pituitary GH, differing by only 13 amino acids. The larger 26kDa hPGH is a splice variant that retains intron 4. The extent of hPGH production is significantly less than that of hPL, and hPGH is not secreted into the fetal compartment.[77] Consequently, circulating levels of hPGH are approximately 1000-fold less than hPL and can be detected in the maternal circulation later in gestation (between 21 and 26 weeks). During the third trimester, maternal hPGH levels increase exponentially, in concert with hPL, and reach a maximum of approximately 20 ng/mL by term.

During the course of human pregnancy, hPGH becomes the dominant GH, and maternal pituitary GH production gradually declines. In the first trimester, pituitary GH is measurable and secreted in a highly pulsatile manner.[78] However, pituitary GH production decreases progressively from about week 15, and by 30 weeks cannot be detected. During the same period, nonpulsatile secretion of hPGH by

the placenta increases markedly. Thus, maternal pituitary GH production declines during the second trimester and is not detectable during the third. By this stage, hPGH serves as the GH of pregnancy.

Biological Actions of hPL and hPGH

Several cases of hPL and hPGH deficiency have revealed their potential roles in human pregnancy.[79] In all cases of complete hPL deficiency (lack of detectable hPL in the mother's blood or in the placenta), pregnancy and fetal development were normal. Thus, despite the remarkably high levels of hPL, it is not essential for normal pregnancy. However, deficiency of both hPL and hPGH due to a mutation in the GH/PL gene cluster, results in severe fetal growth retardation, but an otherwise normal pregnancy. Pregnancies in which only hPGH is deficient have not been identified. These experiments of nature indicate that hPL is not necessary for normal pregnancy, whereas fetal growth is dependent on hPGH. Alternatively, this may indicate that fetal growth is dependent on either hPGH or hPL and that these hormones comprise a redundant system.

The initial identification of hPL was based in its lactogenic activity in bioassays; therefore, it would be reasonable to surmise that hPL acts as a lactogen in human pregnancy. However, it is important to distinguish "activity" in heterologous assays from "function" physiologically. Notably, administration of hPL to nonpregnant women in sufficient quantities to mimic pregnancy levels, did not induce lactation.[80] However, this does rule out hPL as a lactogen, as its actions on the mammary gland may be dependent on other factors in the endocrine milieu of pregnancy (e.g., estrogen and progesterone). Nevertheless, the *in vivo* lactogenic properties, if any, of hPL in human pregnancy remains to be established. In addition, maternal prolactin levels increase significantly in the later stages of pregnancy and, together with estrogen and progesterone, are likely sufficient to induce mammary growth and lactation. Thus, although hPL is produced in very large quantities, it appears to be a redundant hormone.

As hPL and hPGH share a high degree of homology, it would be reasonable to surmise that they act through a common membrane-bound receptor. Indeed, both hPGH and hPL bind the GH receptor, although, the affinity of hPL is around 2000-fold less than that of hGH.[81] However, the high levels of hPL in the maternal circulation compared with those of hPGH would, in part, compensate for the reduced receptor affinity. Both hormones have equal affinity for the prolactin receptor. To date, no specific hPL receptor has been identified.

As hPGH does not enter the fetal compartment, its principal action is likely on the mother. In contrast, hPL is present in the mother and the fetus. However, deficiency in hPL alone has no effect on fetal growth, whereas deficiency in both hPL and hPGH is associated with severe fetal growth retardation. Taken together, these observations indicate that hPL and hPGH influence fetal growth through effects on the mother. This is consistent with the thesis that hPL and hPGH modulate maternal metabolism to meet fetal energy requirements.[82]

Pregnancy is a diabetogenic state, with impaired maternal glucose tolerance, relative insulin insensitivity and elevated level of circulating free fatty-acids. Such a state is advantageous to the fetus, as it increases maternal energy supplies available for fetal use. This is particularly important for the fetal brain, which uses glucose exclusively as an energy source. In non-pregnant subjects, hPL impairs carbohydrate tolerance and increases insulin and free fatty-acid levels,[83] actions consistent with the diabetogenic state of pregnancy. These properties of hPL, together with its high level of secretion into the maternal compartment, suggest that it is an important regulator of metabolic homeostasis during pregnancy.

Soon after birth insulin resistance reverts to the normal nonpregnant state, suggesting that maternal glucose homeostasis is influenced by hormonal factors produced by the fetus-placenta. To maintain glucose homeostasis during pregnancy maternal insulin secretion increases to compensate for the decrease in insulin sensitivity. In women with gestational diabetes, insulin secretion is insufficient to balance the decrease in insulin sensitivity and consequently blood glucose levels increase leading to disorders such as fetal macrosomia.

As pregnancy progresses, the fetus increases its substrate requirements, which leads to an increased functional role for hPL and hPGH in the second and third trimesters. The GH-like and contra-insulin effects of these hormones would lead to impaired glucose uptake and stimulation of free fatty acid release. Free fatty acids can cross the placenta, and the increased ketones induced by their metabolism are an important energy source for the fetus. As a consequence of insulin resistance, muscle proteolysis and the formation of ketones may be enhanced. In addition, the decreased maternal glucose consumption induced by hPL and hPGH would insure a steady supply of glucose for the fetus. These effects of hPL and hPGH on fat and carbohydrate metabolism are similar to those after treatment with GH. In this manner, maternal metabolism would be directed toward mobilization of resources to furnish the needs of the developing fetus.

This hormonal interaction represents an example of fetal-maternal conflict as proposed by David Haig.[40] The model suggests that through natural selection the fetus has acquired traits, e.g., somatotropin production, that favor the extraction of resources from the maternal organism. Conversely, mothers have evolved mechanism to counteract fetal demand. Through natural selection a compromise is attained between fetal requirements and maternal health. Disorders on either side of this equation lead to pathophysiologies of pregnancy.

Placental Corticotropins

POMC and POMC-Derived Hormones

The human placenta expresses proopiomelanocortin (POMC).[84] In pituitary corticotropes, this 31-kDa glycoprotein is the precursor for the adrenocorticotropic hormone (ACTH)-endorphin family of peptides. POMC is enzymatically cleaved into several peptide hormones, including ACTH, β-lipotrophic hormone (β-LPH), α-melanocyte-stimulating hormone (α-MSH), and β-endorphin (β-EP). These neuroendocrine hormones play major roles in the physiological response to stress and the control of behavior. Each of these peptides, including full-length POMC, has been detected in the human placenta.[85-87]

Placental syncytiotrophoblasts express POMC in a transcriptional pattern similar to that of extrapituitary tumors.[86] However, the processing of POMC in placental cells is different than that in pituitary corticotropes. Although some placental POMC is cleaved, a significant amount of intact POMC is secreted by the placenta into the maternal circulation. This is unusual, as POMC, at least in pituitary corticotropes, is processed completely and in nonpregnant adults POMC is undetectable in the circulation.[87] During pregnancy, however, maternal circulating POMC levels are readily detectable by the third month and then increase steadily until midgestation, reaching a plateau of around 300 U/mL between 28 weeks and term. Soon after birth, POMC returns to undetectable levels. Placental POMC production is constant with no diurnal variability—unlike its pattern of secretion by the anterior pituitary—and it is not inhibited by glucocorticoids. Interestingly, maternal POMC levels do not correlate with plasma ACTH or cortisol levels. However, plasma POMC levels are closely correlated with plasma corticotropin releasing hormone (CRH) levels during the third trimester.[87]

The physiological role, if any, of placental ACTH and other POMC-derived proteins in the control of human pregnancy remains to be elucidated. With regard to fetal adrenal growth, placental ACTH plays a negligible role, because it is not sufficient to prevent adrenal hypoplasia in fetal hypopituitarism due to anencephaly. However, placental ACTH may influence maternal physiology and could be responsible for the relative resistance to negative feedback suppression of pituitary ACTH by glucocorticoids during pregnancy.[88]

Immunoreactive β-EP in the maternal circulation remains relatively low throughout pregnancy, with mean levels of approximately 15 pg/mL.[89] Levels rise to approximately 70 pg/mL during late labor and rise further (mean, 113 pg/mL) at delivery.[89] Similar concentrations of β-EP (mean: 105 pg/mL) also are detectable in cord plasma at term, suggesting secretion by the placenta into the fetal compartment or secretion by the fetal pituitary. Many factors that cause an increase in pituitary ACTH (e.g., hypoxia and acidosis) also increase β-EP production.[90]

In addition to β-EP and β-LPH, there are two other families of endogenous opioids: enkephalins and dynorphins. Immunoreactive methionine-enkephalin has been found in the human placenta and is chemically identical to the native molecule. Circulating levels of methionine-enkephalin do not change appreciably throughout pregnancy.[91] Three forms of dynorphin have been found in the human placenta.[92] The amount of dynorphin in the placenta at term is similar to that found in the pituitary gland and brain.[92] Relatively high concentrations of dynorphin are found in amniotic fluid and umbilical venous plasma, and maternal plasma levels in the third trimester and at delivery are higher than in nonpregnant women.[92] Dynorphin binds to kappa opiate receptors, which are abundant in the human placenta and increase at term. Because dynorphin receptor agonists stimulate the release of hPL,[93] it is possible that dynorphin exerts local regulatory effects on hPL production.

Corticotrophin-Releasing Hormone

First identified in the hypothalamus, CRH is a 41-amino acid peptide that stimulates the expression and processing of POMC by pituitary corticotropes and, as its name implies, the secretion of ACTH. The human placenta, fetal membranes, and decidua also express CRH that is identical to that produced by the hypothalamus.[94] Expression of placental CRH can be detected from the seventh week of pregnancy and increases progressively until term. In the last 5 to 7 weeks of pregnancy, placental expression of CRH increases more than twentyfold.[95]

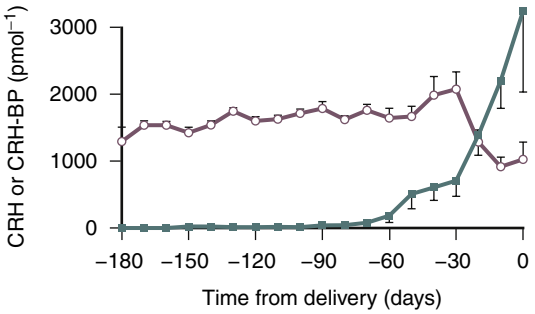


FIGURE 12.5 Levels of corticotropin-releasing hormone (CRH) and CRH binding protein (CRH-BP) in the maternal circulation during human pregnancy. *(From: McLean M, Bisits A, Davies J, et al. A placental clock controlling the length of human pregnancy.* Nature Med *1:460, 1995).*

Placental CRH is released mainly into the maternal compartment. Levels of CRH in the maternal circulation can be detected as early as 15 weeks of gestation and then increase through gestation reaching maximum levels of 1 to 10 ng/mL at term. Remarkably, this is about 1000-fold higher than peripheral CRH levels in nonpregnant women. Placental CRH also is secreted into the fetal circulation, albeit to a lesser extent.[96]

A binding protein (BP) for CRH also exists, and for most of pregnancy it is present in excess of CRH in the maternal circulation. As the CRH-BP binds CRH with greater affinity than the CRH receptor, it effectively suppresses CRH activity. Thus, for most of pregnancy the bulk of the placental CRH is sequestered and rendered inactive by CRH-BP. However, during the last 4 weeks of pregnancy CRH-BP levels decrease markedly.[97] This coincides with the exponential increase in placental CRH production, which could result in a dramatic increase in CRH biological activity (Fig. 12.5).

Despite the elevated concentrations of CRH during pregnancy, maternal ACTH secretion does not increase concordantly. In fact, pituitary ACTH levels remain low throughout pregnancy. The lack of CRH stimulation could be due to inhibition by the CRH-BP. However, maternal ACTH production remains low late in gestation when CRH increases and CRH-BP decreases. *In vivo* studies have shown that responsiveness of the maternal pituitary to CRH is markedly attenuated during pregnancy, and *in vitro* studies have shown that CRH down-regulates expression of its receptor in pituitary corticotropes.[98]

Actions of CRH are mediated by specific cell membrane receptors. Two major CRH receptor (CRH-R) subtypes have been identified, CRH-R1 and CRH-R2.[99-101] CRH binds to CRH-R1 with greater affinity than CRH-R2. These receptors and various subtypes within each group exhibit tissue-specific expression and possibly contribute to differential actions of CRH on different cell types.

In vitro studies indicate that agents that increase CRH production by the hypothalamus also increase CRH production by cultured placental cells.[102] These agents include prostaglandins E_2 and $F_{2\alpha}$ (PGE_2 and $PGF_{2\alpha}$), norepinephrine, acetylcholine, vasopressin, angiotensin-II, oxytocin (OT), interleukin-I, and NPY. In contrast, progesterone[102] and nitric oxide donors[103] inhibit placental CRH expression *in vitro*.

Interestingly, expression and secretion of placental CRH are increased by glucocorticoids. This is in contrast to hypothalamic CRH expression, which is decreased by glucocorticoids. This stimulatory action has been observed *in vivo* in women who receive glucocorticoid treatment during the third trimester[104,105] and *in vitro* in cultured placental cells.[106] As placental and hypothalamic CRH are products of the same gene, the molecular basis for this difference in glucocorticoid regulation is thought to involve placental-specific transcription factors acting through distinct response elements in the CRH promoter region.[107]

The stimulation of placental CRH production by glucocorticoids may result in a positive feedback endocrine loop. Placental CRH may stimulate ACTH production by the fetal pituitary, which would increase cortisol secretion by the fetal adrenals. Fetal adrenal cortisol could then further stimulate placental CRH production. The marked rise in placental CRH during the last 10 weeks of pregnancy could be due to such a positive feedback interaction. This endocrine loop may be involved in the process of parturition (discussed later in the chapter).

CRH also may influence fetal adrenal steroidogenesis by directly increasing dehydroepiandrosterone sulfate (DHEA-S) production.[108,109] Interestingly, CRH is as effective as ACTH in stimulating DHEA-S production, but 70% less potent than ACTH in stimulating cortisol production in primary cultures of midgestation human fetal adrenal cortical cells (Fig. 12.6). The capacity for CRH to act as an adrenal androgen secretagogue also has been demonstrated *in vivo* in adult men.[110] The preferential induction of adrenal androgen synthesis indicates that placental CRH indirectly augments placental estrogen synthesis, because fetal adrenal DHEA-S is the principal substrate for placental estrogen production (discussed later in the chapter). Placental CRH and fetal adrenal DHEA-S increase concordantly during the third trimester, suggesting that production of these two hormones is related. The implication of this endocrine axis in the regulation of parturition is discussed later in this chapter.

Several actions have been ascribed to placental CRH in the control of human pregnancy. CRH may serve an autocrine-paracrine function within the placenta by regulating expression and processing of POMC. The formation of ACTH, β-LPH, β-EP, and α-MSH by cultured placental cells can be stimulated by CRH, an effect that is inhibited by CRH antagonists.[111] Placental CRH may be part of the fetal-placental stress response mechanism. The placenta is comparable to the hypothalamus in its production of CRH in response to stress. Neurotransmitters and neuropeptides activated in response to stress stimulate placental CRH release *in vitro*.[112,113] The physiological implications of this are that the fetus may be able to mount a stress response via placental CRH. This may be critical in conditions such as preeclampsia, placental vascular insufficiency, and intrauterine infection.

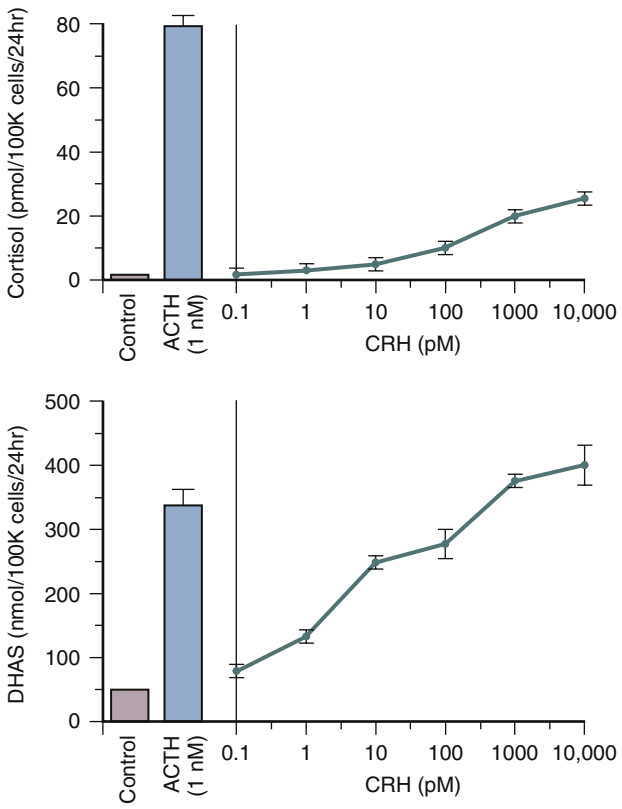


FIGURE 12.6 Effect of corticotropin-releasing hormone (CRH) on cortisol and DHEA-S production in cultured human fetal (midgestation) adrenal cortical cells *(Adapted from Smith R, Mesiano S, Chan EC, Brown S, Jaffe RB. 1998 Corticotropin-releasing hormone directly and preferentially stimulates dehydroepiandrosterone sulfate secretion by human fetal adrenal cortical cells.* J Clin Endocrinol Metab *83:2916–2920, 1998).*

Urocortin

The CRH-family of neuropeptides also includes a group of structurally and functionally related proteins known as urocortins.[114,115] Three urocortins have been identified: urocortin-1,[116] urocortin-2,[117] and urocortin-3.[118] Urocortin-1 binds with high affinity to both CRH receptors, CRH-R1 and CRH-R2, whereas urocortin-2 and urocortin-3 preferentially bind CRH-R2. Each of the three urocortins is expressed by syncytiotrophoblast and extravillous trophoblast cells and by the decidua and fetal membranes.[119,120] Circulating levels of urocortins are low compared with CRH during pregnancy[121] suggesting that the peptide acts mainly as a paracrine regulator within the gestational tissue.

Understanding of the function of placental urocortins in human pregnancy pertains mainly to urocortin-1.[115] Urocortin 1 stimulates ACTH and prostaglandin production by trophoblast cells[122] and causes vasodilation of the utero-placental vasculature via activation of the nitric oxide-cGMP system via CRH-R2.[123,124] In women with decreased uterine artery blood flow during midgestation, circulating urocortin-1 levels are reduced in proportion to the increase in uterine artery resistance.[125] Interestingly, placentas from women with preeclampsia have reduced cGMP-mediated responsiveness to urocortin-1 suggesting that a principal role of urocortin-1 is to facilitate vasodilation in the feto-placental circulation.[126] Studies in animal models also show

the vasodilatory effects of urocortin-1 on placental blood flow and suggest that one of its main functions is to protect the fetus from hypoxic insults.[127,128] Although the roles of placental urocortin-2 and -3 in human pregnancy remain unclear, studies of term myometrial cells show that urocortin-2 acting via CRH-R2 increases contractility[129] suggesting that it plays a role in parturition.

Thyrotropin-Releasing Hormone

A substance similar to the hypothalamic thyrotropin-releasing hormone (TRH) has been found in the human placenta.[54] It stimulates pituitary thyrotropin (TSH) release in the rat both *in vitro* and *in vivo*, but is not identical to hypothalamic TRH.[130] To date, a placental TSH has not been identified. Whether placental TRH plays a role in stimulating fetal or maternal pituitary TSH remains to be ascertained. As discussed above, the thyroid-stimulating activity of the placenta has been ascribed to hCG.

Leptin

Leptin, a 146-amino acid protein produced primarily by adipocytes, is a key regulator of satiety and body mass index and its levels are thought to reflect the amount of energy stores and nutritional state.[131] Leptin decreases food intake and body weight via its hypothalamic receptor.[132] In the reproductive system, leptin is thought to coordinate body mass status with reproductive function.[133] In general, leptin acts as a permissive factor in reproduction–pulsatile hypothalamic GnRH secretion does not occur unless leptin levels reach a threshold value. Such a mechanism may ensure that energy stores are sufficient to support a pregnancy.

The actions of leptin may not be limited to regulation of satiety and energy stores, as its expression and that of its receptor have been identified in a variety of tissue types. This multifocal distribution of expression and targets suggests that leptin plays a paracrine role in a variety of physiological processes.[134]

The placenta is the principal source of leptin during pregnancy.[135] Most of the leptin produced by the placenta is secreted into the maternal circulation, and as a consequence leptin levels are elevated during pregnancy. In the first trimester, maternal plasma leptin levels are double nonpregnant values and continue to increase during the second and third trimesters.[136] In the second and third trimesters, leptin is also expressed by the chorion and amnion.[137] The proportion of placental leptin directed to the fetus is uncertain and its role in fetal development is unknown. Leptin levels decline to normal nonpregnant values within 24 hours of delivery.[138]

The influence of placental leptin on maternal biology is unclear. Current data suggest that abnormally high placental leptin production is associated with maternal diabetes mellitus and hypertension and that umbilical leptin levels correlate with fetal adiposity.[139] Interestingly, leptin levels during pregnancy do not correlate with body mass index as they do in the nonpregnant state.[140] Pregnancy appears to be a state of hyperleptinemia and leptin resistance, with uncoupling of eating behavior, satiety, and metabolic activity.[141] Leptin is lipolytic and favors fatty acid mobilization from adipose tissue. Based on its actions in the hypothalamus, leptin may regulate satiety and maternal energy expenditure during pregnancy. It also may act in the liver, pancreas, and muscle to decrease insulin sensitivity and mobilize glucose. Thus, leptin appears to be another hormone utilized by the placenta to modulate maternal metabolism and partition energy supplies to the fetus.

The human placenta expresses leptin receptors and therefore leptin can act in a paracrine manner to modulate placental function. Leptin induces hCG production in trophoblast cells and is also thought to increase placental growth by augmenting mitogenesis, animo-acid uptake, and extracellular matrix synthesis.[142,143]

Neuropeptide Y

Found in the central and peripheral nervous systems, Neuropeptide Y (NPY) is a 36-amino acid protein that influences neuroendocrine function and behavioral events such as eating and satiety.[144] Immunoreactive NPY and binding sites for NPY have been found in the term human placenta.[145] In women with preeclampsia, placental NPY expression is decreased compared with normal pregnancies and is associated with increased expression of leptin.[146] The inverse relationship between placental NPY and leptin resembles hypothalamic regulation of NPY, whereby leptin suppresses hypothalamic NPY release.[147]

Maternal NPY levels are increased above those of nonpregnant women beginning early in gestation. They remain elevated until term and rise still further during labor, reaching their acme with cervical dilation and labor.[148] There is no significant change in NPY concentrations in the circulation of women undergoing cesarean section who are not in labor.[148] NPY levels decline rapidly after delivery, again suggesting a placental source of this neuropeptide.

Because NPY stimulates CRH release from cultured placental cells,[149] but not the release of GnRH, hCG, or hPL, it may play a role in the regulation of placental CRH production.

Growth Factors and Cytokines

Many growth factors, cytokines, and their cognate receptors have been found in the human placenta.[150-152] These factors likely play a role in controlling the growth and development of the fetus and placenta. In this regard the insulin-like growth factors (IGFs) are notable. Studies in mice, using homologous recombination, have shown that IGF-I and IGF-II are critical for placental and fetal growth. Disruption of placental IGF-II expression[153] or over-expression of decidual IGFBP-1–which inhibits IGF action–leads to restriction of placental and fetal growth.[154]

Interestingly, IGF-II is an imprinted gene only; the paternal allele is expressed.[155] The finding that a paternal gene promotes placental growth supports the concept of genetic conflict between the maternal and fetal genomes.[40] The size and ultimate health of the fetus depends greatly on the size of the placenta. Growth factors that increase placenta size are an advantage to the fetus because they

allow it to more efficiently extract resources from the mother. Passage of paternal genes to the next generation is favored if nutrient supply to the fetus is maximized. Maternal genes, on the other hand, not only must survive to the next generation, but they also must insure that the current pregnancy does not compromise the mother's future reproductive capacity. Maternal genes would therefore be selected to counter and control the effects of paternally imprinted genes such as IGF-II. Interestingly, IGFBP-1 is produced by the decidua, a maternal tissue, and essentially all of the IGFBP-1 in amniotic fluid is maternally derived.[154] Thus, placental, and ultimately fetal, growth appears to be the net result of a balance between factors that stimulate (e.g., IGF-II) and that restrict (e.g., IGFBP-1) growth.

Steroid Hormones and the Fetal-Placental Unit

Steroid hormone production and metabolism by the placenta is dependent on precursors provided by the fetus and mother. The interdependence of fetus, placenta, and mother for steroid hormone production in human pregnancy led to the concept of an integrated fetal-placental–maternal steroidogenic unit. To understand this concept, it will be useful to review the general steroid biosynthetic pathways (Fig. 12.7); (discussed in Chapter 4).

Progesterone

The human placenta produces large amounts of progesterone throughout pregnancy. It does this mainly by converting low-density lipoprotein cholesterol, extracted from the maternal circulation, to pregnenolone, which is then converted to progesterone.[156] Cholesterol is converted to pregnenolone in the mitochondria of trophoblast cells by the cytochrome P450scc enzyme. This is the rate-determining step for placenta progesterone production. The conversion of pregnenolone to progesterone also occurs in the mitochondria and is catalyzed by type-1 3β-hydroxysteroid dehydrogenase (3βHSD-I). The human placenta is highly inefficient at converting progesterone to 17α-hydroxyprogesterone. Consequently, placental steroidogenesis from cholesterol accumulates at progesterone. The original explanation for this block in the steroidogenic pathway was that the human placenta lacks the 17α-hydroxylase/17,20-lyase (P450c17) enzyme responsible for converting progesterone to 17α-hydroxyprogesterone and then androstenedione (i.e., the Δ_4 pathway).[157] However, recent studies have shown that the human placenta does indeed express P450c17 and has an intact Δ_4 steroidogenic pathway.[158] Nonetheless, the efficiency of progesterone synthesis is far greater than its metabolism through the Δ_4 pathway resulting in a net accumulation and secretion of progesterone. Production of progesterone approximates 250 mg/day by the end of pregnancy, at which time circulating levels are on the order of 130 ng/mL.[159]

As its name implies, progesterone is a pro-gestation hormone—it promotes and sustains the pregnant state. Progesterone has been aptly called the "hormone of pregnancy" because it is essential for pregnancy maintenance in all viviparous animals examined so far, and interference of its synthesis or action during pregnancy induces labor and delivery.

A major function of progesterone during pregnancy is to promote myometrial relaxation and quiescence. Progesterone relaxes the pregnancy myometrium genomically by modulating the expression of genes encoding contraction-associated proteins (CAPs) and by inhibiting estrogenic actions on the pregnancy myometrium.[160] It also may promote myometrial relaxation by functioning as an anti-inflammatory factor in the myometrium.[161] It is proposed that progesterone promotes the establishment of pregnancy by suppressing T-lymphocyte cell-mediated responses involved in tissue rejection and thus may contribute to preventing rejection of the conceptus by the maternal immune system.[162] Parturition is thought to be an inflammatory event and the onset of labor is considered to be secondary to tissue level inflammation within the myometrial compartment.[163-165] Thus, progesterone may block labor indirectly

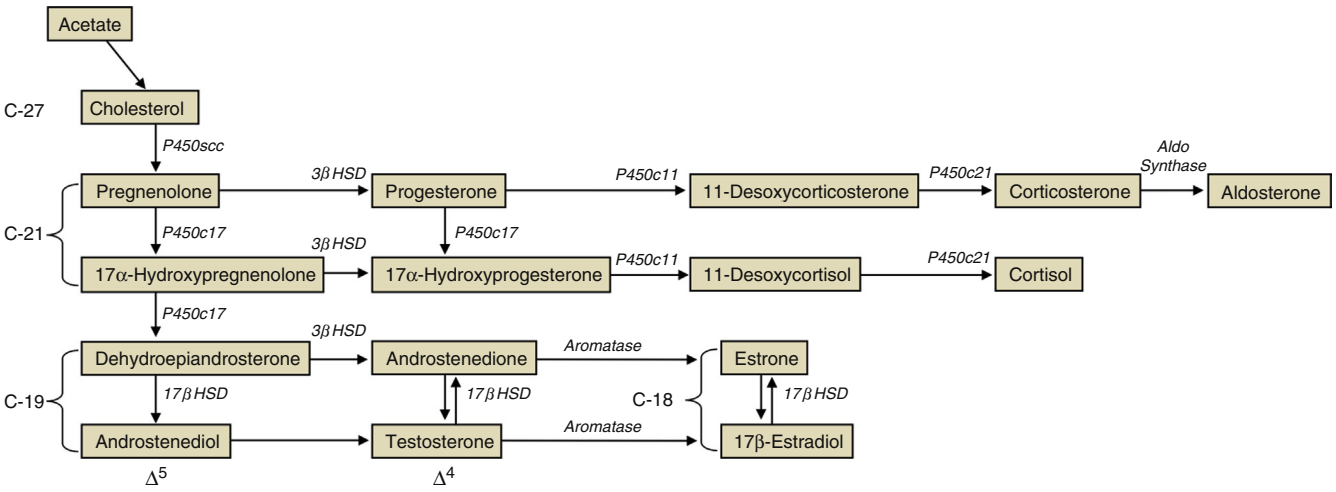


FIGURE 12.7 Biosynthetic pathways in steroid hormone formation. The C-21 compounds include progestins and the adrenal corticosteroids. The C-19 compounds include androgens, and the C-18 compounds include estrogens. Steroids with a double bond between the 5 and 6 positions in the steroid nucleus (Δ_5-steroids) are shown on the left, and those with a double bond between the 4 and 5 positions (Δ_4-steroids) are depicted on the right. The principal steroid metabolizing enzymes are shown in italics.

by repressing inflammation in the myometrium (discussed later in the chapter).

Discovery of the natural pro-gestational actions of progesterone were soon followed by efforts to exploit the activity clinically to prevent preterm birth. Studies in the 1950s and 1960s showed that administration of high doses of progesterone (as a bolus IV or into the amniotic fluid) decreased the frequency of spontaneous contractions and attenuated responsiveness to oxytocin.[166,167] Those findings suggested that progesterone could be used therapeutically to repress labor and prevent preterm birth, however, initial clinical studies to test this hypothesis produced mixed and conflicting results, largely due to insufficient patient numbers. Recent clinical trials of prophylactic progestin therapy to prevent preterm birth have produced more promising, but still somewhat equivocal, outcomes. Prophylactic treatment with natural progesterone administered vaginally[168] or a synthetic caproate ester of 17α-hydroxyprogesterone (17HPC) given as long-acting intramuscular injections[169] beginning at around midgestation decreased the incidence of preterm birth and improved neonatal outcome in women with an increased risk for preterm birth based on a prior preterm birth or a short cervix detected by ultrasound at midgestation.[170-173] Prophylactic vaginal administration of progesterone failed to alter preterm birth rates[174] in the general population and administration of 17HPC to women with twin pregnancies,who generally deliver preterm, failed to decrease the incidence of preterm birth.[175] The reason why progestin therapy is effective in only small subset of at-risk pregnancies is unclear and the value of prophylactic progestin therapy to prevent preterm birth in the general population is yet to be established.[176]

Estrogens

The principal role of estrogens in human pregnancy are to stimulate uterine growth and increase uterine blood flow. Estrogens also affect breast development in preparation for lactation. At parturition, estrogens oppose the actions of progesterone by augmenting uterine contractility and inducing cervical softening. The net physiological affects of estrogens during pregnancy that are central to the maternal adaptations for pregnancy and motherhood.

Estrogens are synthesized by the human placenta from C19 steroids.[177] The principal precursor used for placental estrogen formation is DHEA-S, supplied mainly by the fetal adrenal glands. Because the placenta has an abundance of the sulfatase (sulfate-cleaving) enzyme, DHEA-S is rapidly converted to free (unconjugated) DHEA which is then converted to androstenedione by 3ßHSD-I. The human placenta also expresses high levels of the aromatase enzyme which converts androstenedione estrone. The 17β hydroxysteroid dehydrogenase (17βHSD) enzymes then interconvert estrone and estradiol (Fig. 12.8).

By far the major estrogen formed during human pregnancy is estriol, which has an additional hydroxyl group at position 16. Estriol constitutes more than 90% of the estrogen in pregnancy urine, into which it is excreted as sulfate and glucuronide conjugates. Estriol concentrations increase with advancing gestation, and range from approximately 2 mg/24 hours at 26 weeks to 35 to 45 mg/24 hours at term.[178] At term, the concentration of estriol in the maternal circulation is 8 to 13 ng/dL.[179] In contrast, ovarian production of estriol in nonpregnant women is barely detectable.[179]

Placental estriol is formed by a biosynthetic process unique to human (and higher primate) pregnancy, that begins with DHEA-S from the fetal adrenal glands. When DHEA-S of either fetal or maternal origin reaches the placenta, estrone and estradiol are formed. However, little of either is converted to estriol by the placenta. Instead, some of the DHEA-S undergoes 16α-hydroxylation, primarily in the fetal liver and, to a limited extent, in the fetal adrenal itself. When the newly formed 16α-hydroxyDHEA-S (16α-OH-DHEA-S) reaches the placenta, the placental sulfatase enzyme cleaves the sulfate side chain and the unconjugated 16α-OH-DHEA, after further metabolism, is aromatized to form estriol, which is then secreted into the maternal circulation. Thus, level of estriol in the maternal blood reflects the activity of the fetal HPA axis and especially steroidogenic activity of the fetal zone of the fetal adrenal cortex. In the mother, estriol is conjugated to form estriol sulfate and estriol glucosiduronate in the liver and excreted via the urine.[180] Another placental estrogen derived from a fetal precursor is estetrol, formed after 15-hydroxylation of 16α-OH-DHEA-S. Its function is not known. Relative levels of progesterone and estrogens (estrone, estradiol, estriol, and estetrol) in the maternal circulation during human pregnancy are shown in Figure 12.9.

An interesting facet of steroid hormone metabolism in the human fetus is the formation of sulfate conjugates of the steroid hormones. The process of sulfoconjugation of hydroxylated steroids is ubiquitous in the fetus, occurring in a variety of sites, including lung, gut, liver, and adrenal gland. In the adrenal, sulfurylation leads to the formation of a variety of steroid sulfates, including pregnenolone sulfate, 17α-hydroxypregnenolone sulfate, and DHEA-S—quantitatively the most significant C19-androgen.[181]

Production of estrogens by the human placenta is extraordinarily high and circulating levels during pregnancy are several orders of magnitude higher than physiological levels in non-pregnancy women. A key question therefore is why does the human placenta make so much estrogen? Conditions in which placental estrogen synthesis is markedly decreased (e.g., anencephaly, congenital adrenal lipoid hyperplasia, placental aromatase deficiency, and placenta sulfatase deficiency) have provided some insight into the role of the placental estrogens in human pregnancy. Although pregnancy was prolonged in some cases of placental sulfatase deficiency[182-185] and anencephaly,[186-189] in most cases a marked decrease in placental estrogen synthesis had little effect on fetal and placental development and the timing of parturition. Likewise, there was minimal consequence of lowered estrogen levels in pregnancies with placental aromatase deficiency,[190,191] although mothers and female fetuses exhibited virilization signs due to androgen excess. Thus, high levels of estrogens of placental origin may not be essential for normal pregnancy and parturition, but rather a critical role of placental aromatase is to metabolize circulating androgens to protect the female fetus and mother from virilization.

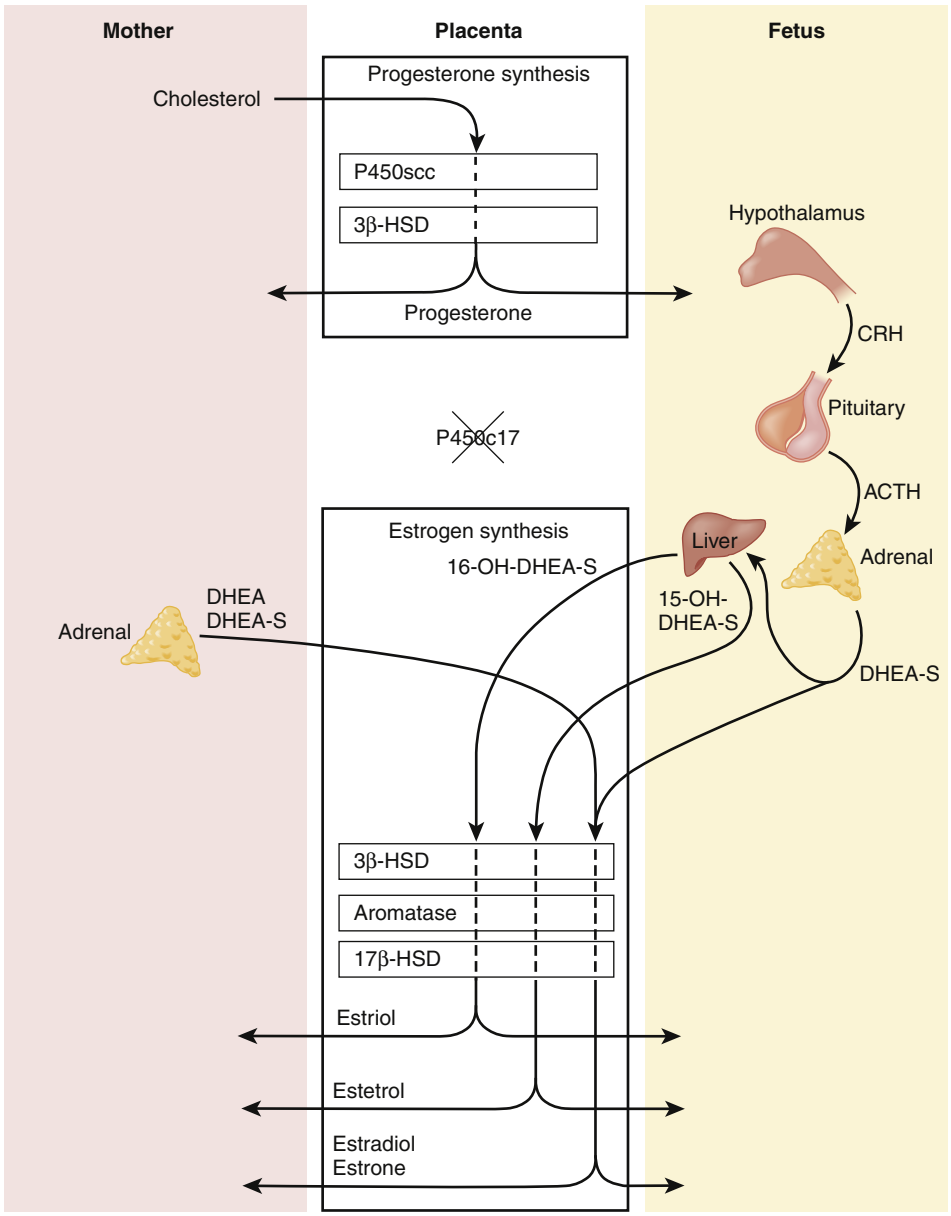


FIGURE 12.8 Biosynthesis of progesterone and estrogens by the human placenta. Progesterone is produced mainly from maternal cholesterol. P450c17 is not expressed in the human placenta and therefore, progesterone cannot be converted to C19 androgens. Instead, estrogens are biosynthesized from C19-androgen precursors (mainly DHEA-S) provided by the maternal and fetal adrenals. *(From Mesiano S. Roles of estrogen and progesterone in human parturition.* Front Horm Res *27:86, 2001.)*

These observations do not exclude a role for estrogen in the control of human pregnancy. Although levels of maternal estrogens in pregnancies with placental estrogen synthesis defects were low compared with normal pregnancies, they were still in a physiologically significant range (1 to 1.6 nmol/L) and comparable to levels reached in the midcycle and luteal phase of the menstrual cycle (0.6 to 2 nmol/L). Thus, despite the inability of the placenta to produce estrogens in certain abnormalities such as aromatase deficiency, the estrogen target tissues were still exposed to moderately high levels of estradiol. This suggests that a minimal level of estrogen is necessary for human pregnancy and that the excessive levels produced by the placenta are redundant. Indeed, no naturally occurring conditions have been described in which human pregnancy exists in the complete absence of estrogens.

The functional role of estriol in pregnancy has attracted a great deal of speculation. In many biological systems, estriol is a weak estrogen with approximately 1% the potency of estradiol and 10% that of estrone, on a weight basis. However, there is one function for which estriol appears to be as effective as the other estrogens: its ability to increase uteroplacental blood flow.[192] Therefore, this may be a primary function of the large amounts of placental estriol.

Placental Glucocorticoid Metabolism

For most of gestation the placenta expresses the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD-2) enzyme, which inactivates cortisol by catalyzing its conversion to cortisone.[193-195] As maternal cortisol levels are three times those of the fetus, this biochemical barrier serves to prevent excess cortisol from entering the fetal compartment. This is important because exposure of the fetus to high levels of cortisol not only could interfere with the fetal hypothalamic-pituitary axis, but also is associated with decreased birth weight and hypertension.[196-198]

Studies in the baboon placenta suggest that estrogen upregulates 11β-HSD-2 expression in the placenta. Because

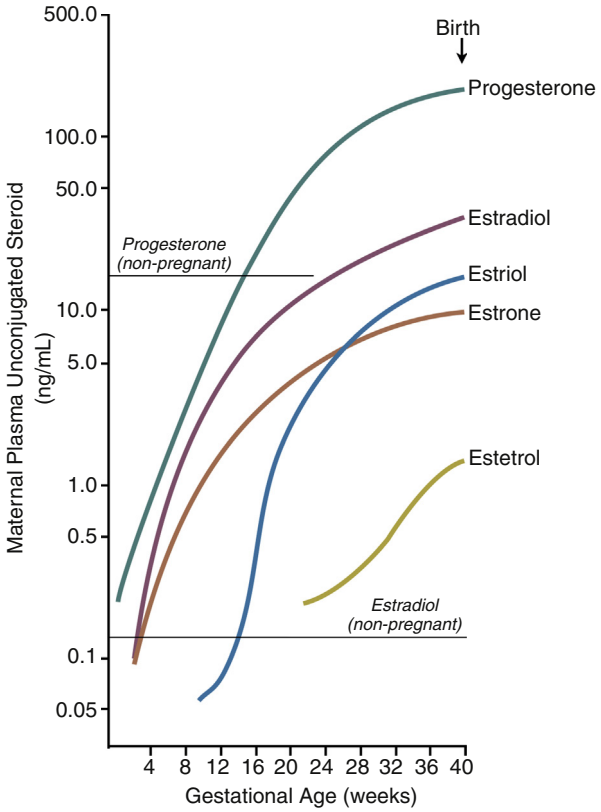


FIGURE 12.9 Schematic depiction of maternal progesterone and estrogen (estradiol, estrone, and estriol) concentrations through human pregnancy compared with average levels in normal cycling women.

estrogen synthesis by the placenta requires fetal adrenal androgen precursors (produced in part by fetal pituitary ACTH stimulation) the estrogen regulation of placental 11β-HSD-2 represents a regulatory loop that ensures that maternal cortisol does not affect the fetal HPA axis.[199]

Development of the Fetal Neuroendocrine System

Hypothalamic Hormones

By the end of the fifth week of pregnancy, the primitive hypothalamus can be identified as a swelling on the inner surface of the diencephalic neural canal. It then differentiates to form a complex of interconnecting nuclei. By 9 to 10 weeks, the median eminence of the hypothalamus is evident, and interconnecting fiber tracts of hypothalamic nuclei can be identified by 15 to 18 weeks. The hypophysiotropic hormones GnRH, TRH, CRH, growth hormone-releasing hormone (GHRH), and SS appear in the fetal hypothalamus during this period (Table 12.2).

Gonadotropin-Releasing Hormone

The human fetal hypothalamus produces immunoreactive and bioactive GnRH by 10 weeks of gestation. Between 10 and 22 weeks, the concentration of GnRH in the fetal hypothalamus remains constant (0.27-13.1 pg/mg) and is not different between the sexes. GnRH release from human fetal hypothalamic explants is pulsatile.[200,201] In male rhesus monkey fetuses during the last third of gestation, the pituitary-gonadal axis is active. The pituitary produces LH in response to GnRH and the testes produce testosterone in response to LH.[202] A similar situation likely exists in the human male fetus. Whether this also is the case for the female fetus is unresolved.

Table 12.2 Ontogeny of Human Fetal Hypothalamic and Pituitary Hormones

Hormone	Age Detected (wk)
Hypothalamic	
Gonadotropin-releasing hormone	14
Thyrotropin-releasing hormone	10
Somatostatin	14
Dopamine	11
Growth hormone–releasing hormone	18
Corticotropin-releasing hormone	16
Pituitary	
Prolactin	16.5
Growth hormone	10.5
Corticotropin (ACTH)	7
Thyroid-stimulating hormone (thyrotropin)	13
Luteinizing hormone	10.5
Follicle-stimulating hormone	10.5

Thyrotropin-Releasing Hormone

Significant levels of immunoreactive TRH are found in the human fetal hypothalamus early in gestation.[201] As with GnRH, fetal hypothalamic TRH levels do not correlate with sex or gestational age. The presence of TRH in the fetal hypothalamus in early gestation and midgestation suggests its role in the regulation of TSH, and possibly PRL, secretion.

Growth Hormone-Releasing Hormone and Somatostatin

GHRH can be detected in fetal hypothalamic neurons and fiber tracts at 18 weeks, with increasing levels found up to 30 weeks.[203] The simultaneous detection of GHRH in neuron cell bodies and fiber tracts indicates its release into portal vessels by midgestation. Immunoreactive SS can be identified in the hypothalamus of human fetuses from 10 to 22 weeks.[201] In contrast to GnRH and TRH, fetal hypothalamic SS increases with advancing gestational age.

Corticotropin-Releasing Hormone and Arginine Vasopressin

CRH is a potent secretagogue for ACTH and β-endorphin release by the human fetal pituitary gland.[204] Arginine vasopressin (AVP) also directly stimulates ACTH secretion by the fetal pituitary and can synergize with CRH.[200] CRH-immunoreactive fibers can be detected in the median eminence between 14 and 16 weeks.[205,206] CRH immunoactivity and bioactivity and AVP immunoactivity have been detected in human hypothalamic extracts from 12 to 13 weeks.[206]

Hypothalamic CRH and AVP increase with gestational age, and the CRH bioactivity of fetal hypothalamic extracts, measured in isolated rat anterior pituitary cells, is augmented by AVP. Thus, the human fetal hypothalamus has the capacity to regulate pituitary ACTH production from early in the second trimester. The extent to which fetal

hypothalamic CRH and AVP and placental CRH interact in regulating fetal ACTH release remains uncertain.

Catecholamine-Dopamine

Catecholamine fluorescence in cells projecting from the arcuate nuclei to the internal and external layers of the median eminence appears during the interval from 12 to 16 weeks.[207] Dopamine is present in the fetal hypothalamus at weeks 11 to 15 at a concentration twice that of the adult. Consistent with the effects of dopamine functioning as the PRL release-inhibiting factor, fetal hypothalami taken within this interval display a PRL release-inhibiting activity.[208]

Pituitary Hormones

The pituitary primordium appears as the epithelial evagination of Rathke pouch arising from a diverticulum of the stomadeum. The evaginated pituitary primordium appears at 4 weeks and separates from the stomadeum by 5 weeks. The floor of the sella turcica is in place by week 7 and separates the pituitary from its epithelial origins. The progenitors of pituitary hormone-secreting cells originate in the ventral neural ridges of the primitive neural tube. This region also gives rise to the diencephalon, suggesting that the hypothalamus and anterior pituitary share a common embryonic origin.

The pituitary increases in size and cell number through proliferation of cell cords into mesenchyme beginning during week 6. Capillaries interdigitate among the mesenchymal tissue of Rathke pouch and the diencephalon at 8 weeks and the median eminence is distinguishable by 9 weeks. The hypothalamic-hypophyseal vascular system in fetuses is intact by 11 to 16 weeks.

The anterior pituitary gland comprises five types of specialized epithelium-derived secretory cells: (1) lactotropes producing PRL; (2) somatotropes producing GH; (3) corticotropes producing ACTH; (4) thyrotropes producing TSH, and (5) gonadotropes producing LH and FSH.

Within the pituitary, cells containing:ACTH have been detected at 7 weeks, β-lipotropin and β-endorphin at 8 weeks, GH-containing and LH-containing cells both at 10.5 weeks, and TSH-containing cells at 13 weeks. Melanocyte-stimulating hormone (MSH)-containing cells, which probably contain β-lipotropin, have also been reported to appear at 14 weeks, and PRL-containing cells appear at 16.5 weeks (Table 12.2).[209] Many of the components required for the normal regulated function of the secretory cells of the anterior pituitary, the hypophysiotropic factors elaborated by the hypothalamus, and the neurohemal link connecting the hypothalamus and pituitary, are present in the fetus well before the end of the first half of pregnancy. The primate fetal pituitary is competent to respond *in vitro* to virtually all of the known hypophysiotropic factors by midgestation. Whether it responds to these compounds *in vivo* remains to be demonstrated directly, although studies in the catheterized rhesus monkey fetus *in utero* suggest that responses to at least one of the hypophysiotropic factors, GnRH, can occur.[202]

The Fetal Pituitary-Adrenal Axis

The anlage of the human adrenal cortex is first identified at about the fourth week of gestation as a thickening of the celomic epithelium in the notch between the primitive urogenital ridge and the dorsal mesentery. By the fifth week, these primitive cells begin to migrate toward the cranial end of the mesonephros where they condense to form the earliest recognizable manifestation of the adrenal gland. Cells destined to become the steroidogenic cells of the adrenal and gonad are derived from neighboring areas of the celomic epithelium and are morphologically identical. In general, the portion medial to the mesonephros produces cells destined for the adrenal cortex, whereas the portion ventral to the mesonephros produces cells destined for the gonad.

During the last two thirds of gestation in humans and higher primates, the fetal adrenal glands are disproportionately enlarged and exhibit extraordinary growth and steroidogenic activity. The growth is attributable to cortical hypertrophy controlled by locally produced growth factors including basic FGF and IGF-II in response to ACTH.[210-212]

For much of gestation, the human fetal adrenal cortex is composed of two morphologically distinct zones, the fetal zone and the definitive zone. The fetal zone accounts for 80% to 90% of the cortex and is the primary site of growth and steroidogenesis. During midgestation, the fetal zone produces 100 to 200 mg/day of DHEA-S, which is quantitatively the principal steroid product of the human fetal adrenal gland throughout gestation. As its name implies, the fetal zone exists only during fetal life; it involutes or undergoes remodeling soon after birth. Clusters of immature neuroblasts are present between the innermost fetal zone cells. These cells aggregate at birth to form a functional adrenal medulla.

The definitive zone (also referred to as the adult cortex, neocortex, or permanent zone) comprises a narrow band of small, tightly packed basophilic cells that exhibit structural characteristics typical of cells in a proliferative state. Its inner layers form arched cords that send fingerlike columns of cells into the outer rim of the fetal zone. Definitive zone cells begin to resemble steroidogenically active cells later in pregnancy, and appear to secrete mineralocorticoids late in the third trimester.

Ultrastructural and functional studies have revealed a third zone between the fetal and definitive zones, referred to as the transitional zone, the cells of which have intermediate characteristics between the fetal and adult zones (Fig. 12.10). After midgestation, transitional zone cells have the capacity to synthesize cortisol and thus may be analogous to cells of the zona fasciculata of the adult adrenal. By week 30 of gestation, the definitive zone and transitional zone begin to take on the appearance of the zona glomerulosa and the zona fasciculata, respectively. Thus, by late gestation, the fetal adrenal cortex resembles a rudimentary form of the adult adrenal cortex.

The postnatal remodeling of the primate adrenal cortex involves a complex wave of differentiation such that the inner portion of the fetal zone atrophies and the zonae glomerulosa and fasciculata develop.[213] Fetal zone remodeling in the human is an apoptotic process.[214] It has generally been thought that the adult cortical zones develop from the persistent definitive zone. However, there is no evidence of adrenal cortical insufficiency during the neonatal period when the postnatal remodeling process is at its peak. It is more likely that the nascent adult cortical zones are present and functional before birth. Indeed, morphologic and

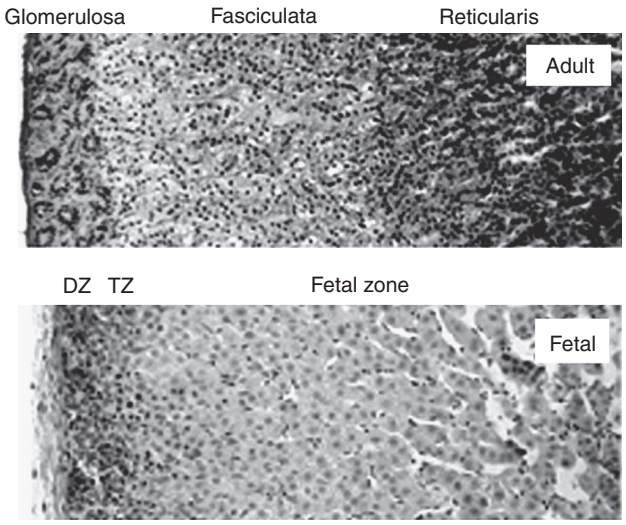


FIGURE 12.10 Morphology of the human adult and fetal adrenal cortex. DZ, definitive zone; TZ, transitional zone.

functional studies have identified rudimentary zonae glomerulosa and fasciculata during late gestation.[215] This lends support to the notion that the postnatal remodeling of the primate adrenal cortex involves apoptosis of a portion of the fetal zone and the simultaneous expansion of pre-existing and functional zonae glomerulosa and fasciculata.

ACTH secreted from the fetal pituitary is the principal trophic regulator of the fetal adrenal cortex. However, ACTH may not be acting directly. During the last two thirds of gestation, the fetal zone grows rapidly and produces large amounts of steroids, even though circulating ACTH concentrations do not rise significantly. Soon after birth the fetal zone rapidly involutes but exposure to ACTH continues, albeit at lower concentrations. Other factors, possibly specific to the intrauterine environment, appear to play a role in the regulation of fetal adrenal cortical growth and function. Substances produced by the placenta (e.g., hCG) have been implicated, and peptide growth factors produced locally within the fetal adrenal[216,217] appear to influence fetal adrenal cortical growth and function by mediating or modulating the trophic actions of ACTH.

Evidence of fetal adrenal steroidogenesis is first seen at 6 to 8 weeks, when the cells in the developing adrenal differentiate and acquire steroidogenic characteristics. At around week 12 of gestation, estriol concentrations in the maternal circulation rapidly increase (approximately 100-fold). This increase coincides with the initiation of fetal zone enlargement and ACTH secretion by the fetal pituitary gland.[209] Production of DHEA-S by the fetal adrenal cortex continues for the remainder of pregnancy and increases considerably during the second and third trimesters. By term the human fetal adrenal produces around 200 mg DHEA-S/day. Thus, placental production of estriol directly reflects the steroidogenic activity of the fetal HPA axis. For this reason, maternal estriol previously was used as an endocrine marker to evaluate fetal well-being.

The point at which the fetal adrenal cortex begins producing physiologically relevant amounts of cortisol has yet to be determined definitively. This question has been partially answered, however, by observations of infants with congenital adrenal hyperplasia (CAH). In CAH due to a deficiency of the 21-hydroxylase (P450c21) enzyme, the fetal adrenals cannot synthesize cortisol.[218] As a result, excess ACTH is produced due to loss of glucocorticoid-negative feedback. Female infants with CAH often are born with urogenital sinus defects and virilization primarily caused by exposure of the urogenital sinus to excessive amounts of adrenal androgen. Because this condition derives from adrenal glucocorticoid deficiency, the observations imply that the adrenal produces enough cortisol in female fetuses before 10 weeks of gestation to regulate ACTH levels and to prevent overproduction of adrenal androgens that may masculinize the female urogenital sinus. Expression of key steroid metabolizing enzymes suggests that the human fetal adrenal cortex does not produce cortisol *de novo* from cholesterol until around week 30 of gestation.[215] However, this does not preclude the possibility that cortisol is produced by using progesterone as a precursor early in gestation.[219]

Because the fate of pregnenolone metabolism is initially determined by the branch point steroidogenic enzymes P450c17 and 3β-HSD, the steroidogenic potential of cells may be inferred by the pattern of expression of these two enzymes (Fig. 12.7). Expression of 3β-HSD by the human fetal adrenal cortex is a critical step in the metabolism of pregnenolone, because it confers on cells the ability to convert Δ5-3β-hydroxysteroids to Δ4-3 ketosteroids essential for mineralocorticoid and glucocorticoid production. Between 12 and 22 weeks of gestation, the human fetal adrenal cortex does not express 3β-HSD. After 22 weeks, 3β-HSD expression can be detected first in the definitive zone cells and later in gestation in the definitive and transitional zone cells. At no time in gestation is 3β-HSD expression detected in the fetal zone. In contrast, expression of P450c17 is highly abundant in the transitional and fetal zones and is lacking in the definitive zone at all gestational ages.[215,220]

The persistent lack of 3β-HSD and expression of large amounts of P450c17 in the fetal zone is consistent with this cortical compartment producing only C19/Δ5 steroids, particularly DHEA. The lack of P450c17 in the definitive zone, and the eventual expression of 3β-HSD in this compartment, is consistent with this zone producing mineralocorticoids late in gestation. The coexpression of 3β-HSD and P450c17 in the transitional zone indicates that this zone has the capacity for glucocorticoid production (Fig. 12.11).

Steroidogenic enzymes downstream of P450c17 and 3β-HSD also have been examined.[221] Their localization and ontogeny are consistent with the concept that the definitive zone develops to form the zona glomerulosa, the transitional zone is analogous to the zona fasciculata, and the fetal zone is analogous to the zona reticularis.

Mineralocorticoid production by the primate fetal adrenal cortex is low early in gestation but increases during the third trimester. At term, 80% of the aldosterone in human and rhesus monkey fetal blood appears to originate from the fetal adrenal.[222] In 18- to 21-week human fetal adrenals, the mineralocorticoid metabolic pathway is localized to the definitive zone, but its activity is low and unresponsive to secretagogues.[223,224]

The angiotensin II receptors, AT1 and AT2, are present on human fetal adrenal cortical cells after 16 weeks.[225] The AT2 receptor is localized mainly on definitive zone cells,

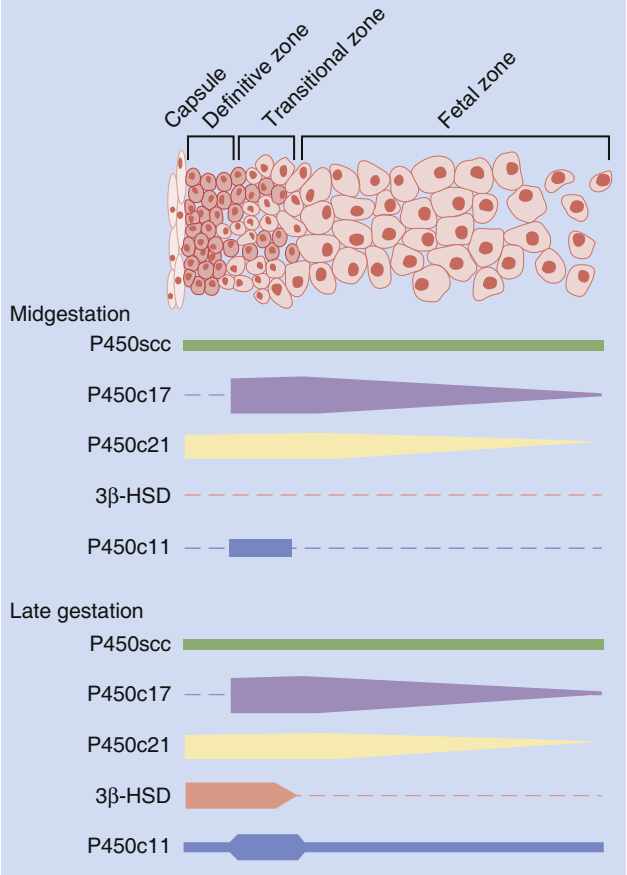


FIGURE 12.11 Schematic representation of the localization of expression of P450scc, 3β-hydroxysteroid dehydrogenase (3βHSD), P450c17, P450c21 and P450c11 in the primate fetal adrenal cortex during midgestation and late gestation. Thickness of the line indicates relative abundance of expression. Dashed line indicates lack of expression. Note the lack of P450c17 expression in the definitive zone at all stages of gestation and the ontogenetic expression of 3βHSD only in the definitive and transitional zones late in gestation. *(From Mesiano S, Jaffe RB. Developmental and functional biology of the primate fetal adrenal cortex.* Endocr Rev *18:378–403).*

whereas the AT1 receptor is detectable to a lesser extent in cells from both fetal and definitive zones. Thus, during the first and second trimesters, the ability of the human fetal adrenal cortex to synthesize mineralocorticoids is minimal even though the cells express AT receptors.

The Fetal Pituitary-Gonadal Axis

The foundation for normal puberty and adult reproductive function is established during fetal life. Intrauterine gonadal development is essential for normal sexual development. The consequence of impairment of this system can be irreparable loss of germ cells and reproductive potential. The development of the fetal testis is described in Chapter 17 and that of the fetal ovary in Chapters 9 and 17.

Studies of anencephalic fetuses indicate that embryonic sexual differentiation and early gonadal development do not depend on fetal pituitary gonadotropes. In anencephalic male fetuses, there is a reduction in Leydig cell number, whereas a reduction in the number of spermatogonia is uncommon.[226] There is also a similar time of appearance of seminiferous tubules in anencephalic and normal fetuses. In female anencephalic fetuses, the ovary appears to develop normally until 32 weeks. At term, ovaries of anencephalic fetuses are smaller than normal, central follicles are absent, and proliferation of oogonia with progression through meiosis until development of primordial follicles is not initiated.[226] Thus, pituitary gonadotropins are necessary for granulosa cell-follicular proliferation near term; they are also needed to a lesser extent, via granulosa cell regulation, for oocyte survival. On the basis of studies in fetal rhesus monkeys, fetal pituitary gonadotropins appear necessary for granulosa cell proliferation and follicular fluid formation during the latter part of pregnancy.[227]

In contrast to the ovary, fetal testes exhibit steroidogenic activity and synthesize testosterone *de novo*.[228] Testosterone secretion by Leydig cells is necessary at different stages of pregnancy for adequate growth of internal and external genitalia. In the fetal rhesus monkey *in utero*, testosterone production can be elicited by hCG injection and by stimulating the fetal pituitary-testicular axis with GnRH.[202,229] Low-density lipoprotein cholesterol can serve as a precursor for steroid synthesis in the fetal testis and may also function as an inducer of testicular steroidogenesis.[230] Levels of aromatase activity, necessary for estrogen formation, are low in the fetal testis.[231]

In contrast to the male, ovarian steroid production is not essential for female phenotypic development. Furthermore, meiosis in the ovary begins relatively early, but significant estrogen production does not occur until late in fetal life, although the capacity for aromatization exists by the eighth week.[232] The preponderance of data indicates that although certain enzymatic activity can be demonstrated in the fetal ovary, it cannot form estrogen *de novo* and is probably steroidogenically quiescent through most of pregnancy.

As with the fetal adrenal cortex, locally produced growth factors, acting in an autocrine-paracrine manner, may play critical roles in controlling the growth and development of the fetal gonads.[233]

The Fetal Pituitary-Thyroid Axis

In the human fetus, the thyroid gland acquires its characteristic morphological appearance and the capacity to concentrate radioiodine and to synthesize iodothyronines by 10 to 12 weeks of gestation. Coincidentally, hypothalamic TRH is detectable at 10 to 12 weeks; by this stage, thyrotropes can be detected in the fetal pituitary and TSH can be found by radioimmunoassay in the fetal pituitary and serum. Thyroxine (T4) has also been found in the fetal circulation.[234]

Thyroid function remains in a basal state until mid gestation. At this time, secretory activity of the thyroid gland and serum T4 concentrations begin to increase. This rise is likely to be related to the establishment of continuity between the hypothalamic and pituitary portions of the portal vascular system. Pituitary and serum TSH concentrations begin to rise shortly before the rise in T4 levels. Maximal TSH concentrations are reached early in the third trimester and do not increase further until term.

In the rhesus monkey, a TSH response to TRH administration is present early in the equivalent of the third trimester.[235] In contrast to the adult, triiodothyronine (T3) administration to the fetus does not suppress the pituitary

TSH response to TRH in primates.[235] The human infant born after 26 to 28 weeks of gestation responds to exogenous administration of TRH with an increase of circulating TSH levels similar to those seen in adults.[236]

At term, TSH secretion by the human fetus can be inhibited by the administration of T4.[236,237] In addition, the elevated cord T4 is associated with marked suppression of the neonatal TSH surge.[237] This inhibitory effect of T4 is presumably mediated through pituitary conversion of T4 to T3. Human fetal serum T4 and free T4 levels increase progressively during the last trimester, although serum TSH levels do not.[237]

Serum T3 concentrations are usually not measurable in the human fetus until approximately the 30th week of gestation.[237] Thereafter, concentrations rise to a mean level of approximately 50 ng/dL at term. The prenatal increase in serum T3 concentration occurs during several weeks and may be related to increased cortisol concentration.[238] Immediately after birth (during the first 4 to 6 hours), circulating T3 levels increase still further to concentrations three to six times those occurring *in utero*.[238,239]

Reverse T3 (3, 3′, 5′-triiodothyronine) reaches concentrations in the human fetus greater than 250 ng/dL early in the third trimester; then decreases progressively until term.[237,239,240] In contrast with T3 levels, serum reverse T3 concentrations remain virtually unchanged during early neonatal life in term infants.

An acute increase in pituitary TSH levels occurs when the term fetus is exposed to the extrauterine environment. This, in turn, stimulates thyroidal iodine uptake and evokes release of thyroid hormones. The maximal TSH concentrations are attained 30 minutes after birth. Thereafter, there is a rapid decrease in serum TSH during the first day of extrauterine life and a slower decrease during the succeeding 2 days. Serum T4 and free T4 levels reach a peak at 24 hours and then decrease slowly during the first weeks of life.[241]

Binding of iodothyronine and maturation of thyroid hormone receptors have not been reported in the human fetus. In the rat, hepatic nuclear T3 receptors mature during the first few weeks after birth.[242] T3 receptor capacity, and possibly affinity, increases during the first 3 to 4 weeks. In the brain (in contrast with the liver) T3 nuclear receptor binding develops early, is comparable with that in the adult brain, and increases still further in the first 3 days of neonatal life.[243]

However, there appears to be a discrepancy between T3 nuclear receptor binding and brain tissue responsiveness in both the neonate and the adult. Neither appears to respond to exogenous T3 administration with an increase in oxygen, α-glycerophosphate dehydrogenase, or malic enzyme consumption.[243] Of interest is the observation that thyroid hormones increase nerve growth factor concentrations in adult and newborn mouse brain[244]; it has been postulated that this nerve growth factor may mediate the effects of thyroid hormones on brain development.[243]

Fetal Pituitary-Growth Hormone

GH is produced and secreted by the human fetal pituitary from around 8 to 10 weeks of gestation.[245] Levels of GH in the fetal circulation peak at around 6 nmol/L at midgestation, then gradually decrease for the remainder of gestation to around 1.5 nmol/L at term. The peak in GH at midgestation is thought to be due to unrestrained secretion because cultured pituitary cells from 9- to 16-week fetuses respond to GHRH with increased GH secretion; they are far less responsive to inhibition by SS.[246] Alternatively, negative feedback pathways for the regulation of GH secretion may develop later then GHRH stimulation. The physiological control of pituitary GH production and secretion is mature by term.

The role of fetal pituitary GH is unclear. Anencephalic fetuses on average are of normal size and weight, suggesting that fetal growth is controlled by factors other than fetal pituitary GH. Placental GH and PL are likely candidates. Clearly, pituitary GH is required for postnatal growth and development, and therefore this system must be mature at birth. However, it likely plays a minimal role in the control of fetal somatic growth.

Fetal Pituitary-Prolactin

Levels of PRL in the fetal circulation are very low during the first half of human pregnancy. The pituitary begins secreting PRL at 25 to 30 weeks, gradually secreting more for the remainder of gestation and reaches a peak at term.[247] The level of PRL expression by the fetal pituitary increases progressively from around 15 weeks. In anencephalic fetuses, pituitary PRL content is within the normal range, indicating that its production by the pituitary is independent of hypothalamic control during most of gestation.[247]

Control of PRL secretion by TRH and dopamine matures late in pregnancy and during the first few months after birth.[245,247] In cultured pituitary cells obtained from midgestation human fetuses, estrogen stimulates and dopamine inhibits PRL secretion.[245] Interestingly, the increase in fetal PRL levels with ongoing gestation coincides with increases in estrogen levels, indicating that its production by the fetal pituitary is influenced by estrogen during most of pregnancy. The role of PRL in fetal development remains uncertain.

Long-Term Effects of Fetal Neuroendocrine Maturation

The appropriate functional maturation of neuroendocrine systems during fetal life has profound effects on long-term health into adulthood. This phenomenon, referred to as developmental origins of adult disease, arose through epidemiological observations that show an association between perturbations of intrauterine nutritional status and the development of hypertension, insulin resistance, and obesity that predispose to cardiovascular disease, diabetes, and the metabolic syndrome in adulthood.[248]

The developmental origins model predicts functional plasticity in the development of fetal neuroendocrine and organ systems such that a single genotype gives rise to multiple "normal" phenotypes in response to environmental cues (e.g., extent of maternal nutrition and stress) mediated through the maternal organism. Thus, the fetus prepares itself for the extrauterine environment by modulating its physiological set points to match the anticipated environment based on maternal cues. Interestingly, the period of neuroendocrine plasticity is limited and the physiological changes appear to be permanent; plasticity does not

continue later in life. For example, studies in animals show that changes in basal and stress-induced fetal HPA activity induced *in utero* by maternal starvation persist after birth. The altered stress-response pathway may underpin pathophysiological conditions that develop during adulthood.[249] Remarkably, studies in sheep indicate that maternal nutrition status before conception influences fetal growth and length of gestation, suggesting that the pre-implantation blastocyst and/or the endometrium are programmed by nutritional status.[250]

The physiological, biochemical, and genetic mechanisms that mediate the reactive plasticity of fetal neuroendocrine development are not clearly defined. The placenta is thought to play an active role, although it remains uncertain how information regarding the environment is transmitted from the mother, through the placenta and to the fetus. As described above, the placenta produces a plethora of hormones that could influence the developmental trajectories of fetal neuroendocrine axes. For example, placental CRH may modulate the responsiveness of the fetal adrenal cortex to ACTH and therefore contribute to the establishment of the HPA set-point.[251]

It is also possible that perturbations in the gestational milieu induce static changes in chromatin structure, referred to as epigenetic modification, in the embryo and fetus that alters gene expression that persist into adult life.[252] The most common epigenetic modifications (also known as marks) are methylation of cytosines in CpG dinucleotides and post-translational modifications (e.g., acetylation) of histones. Methylation in gene promoter regions generally inhibits downstream gene expression and histone modifications alter the chromatin secondary structure affecting activity of the transcriptional machinery.[253] These chemical modifications do not involve changes in the primary DNA sequence and are heritable by mitosis from one cell generation to the next.[254] Importantly, during the preimplantation period embryonic cells undergo waves of demethylation that effectively deprogram all epigenetic marks to generate cells with broad developmental potential. The DNA is then remethylated as the embryo develops. This provides an opportunity for environmental influences to affect the epigenome (genome-wide pattern of epigenetic marks).[255] Thus, epigenetic modification during development is proposed as the main mechanism for environment-induced phenotypic plasticity and provides a mechanistic explanation for the effects of prenatal and perinatal environmental exposures (e.g., malnutrition) on the subsequent disease risk in later life.[256-259]

Fetal Maturation and Timing of Parturition

Fetal Organ Maturation and Preparation for Extrauterine Life

At birth the fetus is abruptly required to establish and maintain physiological homeostasis independently of the placenta and in a markedly altered environment. Survival of the neonate is therefore dependent upon the functional maturation of organ systems essential for extrauterine life. Critical among these are organs that interface with the environment and extract resources (e.g., lungs, gut, and immune system) and those that maintain homeostasis (e.g., the HPA axis, kidneys, liver, and pancreas). The coordination of fetal maturation and the timing of parturition such that birth occurs when the fetus is sufficiently mature to survive as a newborn is therefore critical for neonatal survival.

Many studies in multiple species have demonstrated that glucocorticoids promote the functional maturation of fetal organ systems.[260] Critical processes induced by glucocorticoids include surfactant production by the fetal lungs; activity of enzyme systems in the fetal gut, retina, pancreas, thyroid gland, and brain; and deposition of glycogen in the fetal liver. In sheep, fetal organ maturation is induced by a prepartum surge in cortisol secretion by the fetal adrenals.[261] The cortisol surge also triggers the onset of labor (discussed later in the chapter). Thus, in sheep, the fetal HPA axis, via cortisol, mediates a physiological link between the timing of birth and fetal organ maturation.

The effect of glucocorticoids on the maturation of the fetal lungs is especially important. The inability to exchange gases due to pulmonary immaturity (respiratory distress syndrome) is the leading cause of neonatal morbidity and mortality among preterm infants. Synthetic glucocorticoids that readily cross the placenta are a standard form of therapy administered to women in preterm labor. This treatment significantly increases survival rates among preterm infants mainly by promoting lung maturation and decreasing the severity of respiratory distress syndrome.

The extent to which cortisol from the fetal adrenals regulates fetal organ maturation is uncertain. Experiments of nature suggest that maturation of the human fetus during late gestation is independent of fetal adrenal cortisol production. Fetuses with CAH due to P450c21 deficiency, produce markedly reduced levels of cortisol, yet these infants are usually born at term without any apparent signs of organ immaturity.[262] This observation suggests that cortisol produced by the fetal adrenal gland is not essential for fetal organ maturation; another source of glucocorticoid, possibly from the maternal adrenals, could contribute to the stimulation of fetal organ maturation at the end of human gestation; or maturation of the human fetus is not dependent upon glucocorticoids alone and other factors may be involved.

Although cortisol stimulates maturation of fetal organ systems, it also can have adverse effects on fetal development. To protect the fetus from these negative effects the human placenta prevents maternal cortisol from entering the fetal compartment throughout most of human pregnancy by expressing the 11β-HSD-2 enzyme that converts cortisol to the inactive cortisone.[193] Thus, for most of pregnancy the placenta forms a biochemical barrier to maternal cortisol.

Late in human pregnancy (around 34 to 35 weeks), however, the placental barrier to maternal cortisol weakens. The evidence for this is that estriol levels in the maternal circulation during late pregnancy are inversely related to the circadian changes in circulating maternal cortisol levels.[263] Thus, when maternal cortisol goes up, estriol goes down. This implies that, late in gestation, some maternal cortisol crosses the placenta to the fetal compartment and suppresses ACTH production by the fetal pituitary gland that leads to decreased DHEA-S production by the fetal adrenals and in turn decreased estriol production by the placenta. Increased transfer of maternal cortisol to the fetus

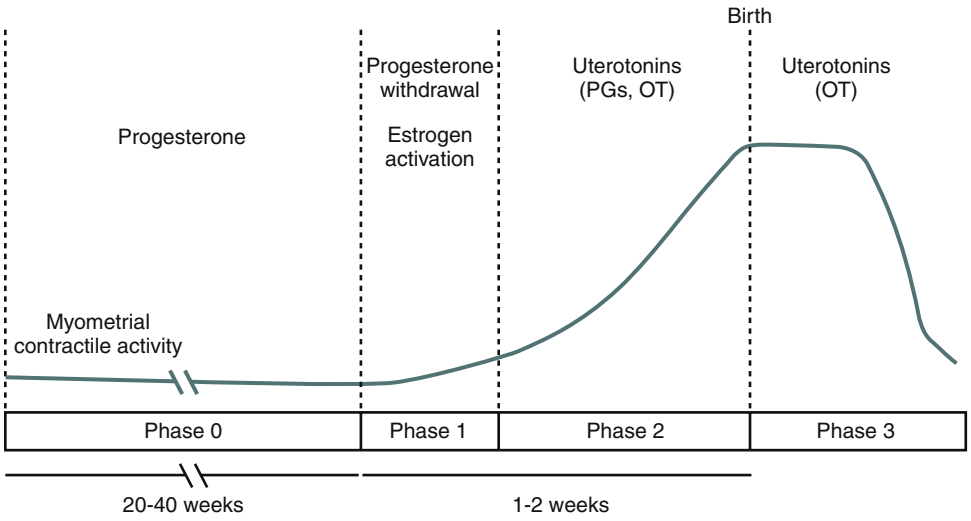


FIGURE 12.12 Phases of human parturition based on myometrial contractile activity and the principal regulatory uterotropins and uterotonins involved. *(Adapted from Casey ML, Macdonald PC. Endocrine changes of pregnancy. In Williams Textbook of Endocrinology, Wilson, JD, Foster, DW, Kronenberg, HM, Larsen, PR, editors. 1998, W.B. Saunders Co: Philadelphia. p 1259.)*

may represent a back-up mechanism to ensure fetal lung maturation. This may explain why fetuses with glucocorticoid deficiency are born without overt signs of organ system immaturity.

The Process of Human Parturition

The process of parturition involves: (1) transformation of the myometrium from a quiescent to a highly contractile phenotype; (2) remodeling of the uterine cervix such that it softens and dilates to allow passage of the fetus; and (3) rupture of the fetal membranes. These temporally coordinated events can be divided into distinct phases based on the contractile activity of the myometrium (Fig. 12.12).[264]

Phase 0 (quiescence): For most of pregnancy the myometrium is in a state of relaxation and relatively insensitive to stimulatory uterotonins (factors that affect uterine contractions); e.g., PGs and OT. In addition the uterine cervix remains closed and rigid. This period, referred to as Phase 0 or quiescence, is controlled by propregnancy relaxatory uterotonins such as β-adrenergic agents, PGI_2, relaxin, CRH, parathyroid hormone-related peptide and nitric oxide, and uterotropins (factors that modulate uterine function and growth), especially progesterone. In general, factors that activate adenylate cyclase or guanylyl cyclases and increase intracellular cAMP and cGMP, respectively (e.g., via $G_{\alpha s}$-protein-coupled receptors) promote myometrial relaxation.

Phase 1 (transformation): As an essential prelude to active labor, the myometrium undergoes a phenotypic transformation, referred to as Phase 1, whereby it gains the capacity to contract forcibly and rhythmically and becomes more responsive to uterotonins. The awakening of the quiescent uterus is initiated by progesterone withdrawal and increased estrogenic effects on gene expression leading to distinct biophysical changes in the myometrium and cervix.

Transformation of the myometrium involves the increased expression of a specific cohort of genes, collectively referred to as *contraction-associated proteins* (CAPs).[265] Some important CAPs include connexins (e.g., connexin-43), which form gap-junctions between myometrial cells, allowing rapid electrical and chemical signaling between adjacent cells to permit coordinated contractions; ion channels (e.g., calcium channels), which determine the resting membrane potential and excitability; uterotonin receptors (e.g., OT and PG receptors); and enzymes that determine the rate of local uterotonin synthesis (e.g., PG endoperoxide synthase-2 [PTGS2]). Concurrently, the uterine cervix gradually softens and becomes more elastic due to increased expression of collagenase and metalloproteinase enzymes. In addition, intrauterine production of PGE_2 and $PGF_{2\alpha}$ increases.

Phase 2 (activation): The increased myometrial exposure to PGs and its augmented responsiveness to PGs and OT eventually initiate Phase 2, which is defined by the onset of active labor. During Phase 2, uterotonic drive induces coordinated rhythmic contractions that eventually become more forceful. At the peak of active labor the myometrium is one of the strongest muscles (on a per weight basis) in the human body. As the cervix effaces and becomes more compliant, the contractions progressively move the fetus towards the birth canal. As contractions become more pronounced the force of the fetus against the lower uterine segment dilates the softened cervix enough for it and the placenta to pass through the birth canal.

Phase 3 (hemostasis and involution): Phase 3 begins after the placenta, fetal membranes and decidua are expelled. Importantly, myometrial contraction is sustained (mainly in response to OT) during this time, which helps constrict the spiral arterioles to facilitate postpartum uterine hemostasis. During the following weeks the uterus gradually reverts back to the menstrual state through a combination of myometrial cell apoptosis and atrophy. In addition, the cervix remodels and reverts to the closed and rigid state.

The Hormonal Control of Human Parturition

The hormonal control of parturition involves mainly progesterone and estrogens that have opposing effect on uterine contractility. For most of pregnancy, progesterone actively blocks parturition by promoting uterine quiescence and at the end of pregnancy labor is initiated by the withdrawal of progesterone's relaxatory effects. In contrast, estrogens (mainly estradiol) oppose the actions of progesterone by stimulating biochemical and physical changes in the uterus

and fetal membranes that facilitate labor. Thus, uterine contractility during pregnancy is determined by the balance between relaxatory actions of progesterone and stimulatory actions of estrogens. During most of pregnancy the relaxatory actions of progesterone (i.e., the progesterone block to labor) prevail. Parturition is initiated by progesterone withdrawal, which permits the stimulatory estrogenic actions to increase myometrial contractility and excitability and promote cervical softening.

Progesterone Withdrawal and Estrogen Activation

A major physiological trigger mechanism for parturition is progesterone withdrawal. In all viviparous species studied so far, parturition can be initiated by any intervention that interferes with progesterone synthesis or action and in most species normal parturition at term is triggered by a prepartum decrease in circulating progesterone levels, referred to as systemic progesterone withdrawal.[266]

The mechanism for progesterone withdrawal and how it is controlled differs between species. In some animals (e.g., rodents) progesterone is supplied by the CL during pregnancy and progesterone withdrawal at parturition is caused by hormonal signals from the uterus that induce luteolysis. In other species (e.g., ovine, bovine) progesterone is produced by the placenta and progesterone withdrawal at parturition is caused by hormonal signals from the fetus that decrease placental progesterone secretion. In sheep, this is induced by a surge in cortisol production by the fetal HPA axis 1 to 2 weeks before term,[267] which stimulates expression of the P450c17 enzyme in the placenta which converts progesterone to androstenedione leading to a dramatic decrease in placental progesterone secretion.[268] This also leads to concomitant increase in placental estrogen production due to increased availability of androstenedione for aromatization (Figure 12.7). The fall in circulating progesterone levels, coupled with a rise in circulating estrogens, transforms the uterus and cervix to the laboring state. As cortisol is the principal stimulator of fetal organ system maturation, the prepartum fetal cortisol surge ensures that parturition is coordinated with fetal maturation.

Although cortisol promotes the functional maturation of fetal organ systems in preparation for extrauterine life in the human, as it does in sheep, its production by the fetal HPA axis does not appear to be necessary for normal human parturition.[262,269] As described above, parturition occurs normally at term in pregnancies where the fetuses has CAH due to the inability to produce cortisol.[262] Transfer of cortisol from the maternal to the fetal compartment may compensate for the lack of fetal adrenal cortisol in CAH pregnancies, but whether it plays a role in triggering parturition is unlikely since administration of glucocorticoid to women in threatened preterm birth does not appear to advance the parturition process.

A distinguishing feature of parturition in humans and some nonhuman primates is that circulating levels of progesterone and estrogens are high for most of pregnancy and during labor and delivery (Figure 12.9).[270] Thus, in human parturition the uterus transitions to the laboring state despite being exposed to high levels of progesterone and for most of pregnancy the uterus remains quiescent despite being exposed to high levels of estrogens. Nonetheless, labor in women can be induced at any stage of pregnancy by interventions (e.g., administration of mifepristone, a progesterone receptor antagonist) that inhibits progesterone action.[271] An explanation for this conundrum is that human parturition involves functional—rather than systemic—progesterone withdrawal, whereby uterine cells become refractory to progesterone actions; and functional estrogen activation, whereby uterine cells become sensitive to the stimulatory prolabor actions of estrogens. The physiological control of human parturition therefore mainly involves hormonal interaction that control progesterone and estrogen responsiveness in uterine target cells (mainly myometrial cells).

Functional Progesterone Withdrawal

Progesterone responsiveness is primarily determined by the extent of expression and transcriptional activity of the nuclear progesterone receptor (nPR) that belong to a family of ligand-activated nuclear transcription factors.[272] Transcription of the human nPR gene is controlled by two promoters that produce two major nPR isoforms, PR-A (83 kD) and PR-B (99 kD). PR-A is a truncated (by 165 N-terminal amino acids) form of PR-B. Other nPR transcripts generated by alternate translational start sites, exon splicing, intronic insertions, and exon deletions have also been described, but their levels of expression in most tissues are low and their physiological significance is uncertain.[273]

All progesterone target tissues examined so far coexpress PR-A and PR-B in varying relative amounts depending on tissue type and pathological condition, and as such genomic responsiveness to progesterone is dependent on the combined actions of PR-A and PR-B. Initial studies examining the transcriptional activities of PR-A and PR-B using the canonical progesterone responsive DNA element, showed that upon exposure to progesterone, PR-B has strong transcriptional activity, whereas PR-A on its own has minimal transcriptional activity and, depending on its amount relative to PR-B, represses the transcriptional activity of PR-B.[274] Those observations led to the concept that PR-A and PR-B comprise a dual system for the control of progesterone responsiveness whereby the genomic action of progesterone is inversely related to the PR-A/PR-B ratio. According to this model, progesterone responsiveness is predominantly mediated PR-B whereas PR-A acts as an endogenous repressor of PR-B.

This concept led to the PR-A/PR-B hypothesis for functional progesterone withdrawal which posits that progesterone withdrawal in human parturition is mediated by an increase in the myometrial cell PR-A/PR-B ratio.[275] Consistent with this hypothesis several studies found that PR-A represses the transcriptional activity of PR-B in human myometrial cells and that the onset of labor is associated with a significant increase in myometrial PR-A expression and a rise in the PR-A/PR-B expression ratio (Fig. 12.13).[275-279] Functional progesterone withdrawal could also be mediated by other mechanisms that inhibit the transcriptional activity of PR-B.[280-283] Thus, current data indicate that functional progesterone withdrawal in human parturition is mediated by a variety of processes that abrogate the capacity for ligand activated PR-B to promote relaxation.

Functional Estrogen Activation

In most species, maternal estrogen levels rise prior to delivery. In humans, this rise begins at midgestation and increases gradually during the remainder of pregnancy and

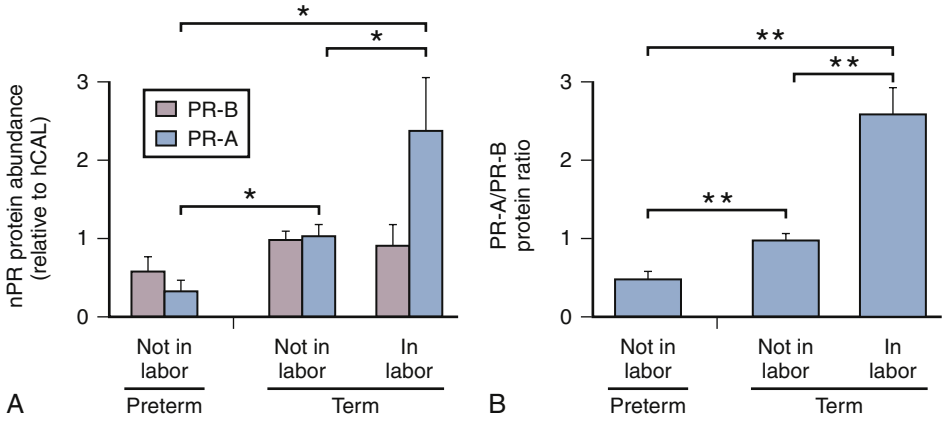


FIGURE 12.13 Relative abundance of PR-A and PR-B proteins **(A)** and the PR-A/PR-B protein ratio **(B)** in lower uterine segment myometrium obtained from term and pre-term c-section deliveries. Each bar is mean ± SEM. (* $P < 0.05$; ** $P < 0.001$). *(From Merlino AA, Welsh TN, Tan H, et al. Nuclear progesterone receptors in the human pregnancy myometrium: evidence that parturition involves functional progesterone withdrawal mediated by increased expression of progesterone receptor-A.* The Journal of Clinical Endocrinology and Metabolism *92:1927, 2007.)*

is dependent mainly on the increased DHEA-S availability to the placenta due to increased activity of the fetal HPA axis (previously discussed in the chapter). The role of placental estrogens in the human parturition process, however, is equivocal because congenital abnormalities affecting estrogen production by the fetal-placental unit, such as anencephaly, congenital adrenal lipoid hyperplasia, placental aromatase deficiency, and placenta sulfatase deficiency, do not affect the timing of birth. It appears that relatively little estrogen is required for normal human pregnancy and parturition.

Increased placental estrogen synthesis also appears to have little effect on the timing of parturition. In pregnancies in which the fetus has CAH due to P450c21 deficiency, cortisol production by the fetal adrenals is markedly reduced, leading to abnormally high ACTH production by the fetal pituitary. This in turn increases production of DHEA-S, which is converted by the placenta to estrogens. Despite markedly elevated placental estrogen production throughout most of pregnancy, individuals with CAH generally do not deliver before term.[262] Thus, increased estrogen synthetic activity by the human fetal-placental unit beyond the already high level does not appear to be a trigger for human parturition. Clearly, the role of estrogens in human parturition is not solely controlled by estrogen levels in the maternal circulation. As with progesterone action, estrogenic activity in the human pregnancy uterus appears to be controlled by changes in estrogen responsiveness.

Functional estrogen activation at parturition could be mediated by changes in estrogen receptor (ER) expression and transcriptional activity in target cells. The human ER exists as two major subtypes, ERα and ERβ (see Chapter 5).[284] Expression of ERα by the human pregnancy myometrium increases significantly at term in association with the onset of labor, whereas ERβ expression in the human myometrium is very low and not affected by the onset of labor.[275] The rise in ERα expression is directly associated with increased expression of connexin-43, a key estrogen-responsive CAP gene. This suggests that the increase in ERα expression at parturition mediates functional estrogen activation by increasing myometrial cell responsiveness to circulating estrogens.

Coordination of Functional Progesterone Withdrawal and Estrogen Activation

Numerous studies in a variety of species have demonstrated functional interaction between the ER and PR systems such that progesterone decreases uterine estrogen responsiveness by decreasing ERα expression, and estrogen increases uterine progesterone responsiveness by increasing PR-B expression. Levels of ERα expression in quiescent (i.e., before labor) term human myometrium correlate positively with the PR-A/PR-B expression ratio,[275] suggesting that myometrial cell expression of ERα increases as progesterone responsiveness decreases. Studies in the rhesus monkey have shown that progesterone inhibits ERα expression in the pregnancy myometrium.[285] Thus, progesterone may decrease uterine estrogen responsiveness by inhibiting myometrial ERα expression. This would explain why the myometrium is refractory to the prolabor actions of estrogens for most of pregnancy. Such a system, in which hormone action is principally controlled by target cell responsiveness, requires circulating hormone to be present; however, the level is not critical, provided that there is sufficient hormone for activation of cognate receptors. This would explain why inhibition of progesterone action alone (e.g., with mifepristone) is sufficient to initiate the full parturition cascade. In addition, suppression of estrogen responsiveness by progesterone may explain why parturition is not initiated prematurely by naturally occurring conditions in which estrogen levels are increased above normal.

Control of Progesterone Withdrawal

The general paradigm regarding the physiological control of human birth timing is that the induction of functional progesterone withdrawal is a pivotal integrative step in the hormonal control of human parturition. Thus, multiple probirth signals converge on the pregnancy uterus to trigger the parturition process by first inducing functional progesterone withdrawal. In particular, proinflammatory stimuli on the myometrium and cervix play a major role in the human parturition cascade.

Multiple studies have shown that human parturition is an inflammatory process and that labor is secondary to inflammation within the myometrium and cervix.[165,286-289] A significant proportion of preterm births are associated with intrauterine infection,[290,291] clinically silent upper genital tract infection, and bacterial vaginosis.[292-296] In the rhesus monkey, activation of the inflammatory system in the gestational tissues, including the myometrium, precedes the onset of labor[297,298] and studies in rhesus monkeys and mice have shown that administration of proinflammatory

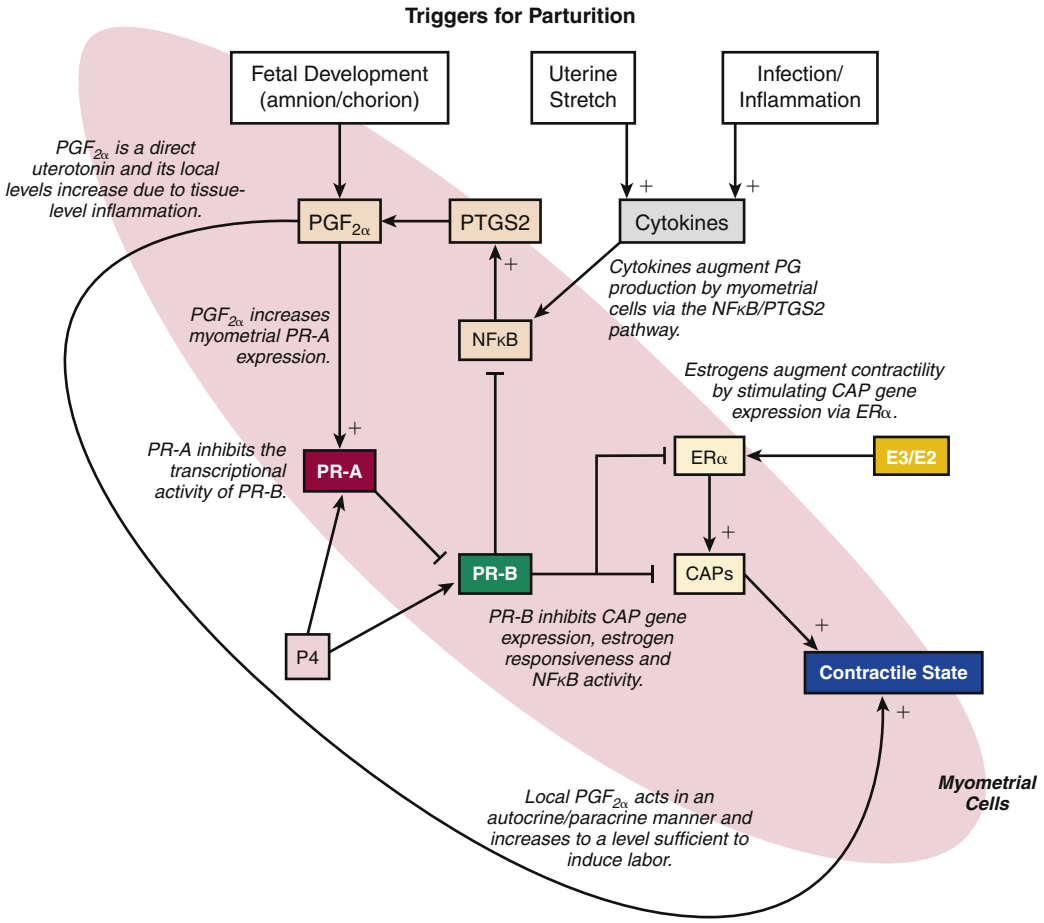


FIGURE 12.14 Theoretical model for how various physiological pro-birth inputs initiate the human parturition cascade by augmenting exposure of myometrial cells to $PGF_{2\alpha}$ which in turn induced functional progesterone withdrawal by increasing PR-A expression which inhibits the anti-inflammatory and anti-estrogenic actions of PR-B. Eventually a NFκB/$PGF_{2\alpha}$-mediated pro-inflammatory state develops in the myometrial compartment which increases local $PGF_{2\alpha}$ to levels that induce labor.

stimuli initiates preterm labor.[299,300] Labor is also associated with, and possibly preceded by, increased PG production by the fetal membranes,[301] which may be an important fetal signal for the induction of human parturition. Studies in human myometrial cell lines showed that $PGF_{2\alpha}$ stimulates PR-A expression and increases the PR-A/PR-B expression ratio.[302] Those findings suggest that $PGF_{2\alpha}$ produced by the gestational tissues in response to proinflammatory stimuli induces functional progesterone withdrawal. In women, administration of $PGF_{2\alpha}$ induces labor and delivery at all stages of pregnancy.[303,304] However, in contrast to its potent and rapid induction of uterine contraction when administered to women in active labor (Phase 2), the induction of labor by $PGF_{2\alpha}$in a quiescent uterus (Phase 0) occurs after a latency of 15 to 20 hours. This likely represents the time needed to augment PR-A expression and induce functional progesterone withdrawal and the downstream transformation of the myometrium to the laboring state.

Implicit in the hypothesis that the uterus transitions to the laboring state as a result of tissue-level inflammation, is the concept that uterine quiescence ensues if proinflammatory pathways in uterine cells are repressed.[162] Recent molecular studies have indeed suggested that this is a principal mechanism by which progesterone promotes myometrial relaxation.[161,305] Progesterone via PR-B inhibits the responsiveness of myometrial cells to proinflammatory stimuli by inhibiting activity of the nuclear factor -κB (NFκB) transcription factor complex, a major mediator of proinflammatory stimuli and regulator of proinflammatory gene expression.[306-308] In human myometrial cells, progesterone via PR-B inhibits the capacity for interleukin-1β (IL-1β) to activate NFκB and induce expression of PTGS2, the key rate limiting and inducible enzyme for the synthesis of $PGF_{2\alpha}$.[305] Importantly, PR-A inhibits the anti-inflammatory effects of PR-B, consistent with the PR-A/PR-B hypothesis.[305] Based on those observations it is possible that with advancing gestation or in response to pathophysiological conditions (e.g., intrauterine infection) $PGF_{2\alpha}$ levels increase in the myometrial compartment and increase PR-A to levels that completely abrogated the anti-inflammatory activity of PR-B leading to a tissue-level positive-feedback proinflammatory state whereby increased local production of $PGF_{2\alpha}$ exerts an autocrine-paracrine effect that further increases inflammation and $PGF_{2\alpha}$ production. Eventually the local $PGF_{2\alpha}$ levels become high enough to exerts potent uterotonic actions to induce labor. This may explain why labor is difficult to stop once it is initiated. A proposed model that integrates myometrial inflammation with functional progesterone withdrawal and local $PGF_{2\alpha}$ production is outlined in Figure 12.14.

Role of Uterine Stretch in Human Parturition

Uterine stretch has also been proposed as a signal for the induction of parturition. Such a mechanism would ensure that the fetus does not grow larger than the pelvic opening. In general, gestation is shorter in twin pregnancies,[309,310] presumably due to the increased stretch imposed on the uterine wall. Studies in rats have shown that distention of a nonpregnant uterine horn induces changes in CAP gene expression similar to those in the pregnant horn and that progesterone inhibits stretch-induced CAP gene expression.[311-313] The stimulatory effect of stretch on CAP gene expression was also observed in cultured human myometrial cells.[314-318] Stretch of cultured uterine myocytes also increased expression and release of IL-8, a potent proinflammatory chemokine that promotes the infiltration of activated macrophage and neutrophils.[315] The human uterus is subjected to a significant amount of distention, especially late in the third trimester. A likely scenario is that a threshold of uterine stretch exists above which the induction of CAP and proinflammatory gene expression contributes to the parturition cascade.

Role of Fetal Lung Maturation in Human Parturition

Studies in rodents have indicated that the fetal lungs contribute to the parturition process.[319] In this species, surfactant protein -A (SP-A), a critical lung surfactant protein, produced by the fetal lung epithelium is secreted into the amniotic fluid where it activates fetal macrophages and causes them to migrate to the myometrium. In the myometrium the increased production of cytokines by the fetal macrophages induces NFκB activation in the myometrial cells leading to functional progesterone withdrawal (discussed later in the text). This interaction ensures that fetal lung maturation, indicated by SP-A production, and birth timing is coordinated. However, evidence for a similar system is not apparent in the human and the trafficking of fetal macrophages in the myometrium in humans and mice differs substantially. However, some studies suggest that SP-A interacts directly with myometrial cells via a specific receptor to increase prostaglandin production and contractile capacity.[320] Thus, SP-A produced by the human fetal lungs may directly affect myometrial contractility and the capacity for the development of an inflammatory response in the gestational tissues. This may impact on the sensitivity of the gestational tissues to inflammation-induced parturition. An interesting twist to this paradigm is the observation that the fetal membranes express SP-A in response to glucocorticoid and that SP-A induces prostaglandin synthesis by chorionic trophoblasts.[321] This may represent an important interaction to coordinate birth timing with fetal lung maturation.

Role of Placental CRH and Urocortins in Human Parturition

A unique feature of primate pregnancy is that the placenta produces CRH in increasing amounts during gestation.[322] Studies of maternal CRH levels during human pregnancy suggest that placental CRH plays a role in the physiology of parturition. Concentrations of CRH in the maternal circulation and the rate of change as gestation progresses are predictive of the gestation length.[97] The trajectory of maternal CRH levels at mid gestation are indicative of whether parturition will occur at term, preterm, or postterm (Fig. 12.15).

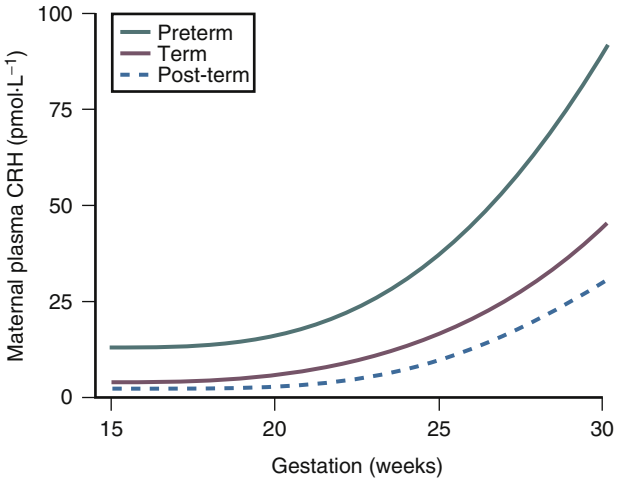


FIGURE 12.15 Schematic representation of the association between changes in maternal plasma CRH levels between 15 and 30 weeks gestation and the timing of birth. *(From McLean M, Bisits A, Davies J, et al. A placental clock controlling the length of human pregnancy.* Nature Med *1:460, 1995.)*

Interestingly, the exponential rise in maternal plasma CRH concentrations with advancing pregnancy is associated with a concomitant fall in the concentrations of the CRH-binding protein in late pregnancy (Fig. 12.5).[97] The implication of these reciprocal concentration curves is that there is a rapid increase in circulating levels of bioavailable CRH concurrent with the onset of parturition.

CRH may be involved directly in the regulation of human parturition by modulating myometrial contractility. Receptors for CRH have been identified in the human myometrium and fetal membranes.[323-326] *In vitro* studies have shown that CRH stimulates the release of PGs from human decidua and amnion,[327,328] and augments the action of OT and $PGF_{2\alpha}$ on myometrial contractility.[329-331] During most of pregnancy, CRH increases adenylate cyclase activity in the quiescent myometrium, leading to an increase in intracellular cAMP, which promotes relaxation. In contrast, at around the time of parturition the capacity for CRH to increase cAMP decreases; especially in laboring myometrium.[332] Thus, for most of pregnancy CRH contributes to the maintenance of myometrial relaxation and this effect decreases as part of the parturition process. Whether changes in CRH receptor signaling contribute to the onset of labor remains unknown.

As previously mentioned, the existence of an extrahypothalamic source of CRH may have profound effects on the activity of the fetal-placental unit. In particular, a positive feedback endocrine loop may develop between placental CRH and the fetal adrenal cortex as a consequence of the direct action of CRH on the fetal adrenal cortex and the stimulation of placental CRH expression by cortisol. In addition, CRH may increase ACTH responsiveness in fetal adrenal cortical cells (especially cells in the transitional zone) by increasing ACTH receptor expression in fetal

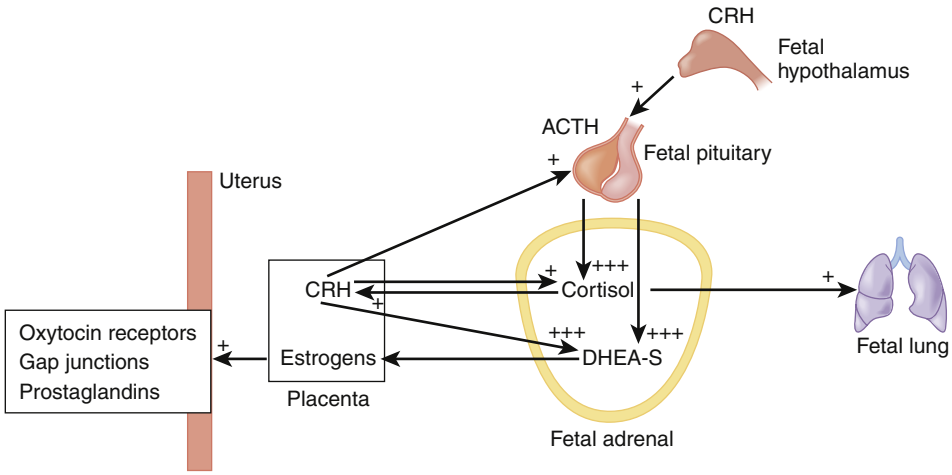


FIGURE 12.16 Schematic model of how placental corticotropin-releasing hormone (CRH) modulates activity of the fetal-placental unit. An increase in bioactive placental CRH at term has effects on both the fetus and the mother. In the fetus, CRH stimulates the fetal adrenal directly, to stimulate production of DHEA-S, which is converted by the placenta to estrogen, which in turn, drives expression of CAPs necessary for uterine contractions and parturition. The placental CRH may also act on the fetal adrenal cortex by up-regulating ACTH receptor expression and increasing responsiveness to ACTH, which evokes fetal adrenal cortisol production from the definitive zone and DHEA-S production from the fetal zone. A feed-forward loop is thus started as fetal adrenal cortisol production, in turn, stimulates CRH production by the placenta. *(From Smith R, Mesiano S, Chan EC, Brown S, Jaffe RB. Corticotropin-releasing hormone directly and preferentially stimulates dehydroepiandrosterone sulfate secretion by human fetal adrenal cortical cells.* J Clin Endocrinol Metab *83:2916–2920, 1998.)*

adrenal cortical cells.[251] As a result, fetal adrenal cortisol and placental estrogen synthesis increase through a positive feedback loop involving placental CRH (Fig. 12.16).

Urocortins, and especially urocortin-II, may also regulate myometrial contractility and the process of human parturition via their interaction with the type-II CRH receptor in myometrial cells.[121,129,333] Other studies have suggested that urocortin augments PG production by the placenta.[334]

Role of Uterotonins in Human Parturition

Uterotonins are substances that modulate myometrial tone and contractility (see also Chapter 28). The main uterotonins involved in human labor are OT and PGs (mainly PGE_2, PGI_2, and $PGF_{2\alpha}$). In addition, PGs (especially PGE_2) also promote cervical ripening. The physiological actions of PGs and OT are thought to be terminal events in the human parturition cascade and occur downstream of progesterone withdrawal and estrogen activation. Therefore, disruption of PG and OT action has attracted considerable attention as tocolytical strategies for the treatment of preterm labor.

Prostaglandins

A considerable body of data has accumulated in the last 30 to 40 years demonstrating that PGs produced by intrauterine tissues are critical regulators of myometrial contractility (see Chapter 6).[335,336] Key among these are following observations: (1) administration of PGE_2 or $PGF_{2\alpha}$ at any stage of human pregnancy will induce uterine contractions and cervical ripening and cause labor and delivery;[337,338] (2) inhibition of PG biosynthesis with aspirin, indomethacin, or specific PTGS2 inhibitors suppresses labor and prolongs gestation,[339,340] and (3) production of PGE_2 and $PGF_{2\alpha}$ by intrauterine tissues (particularly the amnion, chorion, decidua, and myometrium) increases late in gestation and in association with the onset of labor.

PGE_2 and $PGF_{2\alpha}$ stimulate uterine contractions by increasing intracellular calcium, which then triggers the contractile machinery by facilitating myosin light chain phosphorylation. By far the most abundant PG in the gestational tissues, and especially in the myometrium, is PGI_2 which is thought to relax the myometrium by increasing cAMP levels in myometrial cells. However, studies using specific PGI_2 receptor (IP) antagonists have shown that, although activation of IP increases cAMP levels and PK-A activity in myometrial cells, it also increases expression contractile proteins such as connexin-43.[341] This suggests that PGI_2 could contribute to the parturition process even though it is considered to be a relaxatory PG.

The regulation of intrauterine PG production is considered to be a pivotal event in human parturition. The rate-limiting step in PG biosynthesis is catalyzed by two isozymes, PTGS1 and PTGS2 (also known as cyclooxygenase-1 and cyclooxygenase-2) (see Chapter 6). PTGS1 is constitutively expressed in most tissue types, whereas PTGS2 expression is inducible, increasing in response to various physiological conditions and especially in response to proinflammatory stimuli. PGs are metabolized by the prostaglandin dehydrogenase (PGDH) enzyme, which irreversibly converts PGE_2 and $PGF_{2\alpha}$ to inactive forms.

Intrauterine biosynthesis of PGs occurs mainly in the amnion and, to a lesser extent, in the chorion. Both tissues express PTGS2, which increases in association with the onset of term and preterm labor. PGs also are produced in the decidua and in the myometrium, and in both sites, production increases at the time of labor. In the fetal membranes, PTGS2 expression increases during the third trimester and several weeks before the onset of labor.[342] This finding supports the notion that intrauterine PG production rises as part of the parturition process rather than as a consequence of labor.

Exposure of the myometrium to fetal membrane-derived PGs is dependent on the balance between PTGS2

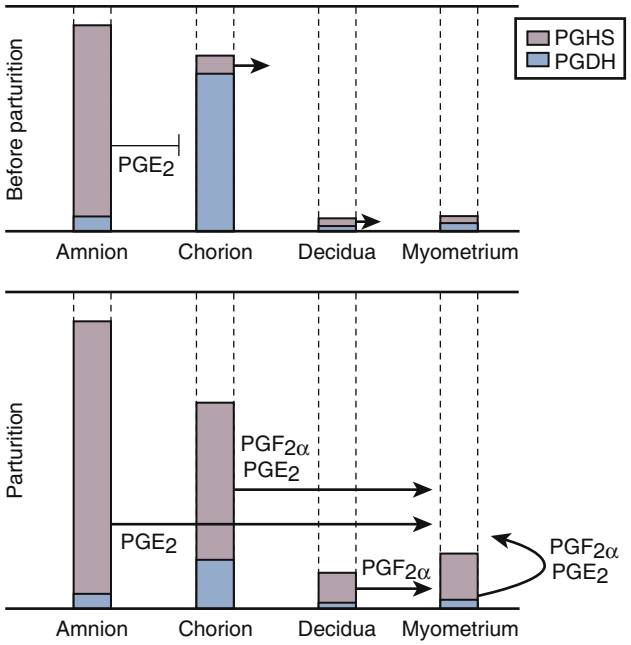


FIGURE 12.17 Changes in prostaglandin endoperoxide H synthase (PGHS) and prostaglandin dehydrogenase (PGDH) activities in the fetal membranes decidua and myometrium associated with the onset of human labor. Parturition is associated with a marked increase in PGHS activity in all tissues. PDGH is predominantly expressed by the chorion and effectively blocks amnion prostaglandins (PGs) from accessing the myometrium. Parturition is associated with decreased PGDH activity in the chorion which could allow more active PG to reach the myometrium.

and PGHD activities in the chorion. PGs produced by the amnion accumulate in amniotic fluid and do not necessarily gain access to target receptors on myometrial cells because the chorion, which lies between the amnion and the maternal tissues of the uterus, expresses high levels of PDGH.[343] Thus, through most of pregnancy the chorion may act as a barrier, preventing amnion-derived PGs from activating the myometrium (Fig. 12.17). Several studies have indicated that propregnancy factors such as progesterone prevent myometrial exposure to PGs by stimulating PGHD and inhibiting PTGS2. In contrast, cortisol, CRH, and several immune cytokines may inhibit PGDH and stimulate PTGS2, leading to a net increase in active PGs accessing the myometrium.[335] Thus, the balance of PG synthesis and metabolizing activities in the fetal membranes may be pivotal in the control of uterine contractility.

During pregnancy, the human myometrium expresses receptors for PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane.[344] The pleiotropic actions of PGs in diverse tissue types can be explained by the existence of multiple receptor species linked to different intracellular signaling pathways.[345] For example, PGE_2 interacts with four subtypes of PGE_2 receptors (EPs): EP1, EP2, EP3, and EP4. In addition, eight splice variants of the EP3 receptor have been identified.[346]

With regard to myometrial contraction, the diversity of PGE_2 receptor types may have important physiological ramifications. Interaction of PGE_2 with EP1 and EP3 increases intracellular calcium and decreases cAMP, leading to contraction. In contrast, interaction of PGE_2 with EP2 and EP4 activates adenylate cyclase, leading to increased cAMP and relaxation. Thus, PGE_2 can cause uterine contraction or relaxation depending on the type and cohort of EPs expressed. In contrast, $PGF_{2\alpha}$ interacts with a single $PGF_{2\alpha}$ receptor (FP) that increases intracellular calcium leading to contraction. PGI_2 binds the PGI_2 receptor (IP) that is linked to adenylate cyclase, leading to increased intracellular cAMP and relaxation; although it may also increase CAP gene expression. Thromboxane binds to the thromboxane receptor (TP) and increases calcium and augments contractility.

The regulation and localization of specific PG receptors in the myometrium and cervix is thought to be an important component in the endocrine control of pregnancy and parturition. Studies of EP and FP expression in the term human uterus have shown that EP2 expression is greater than FP before the onset of labor and decreases with increasing gestation and that expression of the FP receptor is low before labor and increases significantly in association with the onset of labor.[347] These findings indicate that myometrial relaxation through most of pregnancy is maintained at least in part by EP2, whereas the onset of labor involves an increase in myometrial FP, allowing $PGF_{2\alpha}$ to stimulate contraction. The balance between PG receptors mediating relaxation and contraction and their distribution in the uterus (fundal or cervical) may determine the net effect of PGs on the process of labor.

Oxytocin

Oxytocin is one of the most potent stimulators of uterine contraction. It is routinely administered to promote labor and treat postpartum hemorrhage. OT is a 9-amino acid peptide secreted by the posterior pituitary and is also produced in a number of peripheral tissues, including the ovary. During pregnancy, OT is produced by the amnion, chorion, and decidua.[348] OT is not generally considered to be involved in the initiation of labor because its circulating levels do not increase until the expulsive phase of labor.[349] This is not unexpected, because vaginal distention is a principal stimulus for the release of pituitary OT in human labor, via a neurologic feedback mechanism known as the Ferguson reflex.

However, as with PGs, OT produced by intrauterine sites may play a significant paracrine role in regulating uterine contractions at parturition. The mechanisms underlying the control of intrauterine OT production and its potential to stimulate uterine contractions remain uncertain. Despite this, it is clear that OT contributes to the uterotonic milieu of human parturition because inhibition of OT action by specific synthetic antagonists disrupts the normal pattern of labor, decreases uterine contractility in women with threatened preterm labor, and significantly prolongs gestation.[350,351]

OT action appears to be regulated at the level of target tissue responsiveness by changes in OT receptor (OTr) expression. In the human, myometrial and decidual OTr content and expression increase gradually toward the end of pregnancy and then rise significantly in association with the onset of labor.[352] Expression of the OTr by myometrial cells is up-regulated by estrogen and is part of the cassette of CAPs thought to transform the myometrium to the laboring phenotype.[353] Thus, induction of OT responsiveness by increased OTr expression may be the principal mechanism

underlying the uterotonic actions of locally produced and pituitary OT in human pregnancy. After birth, pituitary oxytocin contributes to uterine involution and especially the tonic contraction of the myometrium needed for postpartum hemostasis. OT also affects the preparation of the breast for lactation (see Chapters 3 and 10).

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 13

The Hypothalamo-Pituitary Unit, Testis, and Male Accessory Organs

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The function of the hypothalamo-pituitary-testicular-accessory organ axis is to ensure normal androgenization (including embryonic, infantile, pubertal, and adult sexual maturation), male sexual behavior, and reproductively competent sperm output. Thus, the system is important for both the health of individuals and preservation of humankind. All elements in the system acting together as an integrative regulatory network are critical for male reproductive health. Dysfunction may result in ambiguous genitalia, sex reversal, pubertal delay, eunuchism, impaired spermatogenesis, and reduced systemic androgen exposure. Furthermore, partial deficiency of reproductive hormones may contribute to some of the features of male aging, impair recovery from protracted critical illness, and induce visceral adiposity with insulin resistance. This chapter reviews the hypothalamo-pituitary-testicular axis, its components, and their joint regulation. Where practicable, emphasis is on newer discoveries in humans. Hormonal methods to regulate male fertility and to limit age-associated frailty constitute examples of how knowledge of male reproductive physiology is being translated into clinical practice.

Physiology of the Male Gonadal Axis

Overview: Ensemble Nature of Reproductive System

Male reproductive hormones consist of hypothalamic decapeptides, gonadotropin-releasing hormones (GnRH), pituitary gonadotropins, luteinizing and follicle-stimulating hormones (LH and FSH), testicular steroids, testosterone and estradiol (Te and E_2), and putatively systemically active gonadal peptides like inhibin B. Integration requires repeated incremental and decremental feedforward (stimulatory) and feedback (inhibitory) signaling among neural and hormonal components of the axis (Fig. 13.1). The negative-feedback system can be exploited to regulate male fertility by suppressing spermatogenesis with exogenous sex hormones. Gonadotropin and Te deficiency can result in infertility or subfertility, impaired sexual maturation, reduced muscle and bone mass, insulin resistance, visceral fat accumulation, diminished erythropoiesis, libido, potency, and general well being.[1]

Essential Roles of GnRH and Upstream KiSS1 Neurons

In the adult, specialized neurons (approximately 1200 in the human) located in the arcuate-nucleus of the mediobasal hypothalamus secrete GnRH in a pulsatile fashion into a portal microvasculature system. GnRH pulses stimulate gonadotropes located in the anterior pituitary to secrete LH and FSH.[2] In the embryo, migration of GnRH neurons from the olfactory placode into the upper diencephalon is essential to establish the anatomical proximity required for effectual hypothalmo-pituitary signaling. Several genes (KAL1, KAL2, and PROK2) are critical for both olfactory-bulb morphogenesis and proper GnRH neuronal migration. KAL1, KAL2, and PROK2 are located on chromosomes X, 8, and 3, and respectively encode anosmin-1, fibroblast growth-factor (FGF) receptor 1, and prokineticin 2.[3] Inactivating mutations of any of these genes, including FGF8 which is the ligand for the FGF receptor 1, and less commonly neuroembryonic LHRH factor (NELF) cause Kallmann syndrome.[4] The syndrome is characterized clinically by hyposmia or anosmia and isolated gonadotropin deficiency.[5] Inheritance patterns are respectively X-linked, autosomal dominant and recessive for defects in anosmin, FGF-receptor 1, and prokineticin or its receptor. Recently identified mutations in the chromodomain helicase deoxyribonucleic acid (DNA) binding protein-7, which cause CHARGE syndrome, may also present with Kallmann syndrome and idiopathic hypogonadotropic hypogonadism.[6,7]

Pulsatile GnRH release is both intrinsic to clusters of GnRH neurons and essential to stimulate LH secretion over the long term. Continuous GnRH delivery downregulates gonadotrope GnRH receptors and signaling pathways, which forms the basis for treating prostatic cancer and precocious puberty with long-acting GnRH agonists.[8] Episodic GnRH secretion begins during fetal development when GnRH neurons first populate the mediobasal hypothalamus. A second surge of high-amplitude LH pulsatility emerges in infant boys during the first several months of

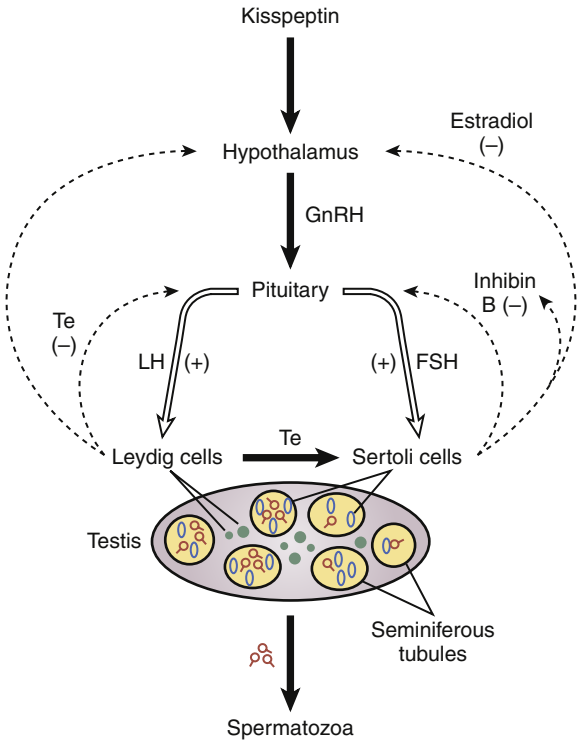


FIGURE 13.1 Schematic representation of the human male gonadal axis. The *left* side highlights feedforward (stimulation) by gonadotropin releasing hormone (GnRH) on luteinizing hormone (LH) secretion and LH on testosterone (Te) secretion as well as feedback (inhibition) by Te onto hypothalamic GnRH and pituitary LH outflow. The *right* side presents GnRH → follicle stimulating hormone (FSH) drive and FSH → inhibin B/estradiol (E_2) feedforward, and conversely feedback by inhibin B and E_2. The inhibin B feedback loop has not been demonstrated directly in men. *(Adapted from Liu PY, Takahashi PY, Nehra AX, et al: Neuroendocrine aging: pituitary-gonadal axis in males. Squire, L. R. Encyclopedia of Neuroscience.* Oxford, Academic Press *6:317–326, 2009, Fig. 6.)*

Table 13.1 Selected Putative Nonsteroidal Regulators of GnRH Neurons

Ligand	Role	Reference
GnRH	Autoinhibit (or stimulate)	316-318
Kisspeptin	Activate GPR54 receptor on GnRH neurons	24,319
Galanin, neuromedin B	Stimulatory of GnRH release	317,320,321
Glutamate	Activate N-methyl-D-aspartate receptor	322,323
Norepinephrine	Excitatory (beta-1 receptor) or inhibitory (alpha-1 receptor)	324-326
GABA*	Inhibit or stimulate	327-331
Nitric oxide	Stimulatory	332,333
Dopamine	Repressor or activator	334,335
Neuropeptide Y	Activate or inhibit via distinct receptor subtypes	336-338
CART†	Stimulate	339,340
Opiatergic peptides	Repress, (e.g., dynorphin)	341
Prolactin	Inhibit via cognate receptor	342
Calcitonin gene-related peptide	Inhibit	343
Ciliary neurotrophic factor	Stimulate	344
Leptin, orexin A/B	Stimulate GnRH outflow fasting	345
Somatostatin	Inhibit GnRH neurons	317
Serotonin	Inhibit or stimulate (via 5HT-1A or 2C and 4)	346
Tachykinins, neurokinin B and Substance P	Stimulatory	347,348

*Gamma-amino butyric acid acts via a type A or type B receptor.
†Cocaine- and amphetamine-regulated transcript.
CRH, AVP, and α-MSH inhibit, whereas melanin-concentrating hormone stimulates, GnRH release.[349,350] (Not shown.)

age.[9] GnRH secretion declines during childhood only to be reinstated at markedly higher amplitude and slightly higher frequency in puberty.[10,11]

The kisspeptin-kiss1R system controls the timing of puberty, and may also regulate GnRH secretion in other epochs of life.[12-14] Kiss1R (formerly known as GPR54) is a G-protein coupled receptor expressed on GnRH neurons.[15] KiSS-1 is the gene that encodes kisspeptin-121, which is proteolytically cleaved to a 54 amino-acid peptide named kisspeptin-54 (also known as metastin) and kisspeptin-14 and -13. Kisspeptin-54 is the natural ligand for the receptor Kiss1R. Inactivating mutations of either KiSS-1 or Kiss1R in mice and of KiSS1 in men result in normosmic hypogonadotropic hypogonadism, thus establishing that kisspeptin and cognate receptor are necessary upstream activators of GnRH neurons.[16] Mutations in the genes tachykinin 3 (TAC3) and TACR3, which encode the peptide neurokinin B and its receptor, respectively, also result in hypogonadatropic hypogonadism.[17] Furthermore, specific neurons (kisspeptin neurokinin B dynorphin [KNDy] neurons) in the arcuate nucleus of the hypothalamus, which coexpress kisspeptin, neurokinin B and the endogenous opioid peptide, dynorphin, are critical for sex-steroid feedback regulation of GnRH secretion. Proximate control of kisspeptin neurons and Kiss1R receptors by sex steroids and neurotransmitters is under intensive study. Table 13.1 highlights some of a myriad of GnRH regulators, whose site(s) of action require more precise definition. Regulators include seasonal, appetitive, anorexigenic, stress-associated, and anabolical (trophic) factors.

Sex steroids, acting in part via estrogen-receptor alpha, regulate KiSS1, neurokinin B, and dynorphin gene expression in a brain region-specific manner.[18] Estrogen and androgen repress both KiSS1 and dynorphin in KNDy neurons in the arcuate nucleus, thereby inferably transducing sex-steroidal inhibition of GnRH outflow.[19-21] This is not true in the anteroventral periventricular nucleus, which directs positive feedback by estrogen in female rodents (Fig. 13.2). The latter site contains few KiSS1 neurons in the male. Other neurotransmitters may be important, since the administration of antihypertensives (particular alpha-adrenergic blockers, selective serotonin-reuptake inhibitors, gamma-aminobutyric acid agonists, and dopamine) can influence LH secretion. However, whether such effects are direct or indirect with respect to KiSS1 and GnRH neurons remains largely unexplored.[22]

Differential Control of LH and FSH Secretion

GnRH preferentially stimulates FSH secretion in childhood and LH secretion in infancy, puberty, and adulthood.[23] Like

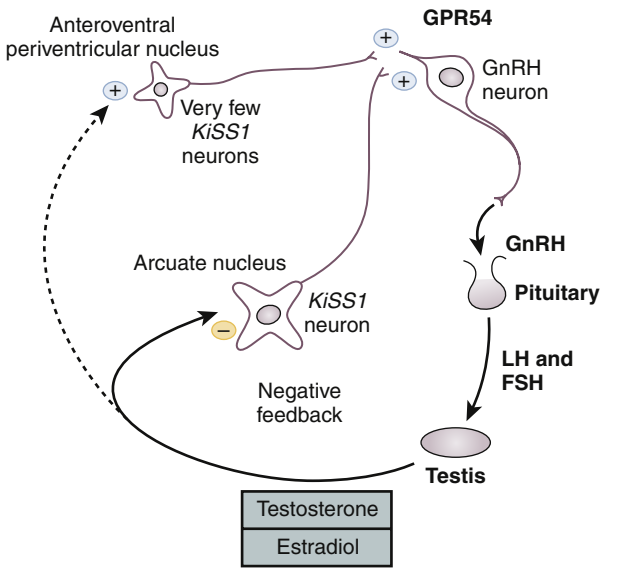


FIGURE 13.2 Model of kisspeptin-GPR54 signaling in the brain of male rodent. KiSS1 neurons in the arcuate nucleus (ARC) secrete kisspeptin, which acts as a stimulatory input to GnRH neurons. Kisspeptin signals via GPR54 expresses on GnRH neurons. KiSS1 neurons also express neurokinin B and dynorphin. The KiSS1 gene is negatively regulated by gonadal sex steroids, thereby providing a plausible mechanistic basis for inhibitory effects of Te and E_2 on hypothalamic GnRH, and secondarily on pituitary LH and FSH, secretion. Males possess very few, whereas females possess numerous, KiSS1 neurons in the anteroventral periventricular nucleus (AVPV). *(Adapted from Kauffman AS, Clifton DK, Steiner RA: Emerging ideas about kisspeptin-GPR54 signaling in the neuroendocrine regulation of reproduction.* Trends Neurosci *30:504–511, 2007, Fig. 1.)*

GnRH, kisspeptin stimulates LH release twofold more than FSH release in healthy men.[24] The putative bases for these distinctions include feedforward and feedback factors. With respect to feedforward, slower GnRH pulse frequencies prior to puberty favor gonadotrope biosynthesis of specific FSH-beta over LH-beta subunits.[2,25,26] In addition, the dimeric glycoprotein, activin A, may stimulate FSH secretion in the prepubertal rat and nonhuman primate.[27-29] At a second level of feedforward, blood-borne FSH acts on Sertoli cells in spermatogenic tubules to drive inhibin B and E_2 secretion, and LH acts on Leydig cells in the testis interstitium to cause Te production.[30] There is some indirect evidence that FSH can also increase Te production, possibly via Sertoli cell-secreted nonsteroidal factors,[31,32] but the effect is quantitatively small.[33] In relation to resultant feedback,[34-36] pubertal elevations in inhibin B and E_2 concentrations would tend to restrict FSH responses to GnRH.[37-39] Elevated Te concentrations would likewise tend to repress the rise in LH concentrations. The fact that the latter does not occur suggests muting of Te's feedback and/or strong central GnRH drive. Both mechanisms are inferable from mathematical feedback analyses performed in transpubertal cohorts of boys.[11]

Te's negative feedback on LH is mediated primarily[40] but not exclusively[41] via aromatization to E_2.[42] Significant feedback occurs at the pituitary level. Sex steroids repress GnRH secretion, but inhibition is not likely to be direct because GnRH neurons do not express the androgen receptor (AR) or estrogen receptor-α(ERα). Although ER-β is detectable and might enhance Ca^{2+}-dependent synchrony of GnRH neurons,[43] transgenic silencing of AR or ERα rather than ERβ is needed to elevate LH and Te concentrations in the adult animal.[44,45] These data favor a feedback role of KiSS1 neurons, which express both AR and ERα.[46] In addition, nongenomic actions of Te and E_2 transduced via membrane-dependent signaling may contribute to feedback in some species.[47]

GnRH and GnRH Receptors

More than 23 different GnRH peptides exist among species.[48] Human GnRH decapeptide exists in at least two forms designated GnRH I and GnRH II, cloned initially in mammals and chickens, respectively.[49] The genes encoding these peptides are located on chromosome 8p11-21 and 20p13, respectively.[50] Rapid and slow GnRH half-lives in men are about 2.6 and 5.2 min.[51] GnRH I is the predominant agonistic peptide regulating gonadotropin secretion in humans.[52] In animals, GnRH II seems to stimulate sexual arousal and courting behavior,[53] whereas GnRH III (lamprey) induces significant FSH release.[54] Truncational mutation of the GnRH-I gene occurs in the *hpg/hpg* mouse, but has not been described in man.[55]

At least 23 GnRH-receptor types have been cloned to date.[49] GnRH receptors are calcium-dependent G-protein coupled receptors (GPR's),[56,57] which control the activity of intracellular heterotrimeric G proteins by promoting guanine-nucleotide exchange and activation of adenylyl cyclase, transmembrane Ca^{2+} influx or intracellular Ca^{2+} mobilization.[56] GPR's contain seven transmembrane segments that form α-helices connected by three extracellular and three intracellular loops. Receptor activation causes a conformational change in the associated G protein α-subunit, promoting the release of GDP and binding of the activator nucleotide, GTP. The GTP-bound α-subunit dissociates from the receptor leaving a stable βγ-dimer. Dissociated subunits modulate cellular signaling as proximate messengers in the cyclic AMP-protein kinase A (α subunit) and phospholipase C-protein kinase C (βγ dimer) pathways. High intrahypothalamic concentrations of GnRH may mediate autoinhibition via a G_i subunit.[58]

Two distinct GnRH receptors have been identified in man. GnRH I receptor is the hypophyseal receptor coupled to LH and FSH synthesis, which is encoded by a gene located on chromosome 4. Two splice variants of the GnRH I receptor exist.[59] Both GnRH I (secreted) and GnRH II (brain) peptides can activate the GnRH I receptor. The GnRH II receptor gene is located on chromosome 1,[50] but is silenced in the human, chimpanzee, cow, and sheep.[53] GnRH regulates neurotransmission and sexual behavior in the rodent via the GnRH II receptor.[49] Truncational, nonsense, and missense mutations in the GnRH receptor I gene or homeobox genes (LHX3) can lead to nonexpression, inactivation, misfolding, and misrouting of nascent receptors, and thereby autosomal recessive hypogonadotropic hypogonadism without anosmia.[60,61] Activating mutations of the GnRH receptor could result in isosexual precocious puberty, but have not yet been identified.[62] Other non-GnRH signals (e.g., IGF-I, galanin, activin A, inhibin B, follistatin, cytokines, gonadotropin-inhibitory hormone) may

potentiate or inhibit GnRH action in the pituitary in animal models.[49,63-68]

Gonadotropins and Cognate Receptors

Gonadotropins comprise lutropins (LH and human chorionic gonadotropin, hCG) and follitropin (FSH). Gonadotropins and thyroid-stimulating hormone (TSH) are oligosaccharide-modified dimeric proteins with a molecular mass of 30-40 kDa. All four corresponding genes are expressed in the human pituitary gland, albeit minimally in the case of hCG.[69] Each contains a common α subunit and a hormone-specific β subunit. Subunits are associated by noncovalent interactions, but cysteine residues (10 in the α subunit and 12 in the β subunit) permit disulfide linkages within subunits. The α subunit is encoded by a single gene localized at 6q12.21.[70] Mutations of this gene have not been identified, possibly because simultaneous loss of all glycoprotein hormones may be lethal *in utero*. The α subunit gene is larger than the β subunit gene because of a noncoding exon 1 and a long first intron, but the translated proteins are comparable in size. Beta-subunit genes are located on chromosomes 19q13.32 (LH-hCG cluster) and 11p13 (FSH). The human LH-hCG cluster consists of one LHβ and 6 hCGβ genes and pseudogenes. Glycosylation and terminal sialylation or sulfation via critical N- and O-linked sites within α (two sites) and β (one site in LH, two in FSH and TSH) subunits influence *in vivo* bioactivity, receptor binding, and metabolical clearance.[71] In particular, four O-linked glycosylation linkages present in an extended C-terminal portion of hCGβ (but not LHβ) increase intact hCG half-life to 24 to 30 hours compared with 1 hour for LH, and augment biopotency by limiting hCG-receptor dissociation.[72]

Exonic polymorphisms and mutations have been described for each gonadotropin β subunit (hCG, LH and FSH) gene.[70] In mice, knockout of LHβ results in decreased production of Te and fewer elongated spermatids.[73] One polymorphism of LHβ, termed variant LHβ, occurs in as many as 30% of some Northern European and Australian aboriginal populations, and contains two amino-acid transversions (Ile15Thr and Trp8Arg) and a supernumerary consensus glycosylation site (Asn13-Ala-Thr) reminiscent of hCGβ. Variant LH is more biopotent, but has a shorter *in vivo* half-life than wild-type LH putatively due to resistance to peptidases or impaired sialylation and sulfation of terminal oligosaccharides.[74] In contrast, mutations of the FSHβ gene are rare in men. There are only three reported cases, each being associated with azoospermia[32,75,76] and one with hypoandrogenism.[76]

The LH-hCG receptor is a G-protein coupled receptor that is encoded by an 11-exon gene located on chromosome 2p21.[77] Unlike the FSH receptor, the lutropin receptor expressed is widely outside the gonad including in the brain, leading to speculative extragonadal actions of LH or hCG.[78,79] Men with inactivating LH-receptor mutations present with male pseudohermaphroditism and a phenotype ranging from micropenis and hypospadias to complete feminization, in each case without an extragonadal phenotype. Activating mutations of the LH-hCG receptor become manifest as gonadotropin-independent (familial) male sexual precocity or testotoxicosis.[80] LH-receptor polymorphisms have also been described (such as Asn291Ser and Asn312Ser) in association with undermasculinization[81] and Leydig cell hypoplasia.[82] A rare splice variant (deleted exon 9) is marked by inhibitory properties.[83] Thus, structural analyses of interactions among LH receptor, ligand, and relevant G proteins should lead to targeted ligand discovery.[84]

The FSH receptor is also a G-protein coupled receptor, but is encoded by a 10-exon gene located on chromosome 2p21.[85] The crystal structure has been determined, revealing a hand-clasp interaction between the receptor and FSH (follitropin) that orients the glycoprotein hormone perpendicular to the long axis of the ligand-binding domain.[86] The follitropin receptors appears to be expressed exclusively on Sertoli cells located within the blood-testis barrier formed by tight junctions.[87] For this reason, FSH is believed to regulate spermatogenesis primarily. A few controversial reports exist of FSH-receptor expression elsewhere, such as on spermatogonia[87] or skeletal osteoclasts.[88] Replication of these preliminary findings is required.[89]

Polymorphisms of the FSH receptor occur, but their influence on signal transduction and spermatogenesis remains unclear.[70] Inactivating mutations of the FSH receptor in five men were associated with subfertility (three cases) and fertility (two cases) with phenotypic variations ranging from oligozoospermia to euspermia.[90] Such data suggest that very low receptor activity is sufficient for spermatogenesis, or that other endocrine or intratesticular factors may compensate to varying degrees.[91] An activating mutation of the FSH receptor was reported,[92] but functional significance has not been corroborated.

In young men, FSH seems necessary for the formation of spermatogonia type B and pachytene spermatocytes, and LH/hCG for round spermatids.[93] In contrast, LH is the dominant agonist of steroidogenesis.[94]

Testicular Steroidogenesis

Testosterone (Te) is the most important local and systemic androgen produced by the testis. Te concentrations are similar by race and ethnicity, but decline with increased abdominal visceral fat and with age.[95-97] In contrast, African-American men have higher E_2 and sex-hormone binding globulin (SHBG) concentrations than their white counterparts. Androgens are crucial for male reproduction and general health. Testosterone triggers the development of male secondary sexual characteristics at puberty, maintains adult sexual behavior and function, and drives late stages of spermatogenesis independently of its metabolite 5α-dihydrotestosterone (DHT).[98] During fetal development, Te is essential for differentiation of the internal and DHT the external male urogenital system. Inactivation of 5α-reductase type I in mice is lethal to the embryo (putatively due to unopposed brain estrogen toxicity), whereas inactivation of 5α-reductase type II in mice and men leads to male pseudohermaphroditism (perineoscrotal pseudovaginal hypospadias, hypoplastic prostate tissue, and decreased male-pattern balding and beard growth).[99] Despite low or absent DHT in such XY individuals, Te is sufficient to ensure wolffian-duct differentiation (*viz.*, epididymides, vasa deferentia, seminal vesicles). Other androgens, such as the reduced metabolite, 5α-androstane-3α,17β-diol, are important for fetal sexual maturation in marsupials, but their roles in primates are unknown.[100,101]

Leydig cells are located in the interstitial compartment of the testis, which allows secretion of Te and E_2 into seminiferous tubules, gonadal lymphatics and venules. Fetal Leydig cells and maternal hCG are responsible for an intrauterine peak in Te secretion at 12 to 14 weeks of gestation. This peak is critical for male urogenital-tract organogenesis. Testosterone production declines during the remainder of *in utero* life as fetal Leydig cells begin to degenerate. Augmented pulsatile LH secretion and neonatal proliferation of Leydig cells result in a second peak of androgen production at 2 to 3 months of postnatal age.[9] The neonatal peak is putatively important for imprinting masculine behavior at least in animals. Fetal Leydig cells undergo apoptosis, but are slowly replaced as mesenchymal stem cells populate the interstitium, and differentiate into initially immature Leydig cells that secrete 3α- and 5α-reduced androgens rather than Te. Further division and differentiation occur in puberty, when mature (adult) Leydig cells secrete large amounts of Te (Fig. 13.3). Beginning in young adulthood and progressively thereafter, total Te concentrations fall gradually by 0.6% to 1.1% annually.[102,103] resulting in a net decrement of 35% to 50% from the young-adult maximum by the eighth decade in life.[96,104]

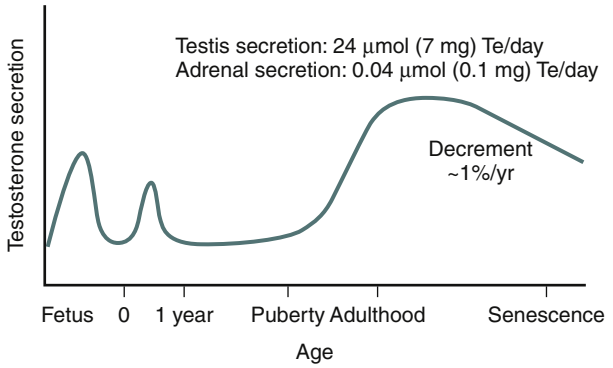


FIGURE 13.3 Schematic profile of testosterone (Te) secretion over the human male lifetime. There are three prominent intervals of amplified Te production.

Testosterone is synthesized from cholesterol through sequential reactions that are catalyzed by cytochrome P450-containing complexes. A defect in any step results in variably severe male pseudohermaphroditism (46XY infant with undermasculinization of the external genitalia). Five major steps are involved, namely cholesterol C20,22-desmolase (CYP11A cholesterol side-chain cleavage), 3 β-hydroxysteroid dehydrogenase/$\Delta^{4,5}$-isomerase, (3 β-OHSDH), C17,20-desmolase/17α-hydroxylase (CYP17A), and 17 β-hydroxysteroid dehydrogenase type III (Fig. 13.4). Cytochrome P450 refers to a 450-nm light-absorbing heme-binding peptide sequence that is critical for stoichiometry. The enzymatically rate-limiting step in steroidogenesis is encoded by CYP11A, which converts cholesterol to pregnenolone in the inner mitochondrial membrane. However, the kinetically rate-limiting step is delivery of LDL-, membrane, and cholesteryl ester-derived free cholesterol to the inner mitochondrial leaflet by steroidogenic acute regulatory (StAR) protein.[105,106] LH activates StAR rapidly via posttranslational modification and slowly via increased gene transcription. Combined responses ensure both immediate and sustained access of cholesterol to mitochondrial side-chain cleavage system.

Pregnenolone leaves mitochondria and enters endoplasmic reticulum, where subsequent enzymatic modifications occur. Pregnenolone (or its 3 β-reduced metabolite, progesterone) undergoes sequential cleavage and hydroxylation via CYP17A-encoded C17,20 lyase/17α-hydroxylase (a single enzyme with dual functions), which constitutes the androgen-committing step. The product is a weak ketosteroid, androstenedione,

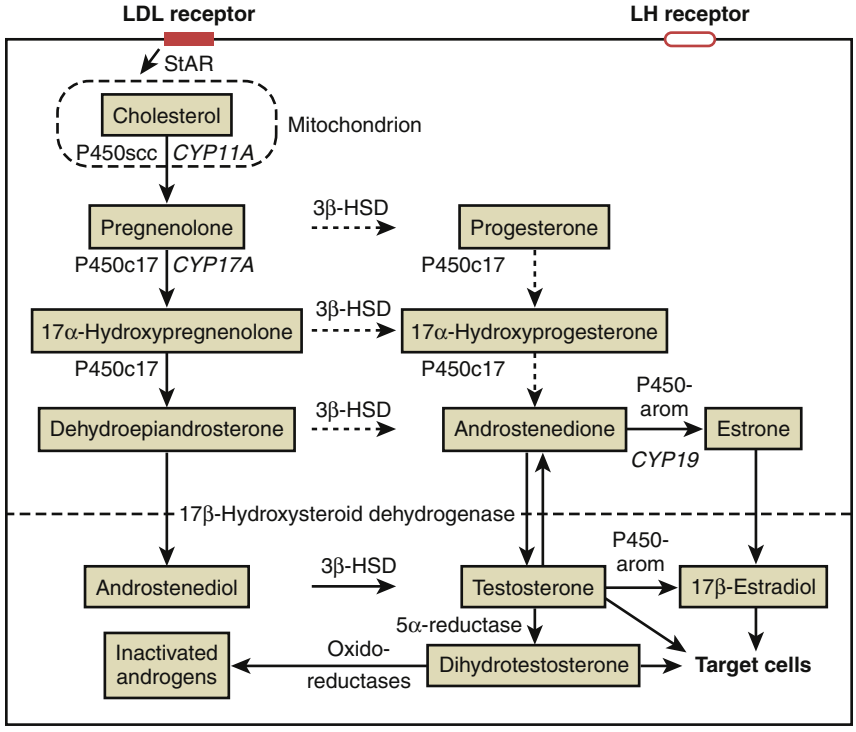


FIGURE 13.4 Testosterone (Te) biosynthesis in Leydig cells. The principal (Δ^5) pathway in humans is indicated by continuous arrows. The Δ^4 route *(dashed arrows)* is more important in other species. Some Te is taken up by Sertoli cells, which convert the androgen to E_2 and DHT. LDL, low-density lipoprotein; P450scc, cytochrome P450 cholesterol side-chain-cleavage enzyme (CYP11A); 3β-HSDH, 3β-hydroxysteroid dehydrogenase; P450c17, C17,20-lyase/17α-hydroxylase (CYP17A); P450arom, aromatase (CYP19); StAR, steroidogenic acute regulatory protein. DHT is inactivated to 3α- and 3β-diol metabolites.

which must be converted to the potent hydroxysteroid, Te, via 17β-hydroxysteroid dehydrogenase type 3.[107,108]

In men, gonadal steroidogenesis accounts for over 95% of Te production with the remainder produced by the adrenal gland and extraglandular conversion of the weak androgenic precursors, dehydroepiandrosterone and androstenedione, to Te.[109,110] Te production in young men averages 15 to 30 μmol (4 to 9 mg) per day. Estimates are consistent when determined by infusion of stable or radioactive isotopes and recent noninvasive analytical methods.[111-117] Testosterone secretion is determined genetically and environmentally. For example, men convert more Te to E_2 by peripheral aromatization and more androstenedione to Te via 17β-hydroxysteroid dehydrogenation than women.[118] In addition, Te concentrations differ between ethnic Chinese men living in an Asian compared with a Western environment.[113,119] Combined factors also may explain some populational differences in 5-α reduced metabolites and prostate volume.[120]

Intratesticular regulation of Leydig cells is complex, and not yet fully understood.[121] All three of spermatogonial, peritubular myoid, and Sertoli cells exert effects on Leydig-cell steroidogenesis.[94,122] Central nervous-system and spinal-cord pathways that innervate the testis further modulate testicular steroid production, particularly in the settings of inflammatory stress, alcohol intoxication and central adrenergic modulation.[123]

Aromatization, 5α-Reduction and Inactivation of Te

Untransformed Te exerts potent anabolical effects. Te also functions as a prohormone, which undergoes conversion to other steroids, such as E_2 and DHT.[1] Converted steroids can mute or amplify, and thus diversify, Te's actions through ligand-receptor and coactivator specificities (Fig. 13.5). In particular, DHT binds ERα negligibly,[124] whereas E_2 binds AR with an affinity one order of magnitude less than that of Te.[124] Testosterone directly stimulates AR, but with a potency two to thirtyfold less than that of DHT depending upon target tissue. Thus, by being reduced or aromatized, Te may oppose or potentiate the actions of E_2 on certain genes.

Estradiol acts via two primary (and multiple variant) ERs, whereas Te and DHT act via a single AR. Heterogeneity of tissue responses is presumably conferred via cellular differences in the expression of AR and important corepressors and coactivators. The existence of tissue-selective modulators of nuclear AR action confers a basis for designing pharmacologically selective AR modulators (SARM's) to target anabolism in osteoblasts, erythropoietic precursors, stellate cells in skeletal muscle and brain while exerting little or no effect on adipocytes or the prostate gland.[125-128] A subset of SARM's may be suitable for use in male contraception.[127,129] Compared with Te, DHT is more potent due to both higher affinity for and slower dissociation from AR.[130-132] DHT is considered a pure androgen in that it is nonaromatizable. Only 4% of blood Te is converted to DHT,[119,133,134] less than 1% is transformed to E_2,[114,135] and approximately 2% to androstenedione (Fig. 13.5).[119] Although systemic transformation is minimal, tissue-specific conversion of androstenedione to Te and of Te to DHT or E_2 may be critical in organs expressing AR or ER.[132] For example, systemic Te administration elevates intraprostatic DHT concentrations by providing substrate for local 5α-reductase type II, whereas exogenous DHT lowers prostatic DHT concentrations by suppressing LH and thereby Te secretion.[136] Other hormones such as vitamin D, glucocorticoids, and progesterone also modify 5α-reductase under pharmacological conditions.

The CYP19 gene, located on chromosome 15q21.1, encodes the enzyme aromatase, which converts Te to E_2 and androstenedione to estrone.[137,138] Aromatase is widely distributed in the testis (Leydig cells, Sertoli cells, spermatid lineage), bone, brain, pituitary, liver, intestine, skin fibroblasts, adipocytes, and breast stromal cells.[139] Systemic aromatization predominantly (>60%) proceeds in extraabdominal adipose tissue.[114,140] The coexistence of CYP19 and ER in the brain, pituitary, and testis suggests that locally produced E_2 is physiologically important in these key sites. Evidence favors a role for E_2 and ERα in stimulating libido and mating frequency especially in animals, inhibiting testis descent into the scrotum by repressing the INSL-3 gene, opposing germ-cell apoptosis in older animals, decreasing LH secretion, inhibiting Leydig-cell growth and differentiation, maintaining efferent spermatogenic ductule water reabsorption and endocytosis, and stimulating male breast anlagen (Table 13.2).[141-143] Moreover, transgenic silencing and rare human mutations of CYP19 and ERα have unmasked important roles for E_2 in bone-mineral density, fat-deposition patterns, appetitive behavior, and feedback on LH and FSH. Splice variants and polymorphisms of CYP19 exist,[144,145] which might modify the risks of prostatic cancer or osteoporosis.[145,146]

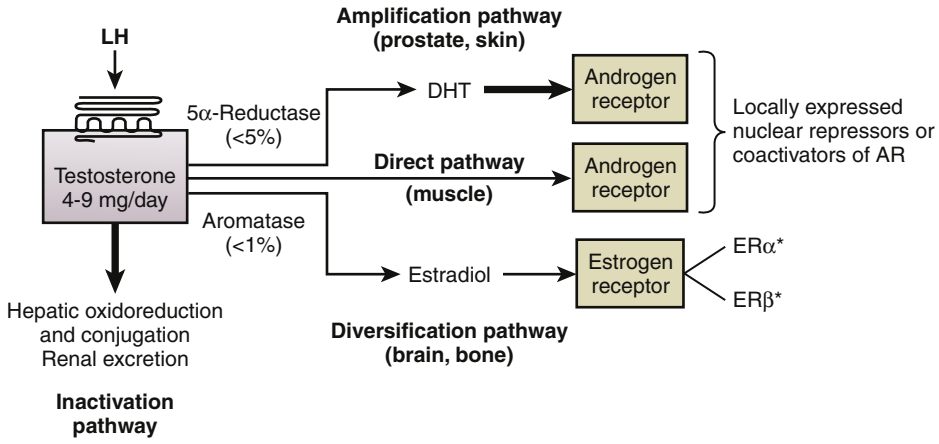


FIGURE 13.5 Amplification, diversification and inactivation of testosterone (Te) via its conversion to dihydrotestosterone (DHT) or E_2, and subsequent receptor-specific drive. *, Potentially activated by DHT metabolites. *(Adapted from Liu PY, Death AK, Handelsman DJ: Androgens and cardiovascular disease.* Endocr Rev *24:313–340, 2003.)*

Table 13.2 Estrogen Effects on Male Gonadal Axis

Action	Mechanism*
↓ GnRH gene transcription	GnRH neuronal ERβ and kisspeptin ERα[351]
↓ Gonadotropin concentrations	↓ LH and FSH pulse size
↓ GnRH-stimulated LH secretion	↓ GnRH efficacy at pituitary level
↓ Te biosynthesis	↓ CYP17A, StAR, and LH receptor[352]
↓ Testis descent	↓ Insulin-like factor 3 in Leydig cells via ERα
↓ Spermatogenesis	↓ FSH by feedback in adult
↓ Epididymal and seminiferous-tubule fluid absorption	Putatively via ERα, which maintains aquaporin-1 expression[355]
↓ Germ-cell apoptosis	Older male animal[356]
↓ Leydig cell proliferation	Fetal and puberal testis[357]
↑ Male sexual arousal and aggressive behavior	Neonatal brain ERα pathways[358]

*Male ERβ-knockout mice are fertile.[202]

Two isoforms (type I and type II) of 5α-reductase are present in the human, encoded by separate genes located on chromosomes 5p15 and 2p23.[131,147] Type I is found mainly in skin, liver, and brain, and type II primarily in male accessory sex glands, liver, and brain.[148] The isoenzymes share approximately 50% structural peptide homology. Polymorphisms of both isoenzymes have been reported, some of which (e.g., V89L) alter enzymatic activity[149] or correlate with sperm output (A49TT and V89LV).[150] Genetic variations in DHT synthesis or catabolism may thus contribute to interindividual variability in spermatogenic suppression by Te in male contraceptive regimens.[151] Prospective assessment of this postulate is needed.[152,153]

Testosterone and DHT are inactivated in the liver, kidney, and prostate by mixed-function oxidoreductases (e.g., 3α- and 3β-hydroxysteroid dehydrogenases) or glucuronidases (conjugation reactions).[154] Three-alpha reduction of DHT decreases its affinity for AR by a factor of 100,000-fold. The oxidoreduced and conjugated metabolites are lost into urine and bile, respectively. Certain metabolites of DHT, such as 5 alpha-androstane-3 beta, 17 beta-diol may mimic E_2 action via ERβ or modulate neurotransmission (Fig. 13.5).[101]

Progesterone

Although gonadotropes and KiSS1 neurons express nuclear progesterone receptors, there is no established role of progesterone in adult human male reproductive physiology. Exogenous progesterone and synthetic progestins can suppress gonadotropin output *in vivo* through the hypothalamic progesterone receptor,[155] and inhibit Te biosynthesis *in vitro* by down-regulating Leydig-cell LH receptors.[156] Reduced metabolites of pregnenolone and progesterone exert inhibitory effects on neurotransmission in experimental models, but the relevance of these actions in men is unknown.

Sex-Steroid Receptors

Sex-steroid hormones are lipophilic, thus allowing passive transmembrane diffusion into the cell before interaction with specific high-affinity nuclear receptors. Whether active transmembrane transport also occurs is possible but unproven. Ligand-receptor binding prompts dissociation of chaperone proteins (heat shock protein [Hsp] 90 and immunophilin Hsp 56), receptor dimerization, and interaction with distinct palindromic DNA sequences known as steroid-responsive elements.[157] Typical motifs consist of hexanucleotide sequences arranged as inverted repeats that are separated by three nonconserved base pairs (GGTACAnnnTGTTCT). The classical genomic mode of action is represented by this pathway, in which steroid-hormone receptors function as ligand-activated transcription factors.

Circumstantial evidence in many cell systems suggests that steroids may also act through nongenomic membrane-associated signaling mechanisms.[158-162] In general, nongenomic pathways are inferred when responses are: (1) too rapid to arise by nuclear effects; (2) evident in cells lacking sex-steroid receptors; (3) persistent in the presence of inhibitors of transcription and protein synthesis; or (4) elicited by steroids bound to substances that preclude entry into the cell.[158-162] When defined in this manner, membrane-associated pathways trigger calcium flux, cyclic AMP generation, and extracellular-regulated or mitogen-activated protein kinases. However, the precise molecular relationship between putative membrane and known nuclear receptors is not clear.

Androgen Receptor in Testes

The androgen receptor (AR) is encoded by a gene located on the X chromosome.[163] Splice variants of the AR exist,[163] but the full-length isoform is the predominant or only form expressed in many tissues including male reproductive organs.[164] In the testis, AR expression is prominent in early-stage Sertoli cells associated with stage III of the spermatogenic cycle,[165] and to a lesser extent Leydig and myoid but not germ cells.[165] Murine knockout models indicate that AR activity is essential in Sertoli and Leydig cells for fertility due to AR's role in spermatogenesis and steroidogenesis, respectively. In contrast, functional AR in peritubular myoid cells is not critical for reproduction.[166-169] Other tissues expressing AR include prostate, seminal vesicles, skin, fat, liver, all three of smooth, skeletal, and cardiac muscle, brain, pineal gland, bone, and cartilage.[139,170-175] Leydig-cell Te stimulates AR in spinal motoneurons, thereby activating gonadal descent into the scrotum via muscles in the outer layer of the gubernaculum (rudder) of the testis.[176] A Leydig cell-derived peptide, insulin-like factor (INSL3), albeit insufficient without Te, is necessary for development of gubernacular ligaments, and prevention of cryptorchidism.[177] INSL3 is a relaxinlike ligand, which signals by the LGR8-GREAT receptor.[178,179]

Inactivating mutations of AR result in the androgen-insensitivity (testicular-feminization) syndrome in the genetic male, and are the most common cause of male intersex.[163,180] Genotype and phenotype are strongly correlated, since mutations that increasingly impair receptor function result in more marked androgen insensitivity. A regularly updated electronic database of AR mutations is available at http://www.androgendb.mcgill.ca. (Accessed 19 July 2012.)

Polymorphisms that modify AR function have been described, such as polymorphic polyglycine (GGC) and polyglutamine (CAG) repeats. Increasing polyglutamine-repeat length is associated with decreasing AR transcriptional activity,[181] impaired spermatogenesis or subfertility,[182] and

genital abnormalities[183] in some but not all studies.[184,185] Polyglutamine-repeat length may contribute to nonuniform suppression of sperm output during male hormonal contraception.[185,186] Selected nuclear coregulators of AR could be targets of tissue-specific androgen-receptor modulators.[187-192]

Estrogen Receptors

The genes encoding ERα and ERβ have been mapped to chromosomes 6 and 14, respectively.[193,194] There are striking similarities in both the DNA-recognition and ligand-binding domains of the expressed receptor proteins. ERα is expressed in pituitary, brain, kisspeptin (but not GnRH) neurons, fat, skin, bone, seminal vesicles, Leydig cells, round spermatocytes, and spermatids,[139,195] whereas ERβ is detectable in gonadotropes; kisspeptin and GnRH neurons; prostatic epithelium; and Sertoli, Leydig, and spermatogonial cells in several mammals.[195-197] Male ERα-knockout mice exhibit elevated LH concentrations due to impaired negative feedback, enhanced Leydig-cell steroidogenesis, and oligo-azoospermia associated with efferent ductule occlusion.[198,199] In contrast, ERβ-disabled mice maintain normal LH and Te concentrations, have decreased germ-cell apoptosis in later adulthood, Leydig-cell hyperplasia, and normal fertility.[194,200-202] ERα and ERβ can act individually or as heterodimers, and can exert opposing or synergistic effects upon target genes, thus creating signaling diversity (Fig. 13.5). Splice variants and polymorphisms are described for both ER subtypes, some of which may be linked to reduced sperm output and infertility.[203,204] Environmental xenoestrogens and phytoestrogens could in principle influence spermatogenesis further in genotypically susceptible individuals.[205] Estradiol may also activate membrane-bound receptors, such as G-protein coupled receptor 30 (GRP30) linked to cyclic adenosine-5-monophosphate (cAMP), [206-207] and others coupled to extracellular-regulated kinases or Ca^{2+}.[208-210] Some but not all E_2 actions depend upon estrogen-response elements (ERE). For example, ERα and ERE are together necessary to maintain male sexual behavior, whereas either ERα or non-ERE-mediated pathways can suppress LH and Te secretion in mice.[211]

Progesterone Receptor

A single gene located on chromosome 11q22-3 encodes two progesterone receptor isoforms (termed A and B) through alternative transcriptional initiation sites.[212-214] The isoforms exert reciprocal effects via interactions with selected DNA motifs, defined as progesterone-responsive elements. Progesterone receptors are expressed widely including in KiSS1 neurons, gonadotropes, breast, brain, and adipose tissue.[213,215] Whether progesterone receptors are expressed in the testis is debated. Although physiological actions of progesterone remain to be elucidated in men, pharmacological amounts of progesterone and progestins suppress gonadotropin output, which has utility in the design of male hormonal contraception.[155]

Sex Hormone-Binding Globulin (SHBG): Steroid Transporter and Putative Ligand

SHBG in the male primate is expressed in the liver, adrenal cortex, prostatic alveoli, epididymides, and seminiferous tubules.[216] Infusion of SHBG acutely reduces the metabolical clearance of Te and E_2 in male monkeys.[217] Crystallographic studies show that SHBG is a homodimer structurally similar to the pentraxin family of proteins. Each monomeric subunit contains one hydrophobic steroid-binding pocket as well as tandem laminin G-like domains near the amino (N) terminus that are critical for dimerization.[218-220] Homodimerization is induced by the presence of steroid ligand, and stabilized by calcium ions. Both potential steroid-binding regions in the dimer are occupied by Te.[221] SHBG contains several glycosylation sites, which give rise to multiple posttranslational isoelectric isoforms,[222] and by common variants (polymorphisms) or mutations that alter glycosylation sites resulting in inefficient secretion or metabolical clearance of secreted SHBG.[223-225] Mutagenesis studies suggest that glycosylation sites in the C-terminus of SHBG may be important for cell-surface recognition.[218]

Te, E_2, and progesterone bind SHBG[218,226] and to a lesser degree corticosteroid-binding globulin.[227] About 45% of blood Te is bound to high-affinity SHBG, 4% to corticosteroid-binding globulin, and the remaining 50% to lower-affinity high-capacity binding proteins, such as albumin. Approximately 1% to 2% remains free-unbound.[228] DHT and anabolical steroids have greater affinity for SHBG than Te.[228] Although of higher affinity, the 1000-fold lower molarity of plasma E_2 than Te in men limits any substantial depression by E_2 of Te's binding to SHBG at physiological pH and temperature.[229,230] Low-affinity binding of Te (or E_2) to albumin may create a reservoir from which free Te (or E_2) arises rapidly due to an equilibrium dissociation half-time of about 0.2 second compared with 3.8 seconds for SHBG.[226,231] Significant one-pass dissociation is inferable in the liver, brain, and splanchnic circulation,[118] but whether capillary-bed dissociation kinetics are organ selective is unknown. In one study, experimentally controlled free, bioavailable, and total Te concentrations equally predicted feedback onto GnRH-LH outflow in healthy men.[232]

The traditional perspective is that protein binding mediates steroid-hormone retention and transportation in plasma, and restricts metabolism and inactivation.[233] This view has been challenged by recent experimental data,[234-236] since SHBG and albumin-bound steroids may also be biologically available for tissue-specific uptake.[226,231,237] Although only free (unbound) hormone is believed to enter the cell by diffusion, SHBG may associate with presumptive high-affinity membrane-binding receptors, such as megalin, which may facilitate SHBG-bound Te's entry into cells.[238] Mutagenetic studies suggest that certain glycosylation sites in the C-terminus of SHBG are important for cell-surface (albeit not necessarily megalin) receptor recognition.[218] Megalin-knockout mice exhibit apparent androgen resistance, inferable from impaired testicular descent into the scrotum.[235] Genetic studies also show that the risk of type 2 diabetes is influenced by certain polymorphisms of the SHBG gene,[239] which may not be related to SHBG-steroid binding. In addition, occupancy of membrane-bound SHBG by Te, DHT, or E_2 may activate intracellular Ca^{2+} or cAMP signaling purportedly via G-protein coupled and mitogen-kinase pathways.[240]

SHBG and testicular androgen-binding protein (ABP) are both encoded by a single gene located on the short arm of chromosome 17.[241] The proteins differ in their sites of production (SHBG by hepatocytes, brain, and placenta and ABP by Sertoli cells and possibly brain), as well as oligosaccharide composition.[218,242] SHBG secretion by hepatocytes is induced prominently by estrogens, thyroxine, and some anticonvulsant drugs and repressed by insulin, growth hormone-IGF-I, and nonaromatizable androgens. The presumptive intratesticular role of testicular ABP is to maintain high intratubular total Te concentrations required for spermatogenesis.[243-245] This notion is endorsed by the findings of increased germ-cell apoptosis and decreased fertility after transgenic overexpression of ABP in mouse testes.[243]

Physiology of Hypothalamo-Pituitary-Testicular Network

None of kisspeptin, GnRH, LH, or Te acts alone. Rather all four signals are interlinked in a network or ensemble (Fig. 13.6A). Pulsatile GnRH secretion drives pulsatile LH secretion, which stimulates pulsatile Te production.[246-248] Direct sampling of human spermatic-vein blood suggests that: (1) Te feeds back onto both LH and FSH secretion within 30 to 45 minutes (LH) or longer (FSH); (2) LH, but not FSH, feeds forward onto Te secretion after a delay of 40 to 90 minutes; and (3) secreted Te distributes rapidly among free (aqueous), albumin-bound and SHBG-bound compartments (Fig. 13.6B).[111,248-250] These concepts form the basis for constructing biomathematical models of time-delayed, nonlinear interactions among GnRH, LH, and Te with ultradian (pulsatile) and circadian (24-hour rhythmic) features.[111,232,251] Further model advances require inclusion of locally modulated Te actions via ER subtypes, AR, and membrane receptors.

Both GnRH outflow and LH secretion are subject to feedback inhibition by Te and E_2. Te feedback is substantially,[40,252] but not completely,[41,253] mediated by aromatization to E_2 systemically and/or within the hypothalamo-pituitary unit. Exogenous E_2 by acting on

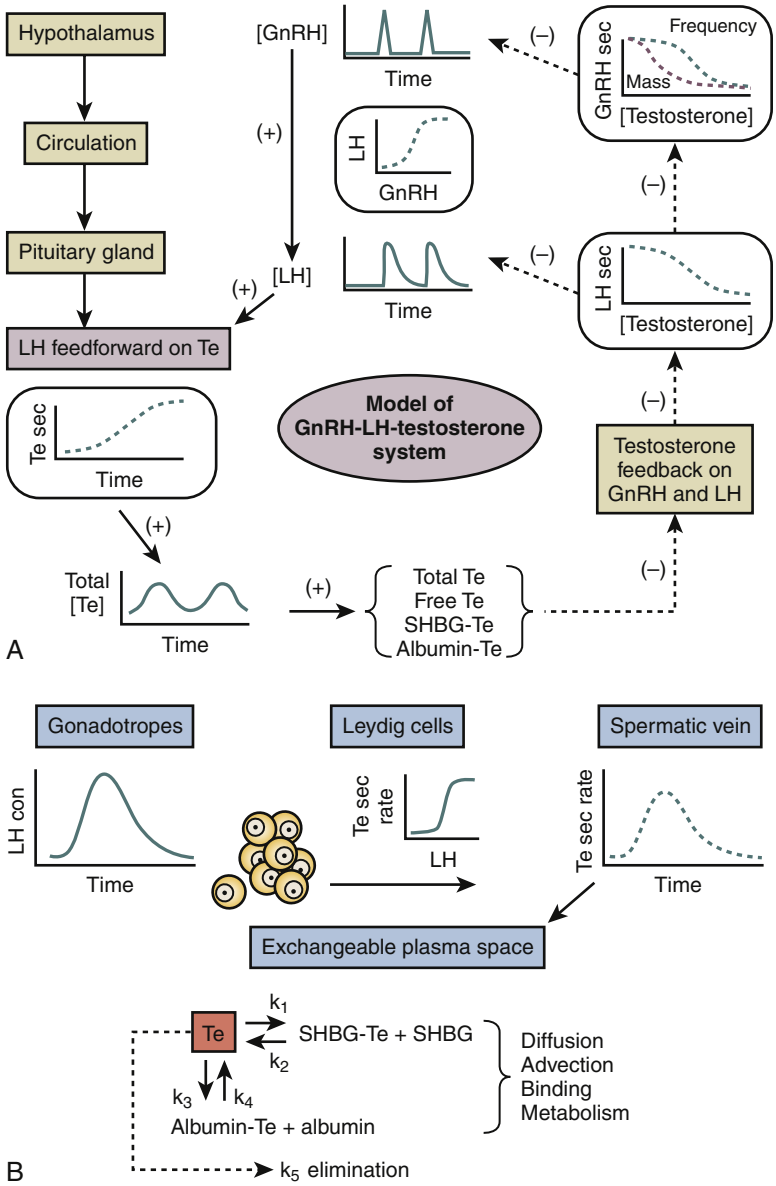


FIGURE 13.6 **A,** Ensemble construct of GnRH → LH and LH → Te feedforward and time-delayed Te → LH and Te → GnRH feedback. No single pathway or signal acts alone to maintain male reproductive function. **B,** Schema of regulated secretion, plasma distribution, protein binding, and elimination of secreted Te by LH pulses. GnRH, gonadotropin-releasing hormoe; LH, luteinizing hormone; sec, secretion; SHBG, sex hormone–binding globulin; Te, testosterone. *(From Keenan DM, Veldhuis JD: Divergent gonadotropin-gonadal dose-responsive coupling in healthy young and aging men. Am J Physiol 2004; 286:R381–R389. From Keenan DM, Veldhuis JD: Divergent gonadotropin-gonadal dose-responsive coupling in healthy young and aging men.* Am J Physiol *286:R381–R389, 2004.)*

the pituitary reduces LH pulse amplitude without altering pulse frequency,[254] whereas exogenous Te by acting on the hypothalamus and the pituitary gland decreases GnRH-LH pulse frequency and amplitude.[41] Conversely, depletion of endogenous E_2 concentrations with an aromatase inhibitor or blockade of E_2 action with an ERα antagonist augments LH pulse size and number,[40,255,256] therein mimicking the effects of nonsteroidal antiandrogens.[253,257] Inasmuch as E_2 depletion but not E_2 excess modulates GnRH-LH pulse frequency, endogenous estrogenic inhibition of the GnRH pulsing process (but not GnRH-driven LH pulse size) may be already maximal in normal men.

The characteristics of Te delivery may modulate inhibition of LH, since greater suppression is enforced by continuous than pulsatile Te infusions.[258] This outcome supports the male hormonal-contraceptive approach of sustained Te administration to suppress LH and thereby intragonadal Te concentrations. Endogenous DHT plays a lesser role in feedback onto GnRH and LH, given that patients with 5α-reductase type II deficiency exhibit minimally elevated LH concentrations.[259] Administration of a potent combined type I and II 5α-reductase inhibitor, dutasteride, in healthy young men, reduced systemic DHT concentrations by 95% and stimulated basal (constitutive) LH (and Te) secretion.[260] This mechanism could explain the rise in all three of mean, nadir, and peak LH concentrations in patients with 5α-reductase deficiency.

FSH concentrations are also regulated by negative feedback. Aromatization of Te to E_2 appears to be critical because: (1) combined deprivation of Te and E_2 and isolated depletion of E_2 elevate FSH concentrations to comparable degrees[36]; (2) physiological replacement of either Te or E_2 in hypogonadal men normalizes FSH concentrations; and (3) an aromatase inhibitor fully reverses suppression of FSH by Te.[40] Although nonaromatizable androgens can also suppress FSH and LH secretion, the study paradigms have been pharmacological rather than physiological.[41,261-263]

Nonsteroidal testicular products, such as inhibin B secreted by Sertoli cells, may be involved in feedback regulation in animals.[37] In humans, inhibin B concentrations exhibit a multimodal lifetime pattern. Values in umbilical cord blood are comparable to peripheral-venous levels in men, increase further in infancy, decline during subsequent childhood, rise in puberty, and fall by 25% by the eighth decade of life.[264] The changes are paradoxically concordant (birth through puberty) and reciprocal (adulthood) with FSH, LH, and Te secretion, indicating that other factors modulate both FSH and inhibin B. Albeit supported by corollary data, the role of inhibin B in restraining FSH production *in vivo* has been inferable only in the adult male rodent and monkey. To date, no clinical studies have infused or neutralized inhibin B in men, and no human mutations of inhibin B or its receptor have been described. The same qualifications apply to the roles of activin A, a homodimer that stimulates FSH-beta gene expression, and follistatin, a linear peptide that binds to and antagonizes feedforward by activin.[265,266] Inhibins and activins signal via transforming growth-factor (TGF) beta receptors and β-glycan coreceptors.[265] TGF receptors also strongly regulate cell proliferation, apoptosis, tumorigenesis, and hematopoiesis.[267] Contraceptive applications of safe and effective mimetics of inhibin B and antagonists of activin A remain to be exploited.

The Testis

The adult testes are paired ovoid structures suspended in the scrotum by the spermatic cord. Prepubertal testis volume is less than 7 mL, and postpubertal greater than 12 mL. Each is encapsulated by the fibrous tunica albuginea (white cloak), which is invaginated posteriorly to form the mediastinum testis perforated by neurovascular and lymphatic vessels and efferent ductules. Efferent ductules form from the rete (net-like) testis, which represents the convergence of all seminiferous tubules. Fibrous septa project from the tunica albuginea into the testis dividing it into 250 to 300 lobules. Each lobule contains 1 to 4 segments of seminiferous tubules. The latter develop at the time of puberty through canalization of the seminiferous cords. Peritubular-myoid and Sertoli-cell interactions mediate formation of seminiferous cords. Spermatogenic tubules are lined internally by Sertoli cells, which support the proliferation and differentiation of spermatogonial cells by secreting trophic and prosurvival factors, like stem-cell factor (*c-kit* ligand), which nourish and inhibit apoptosis of germ cells.[268] Sertoli cells protect spermatogonia by maintaining a unique tubular microenvironment through tight junctional complexes that constitute the blood-testis barrier. Conversely, spermatogonia and peritubular myoid cells both signal to Sertoli cells.[269,270] Sertoli cells abut a basement membrane, which is lined externally by peritubular myoid cells. Myoid cells are surrounded by an interstitium containing Leydig cells, lymphatics, venules, and nerve fibers. Peritubular myoid, Leydig, and Sertoli cells together regulate spermatogenesis.

Testes develop intraabdominally, and descend into the scrotum perinatally. Insulin-like 3 induction of gubernacular development is essential for transinguinal migration.[271] Male-sex determination and testicular morphogenesis are orchestrated by the SRY (sex-determining region of the Y chromosome), which is both necessary and sufficient for testis formation (Fig. 13.7). SRY in turn activates a family of homeobox-related genes, in particular Lim1 and SOX9, as well as steroidogenic-factor 1 (SF1). SF1 is a member of the NR5A subfamily of nuclear receptors required for testis, adrenal, and gonadotrope development. Mutations in SRY, SOX9, and SF-1 or its repressor, the dosage-sensitive X-linked sex-reveral (DAX1) gene, are responsible for the majority of known causes of aberrant sexual development.

Accessory Organs

Under the influence of Te, embryonic wolffian (mesonephric) ducts develop into the internal male reproductive tract, namely the epididymis (upper segment), vas deferens (middle) and ejaculatory duct, and seminal vesicles (lower). By the fourth week of embryonic life, the ejaculatory ducts arch anteriorly to join the ventral portion of the cloaca. The cloaca subsequently differentiates into the urogenital sinus. DHT is required for development of the prostate gland, urethra, scrotum, and penis from the urogenital sinus.

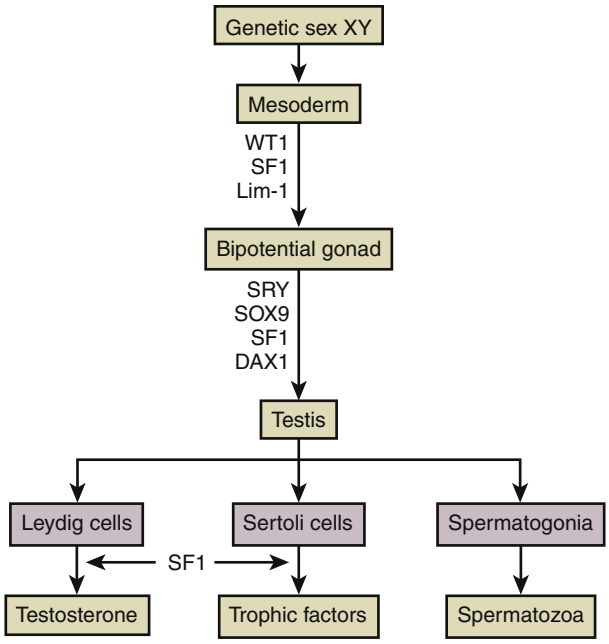


FIGURE 13.7 Critical embryogenetic determinants of testis formation given the genetic sex, 46 XY. The bipotential gonad of mesonephric origin must be directed definitively by SRY (sex-determining region of Y chromosome). SOX9 denotes SRY-related HMG-box 9, WT1 Wilms' tumor, DAX1 dosage-sensitive sex-reversal, SF1 steroidogenic factor 1, and DMRT sex-reversal gene on 9p. Further differentiation of the primitive male gonad entails development of Leydig, Sertoli, and germ cells. Testis formation and descent can be disrupted via estrogen.[353,354]

Vas Deferens

Vasa deferentia transport sperm by means of muscular layers that propel spermatozoa during ejaculation. In the absence of ejaculation, spermatozoa stored in each vas deferens dribble through the terminal dilated ampulla into the ejaculatory duct where they are washed away during micturition. The distal urethral meatus discharges urine or semen from the body.

Epididymis

The epididymis is formed from a single duct through which all spermatozoa pass, mature, gain fertilizing capability, and become motile before entering the vas deferens and ejaculatory duct. The human epididymis is 4 to 5 cm long and consists of the caput, corpus, and cauda. The cauda in man appears to differ from that in other species by way of more rapid transit of spermatozoa (2 to 4 days) and a less differentiated initial segment. Peptides and non-proteinaceous molecules are secreted into the epididymal lumen by principal cells. Although little is known in any species about precise regulation of luminal fluids that modulate sperm maturation and motility, microarray profiling of human epididymal tissue has demonstrated highly regionalized gene expression with maximal complexity in the caput and least in the corpus.[272] The maturation process is complete by the time spermatozoa enter the proximal (caudal) epididymis and are able to hyperactivate, bind the zona pellucida of the oocyte, and undergo the acrosome reaction.

The micromilieu of spermatozoa stored in the cauda epididymidis remains poorly understood. Ductal mucosa must play a critical role in controlling solute concentrations and degree of sperm hydration. Muscular cells exist only in the cauda epididymides and ductus deferens, and hence repeated ejaculation increases the rate of sperm transport from these regions only.

Epididymal growth, development, and function are dependent on androgens, estrogens, and other nonsteroidal luminal factors. Luminal androgen concentrations exceed those in peripheral blood, and for this reason systemic androgen exposure may not be critical for epididymal development and function. Amplification of luminal Te effects occurs by local synthesis of DHT via gonadal 5 α-reductase type II.

Seminal Vesicles

The seminal vesicles secrete the majority of fluid found in the ejaculate. Secretion includes fructose, prostaglandin, semenogelin I (responsible for coagulation of the seminal fluid), reactive-oxygen scavengers (superoxide dismutase, catalase), and immunoglobulin G receptor III. During embryogenesis, androgens transform simple saccular anlagen into active secretory epithelium by branching morphogenesis, yielding a highly folded internal structure with an expanded surface area. The precise molecular mechanisms are not yet defined.

Prostate Gland

The prostate is the largest accessory gland, but unlike the epididymis and seminal vesicles, it arises from the urogenital sinus rather than mesonephric ducts and requires DHT action (Fig. 13.8). Prostatic fluids, which comprise about one-third of the volume of the ejaculate, contain high concentrations of zinc, citric acid, choline, and proteins such as acid phosphatase, seminin, plasminogen activator, and prostate specific antigen (PSA). PSA is an important blood marker of prostate size and proliferation. PSA concentrations are elevated in benign prostate hypertrophy, inflammation, and prostatic cancer. The prostate gland develops from distinct sets of tubules that initially form discrete lobes, which become confluent by adulthood. Thus, the adult prostate gland is generally divided into peripheral, central, and transitional zones according to morphological and functional properties rather than lobular structure. The peripheral zone is subcapsular and surrounds the distal urethra, the central zone encircles the ejaculatory ducts, and the transition zone envelops the proximal urethra. The peripheral and central zones comprise respectively 70% and 25% of total prostate-gland volume in young men, accounting for equivalent proportions of prostate cancer in later life. In contrast, prostate cancer rarely arises from the transition zone (5% of volume), where benign prostate hypertrophy usually develops.

Spermatogenesis

Spermatogenesis is a dynamic, complex process contingent on systemic hormone support and local cellular, autocrine, and paracrine interactions directed *ab initio* via SRY and SOX9.[273,274] Sperm production proceeds in an orderly stage-specific fashion in all species (Fig. 13.9).

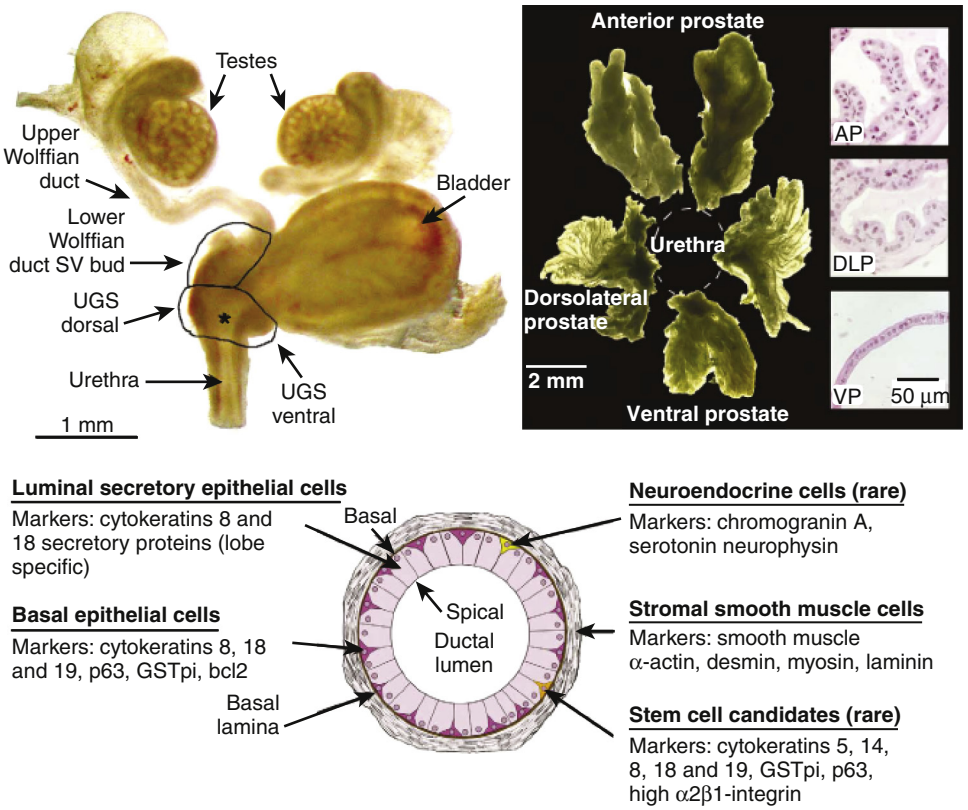


FIGURE 13.8 Micromorphometric features of the prostate gland. Portion of the urogenital tract from a male embryonic mouse day 16.5 *(top left)*. The prostate develops from the urogenital sinus (UGS), which is located at the base of the developing bladder. UGS epithelium is visible as a dilation of the urethra *(lighter area containing asterisk)* surrounded on the dorsal and ventral sides by condensed UGS mesenchyme *(darker areas)*. AP, anterior prostate; DLP, dorsolateral prostate; SV, superoventral; VP, ventral prostate. Lobes of the adult mouse prostate *(top right)* together with hematoxalin and eosin-stained sections of prostatic ducts from each lobe *(inset micrographs)*. Each lobe has a distinct shape and histological appearance. Diagram of a ductal cross-section *(bottom left)* with labels indicating cell types present in prostatic ducts, *viz*., luminal secretory epithelial cells, basal epithelial cells, neuroendocrine cells, stromal smooth-muscle cells, and stem-cell candidates. Below the label for each cell type is a list of differentiation markers commonly used to distinguish these cell types. Prostatic epithelial stem-cell candidate markers include cytokeratins 5, 14, 8, 18 and 19, GSTpi, p63, and α2β1-integrin. *(Adapted from Marker PC, Donjacour AA, Dahiya R, Cunha GR. Hormonal, cellular, and molecular control of prostatic development.* Dev Biol. *2003;253[2]:165–174, Fig. 1.)*

Seminiferous tubules exhibit a stratified and layered arrangement of germ cells wherein spermatogonia rest on the basement membrane and more mature germ cells fill the apical luminal regions. In humans and some other primates, stages follow an intertwined helical pattern so that a single tubular cross-section may contain up to six distinguishable stages.

Embryonic spermatogonia develop from primordial germ cells, which first appear in the yolk sac and then migrate to the hindgut and genital ridge during the fourth week of fetal life to form sex cords. The resultant seminiferous tubule permits spermatogenesis to proceed via four basic stages: development and repopulation of spermatogonia from stem cells accomplished via mitotic divisions that renew the stem cell-population and yield type A and B spermatogonia; meiosis requiring DNA synthesis and two meiotic divisions to produce haploid spermatids, specified as primary and secondary spermatocytes; spermiogenesis, defined by development and differentiation of the spermatid head and tail; and spermiation, denoting release of mature spermatozoa into the seminiferous tubule lumen.[275-279]

Type A spermatogonia are divided into A dark and A pale according to morphological criteria of chromatin staining. The A-dark spermatogonia exhibit uniform dark nuclei and are presumptive adult stem cells, but other theories claim that both A-pale and A-dark spermatogonia are capable of self-renewal.[275] Spermatogonial niches in the seminiferous tubules are thought to provide the necessary microenvironment to maintain self-renewal in the presence of relevant systemic and locally secreted Sertoli-cell factors. Endocrine, paracrine, and autocrine signals to spermatogonial mitosis include FSH, retinoic acid, stem-cell factor (*kit* ligand) and glial cell line-derived neurotropic factor. The last two peptides are produced by Sertoli cells. Inactivating mutations of the C-KIT gene (stem-cell factor receptor) cause azoospermia, bone-marrow failure, and absence of mast cells.[280,281]

Type B spermatogonia are characterized by granulated nuclear chromatin and a nucleolus, consistent with greater differentiation and commitment to meiosis. Meiosis requires 3.5 weeks in man, comprising a prophase (leptotene, zygotene, pachytene, and diplotene), metaphase and anaphase. Cells undergoing meiosis are primary spermatocytes, in which the preleptotene stage is characterized by DNA synthesis (occurring for the last time during spermatogenesis). Chromosome number remains diploid but DNA content doubles in association with finely granulated and uniformly

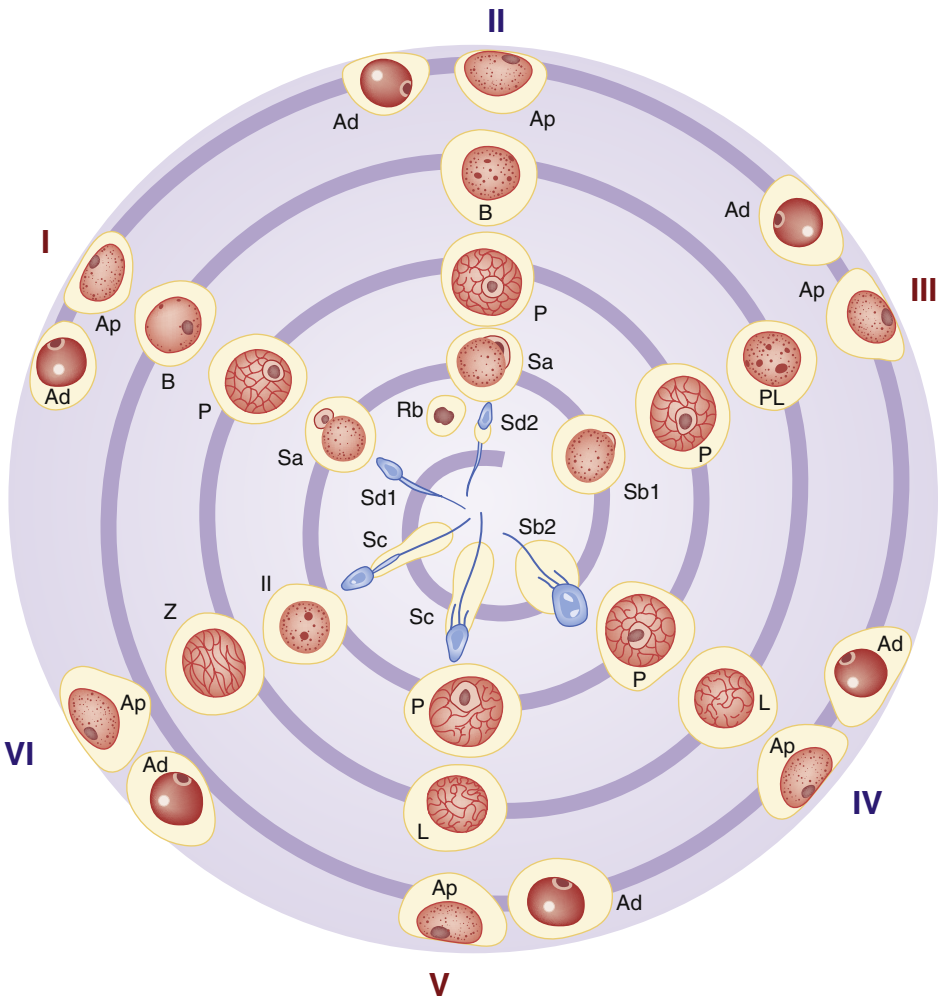


FIGURE 13.9 Spermatogenic cycle. Schematic representation of the six stages of the human spermatogenic cycle and with associated germ cells positioned to show sequence of development as a spiral pathway of ever-increasing radius. In actual tubules, some cell associations would be more superficial or deeper levels. Ad, Ap and B denote, respectively, dark and pale type A and B spermatogonia; PL, L, Z, P and II, preleptotene, leptotene, zygotene, pachytene and meiotic division I and II spermatocytes; Sa, Sb1, Sb2, Sc, Sd1 and Sd2, steps in spermiogenesis; Rb, excess residual body cytoplasm. *(From Kerr JB. Functional cytology of the human testis.* Baillieres Clin Endocrinol Metab *6[2]:235–250, 1992, Fig. 4.)*

distributed nuclear chromatin. Spermatocytes then lose contact with the basement membrane of the tubule (Fig. 13.9). Leptotene (thin and thread-like) spermatocytes begin in prophase, and then gradually enter the zygotene phase when homologous chromosomes pair and synapse. Cells continue to increase in volume until the pachytene (large size) stage of premeiotic chromosomal recombination that introduces genetic diversity. The subsequent first meiotic division produces secondary spermatocytes, in which chromosome number is halved thereby defining haploidy, and DNA content per cell is reduced from 4N to 2N. Secondary spermatocytes are rarely identified histologically, because the second meiotic division follows rapidly without an intervening S phase of DNA synthesis to form 1N spermatids.

Round and elongated spermatids undergo spermiogenesis, which can be divided into three interlinked phases referred to as Golgi, acrosomal, and maturational. Protein synthesis increases markedly during the Golgi stage, and the acrosome forms by coalescence of proacrosomic vesicles from the Golgi apparatus. Concomitantly, the nucleus of round spermatids condenses. Elongated spermatids emerge by further structural remodeling and differentiation. Although morphological features are used to stage spermatid development, spermiogenesis is a continuous, not a discrete, process. As acrosome formation proceeds at one nuclear pole to eventually form the head of the spermatozoon, centrioles gather at the opposite pole to form the flagellum (tail). The tail consists of the middle piece, principal piece, and end piece. Mitochondria migrate along the developing axoneme, the main cytoskeletal component of the sperm tail. Collapse of cytoplasmic volume results in a residual cytoplasmic body containing obsolete organelles and redundant nuclear membranes.

As the spermatid elongates, an ectoplasmic specialization orients the flagellum toward the seminiferous-tubule lumen. The ectoplasmic specialization is a unique cell to cell junction joining the plasma membranes of the spermatid acrosome and the Sertoli cell. Spermiation, or release of spermatids into the lumen of the seminiferous tubules, involves disassembly of these ectoplasmic specializations. During spermation, the residual cytoplasmic body is retained by Sertoli cells, and eventually phagocytosed.

Meiosis and spermiogenesis are hormonally regulated in rodents, whereas spermatogonial division and spermiation are so regulated in primates. Adequate FSH and LH secretion and action are required to initiate spermatogenesis in primates. The relative importance of FSH unlike Te in maintaining spermatogenesis remains controversial.[282,283] LH elevates intratesticular Te concentrations by sixtyfivefold more

than systemic Te concentrations, as is necessary to maintain spermatogenesis. Sertoli cells express an FSH-inducible aromatase system, thus permitting intratubular synthesis of E_2 as well. Te stimulates Sertoli cells expressing AR rather than germ cells, which probably do not express AR.[284]

Sertoli Cells

Adult Sertoli cells are nondividing asymmetric columnar cells with an elongated typically tripartite nucleus, a prominent nucleolus, abundant smooth endoplasmic reticulum, Golgi complexes, mitochondria, lysosomes, and cytoskeletal components. Immature Sertoli cells proliferate during fetal and neonatal life until the age of 12 to 18 months, and then again during the peripubertal period.[285] Secretion of müellerian-inhibiting substance by fetal Sertoli cells is critical for the regression of embryonic müellerian ducts, leaving the prostatic utricle as a remnant. Proliferation and maturation are subject to endocrine control. FSH stimulates proliferation whereas Te and thyroid hormones regulate maturation of Sertoli cells. With the neonatal surge of LH, FSH, and Te, the two Sertoli-cell products, inhibin B and MIS, both rise.[286] Sertoli cell number dictates maximal spermatogenic capacity in the adult, because each Sertoli cell can support only a fixed species-defined number of germ cells.

Estradiol and multiple proteins are secreted by Sertoli cells and serve as local paracrine signals, which further regulate spermatogenesis or Leydig-cell steroidogenesis.[91] For example, Sertoli cell-derived IGF-I induced by systemic delivery of FSH to the testis enhances LH-stimulated Te production synergistically, whereas Sertoli cell-secreted IGFBP-3 mutes IGF-I action.[121] Other systemic hormones that can act on the testis include interleukins, leptin, growth hormone, l-thyroxine, and cortisol.[287-289] In the human, a large amount of cortisol inhibits whereas growth hormone does not affect Te secretion.[290] Intratesticular mediators may further direct developmental and stress-related adaptations in gonadal function via local release of E_2, activin A, inhibin A, follistatin, atrial natriuretic hormone, catecholamines, ghrelin, growth-hormone releasing hormone, corticotrophin-releasing hormone, neuropeptide Y, serotonin, and arginine vasopressin.[291-295] In particular, E_2 is required for spermatogenesis, in contrast to its better known inhibitory effects on the hypothalamo-pituitary unit,[202] whereas activins influence apoptosis, proliferation, and differentiation.[267]

In humans, the spermatogenic cycle (transit time from spermatogonia to ejaculated spermatozoa) is 60 to 70 days according to kinetic studies using stable isotopes.[276,296] This cycle length is shorter than earlier estimates of 90 days.[297] Cross-species germ-cell transplantation experiments suggest that cycle length is dictated by the germ cell, rather than Sertoli cell.[298] Physiological principles of spermatogenesis confer a basis for understanding male-directed (hormonal and nonhormonal) contraceptive methods, since repression of spermatogonial proliferation only becomes evident 2 months later in ejaculated sperm.

Regulation of Male Fertility

Child rearing imposes major life demands, and consequently fertile couples practice family planning to obviate unintended pregnancies and to conform childbearing with personal desires and financial abilities. Accordingly, broadening contraceptive choices to allow men and women the option to share family-planning responsibilities satisfies important individual and societal needs. Surveys reveal that effective and safe male-directed contraceptive methods, if available and reversible, would be acceptable to many men and women.[299,300]

Hormonal methods that exploit exogenous feedback-based suppression of gonadotropin secretion, analogous to ovulation inhibition by combined estrogen-progestin contraceptives, are effective, reversible, and safe in the short term.[301-306] Androgen or androgen-progestin treatment combinations promote germ-cell apoptosis and inhibit spermatogenesis, resulting in azoospermia (no sperm in ejaculate) or near-azoospermia (≤1 million sperm/mL semen) with acceptable safety parameters.[307] Dual-hormone regimens are more effective and Caucasian populations more responsive than Asian cohorts.[308] Marked suppression of sperm output confers contraception with efficacy rates of 97% to 100%.[301-304] Subsequent restoration of spermatogenes to levels consistent with normal male fertility has been observed in all men.[305]

Additional approaches to male contraception are under active investigation. Long-acting GnRH-receptor antagonists with Te addback are candidate agents. Epididymal targets are plausible, because spermatozoal maturation is critical for fully motile sperm.[309,310] Other studies have explored contraceptive vaccines using GnRH or zona-pellucida (oocyte) proteins as antigenic targets.

Decremental Changes in GNRH-LH-TE Axis in Aging

A syndromic definition of late-onset hypogonadism requiring three sexual symptoms (decreased sexual interest and morning erections and erectile dysfunction) in conjunction with both total testosterone below 11 nmol/liter and free testosterone below 220 pmol/liter has been suggested.[311] However, the fundamental mechanisms that cause hypoandrogenemia in the aging male remain unknown. In fact, recent animal experiments and clinical investigations have disclosed multiple alterations in the aging gonadal axis, *viz.*: (1) lower amplitude LH pulses, suggesting reduced drive by GnRH or excessive sex-steroid inhibition; (2) more frequent LH pulses and less orderly patterns of LH release, pointing to diminished negative feedback; (3) preserved LH and heightened FSH responses to exogenous GnRH pulses, indicating intact gonadotrope function; (4) reduced pulsatile and thereby total daily Te secretion; and (5) impaired Te secretion in response to both elevated endogenous LH concentrations (stimulated by flutamide, tamoxifen, GnRH, or anastrozole) and infused pulses of recombinant human LH.[96,312,313] These alterations are summarized in Figure 13.10.

Collective clinical findings allow the hypothesis that relative androgen deficiency in older men reflects multisite failure of the GnRH-LH-Te axis. This postulate has not yet been tested in individual men. One noninvasive strategy is to exploit an integrative biomathematical model of reciprocal interactions among GnRH, LH, and Te via estimable feedback and feedforward interactions.[251] The analytical rationale is to reconstruct aging-related adaptations among all three interlinked signals, rather than any one

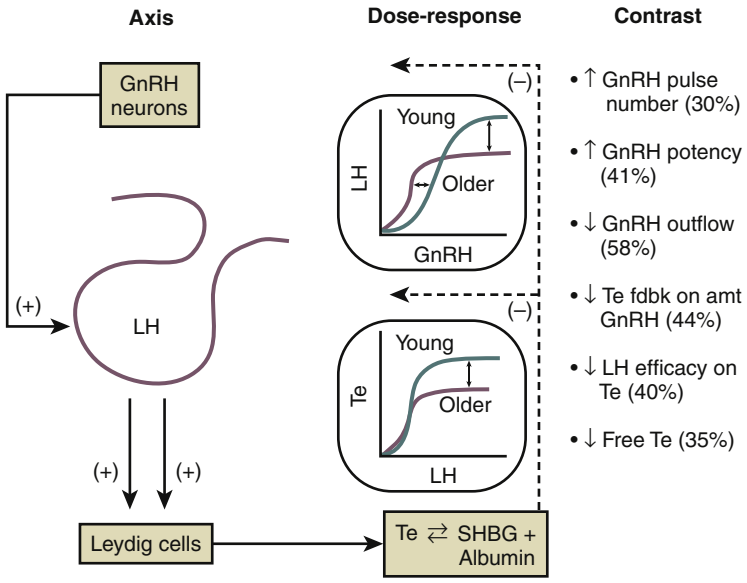


FIGURE 13.10 Schema of inferred anatomical sites *(left)* and adaptive mechanisms *(right)* that subserve relative testosterone deficiency in healthy older (age >60 years) compared with young (age <35) men. Arrows define stimulation (+) and inhibition (–). The two circled insets depict age-related contrasts in GnRH→LH drive *(top center)* and LH→Te feedforward *(bottom center)*. *(Adapted from Liu PY, Takahashi PY, Nehra AX, et al. Neuroendocrine aging: pituitary-gonadal axis in males. In Squire, LR. Encyclopedia of Neuroscience.* Oxford, Academic Press *6:317–326, 2009, Fig. 8.)*

signal in isolation.[232] According to such ensemble analyses, the most parsimonious explication of available clinical data is that aging (1) attenuates hypothalamic GnRH outflow, (2) impairs testicular responsiveness to LH pulses, and (3) decreases androgenic negative feedback.[111,232] Longitudinal studies will be required to establish the relative importance of inferred regulatory deficits in healthy aging men. In addition, the degree to which mechanisms associated with aging emerge in other clinical syndromes of hypogonadotropic hypogonadism, such as those associated with cardiovascular disease,[314] visceral adiposity, type II diabetes mellitus, uremia, hepatic failure genetic anomalies,[315] or chronic stress-anxiety states, has not been investigated.

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The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 14

Immunology and Reproduction

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The immune system may act as friend or foe. Fortunately, the human body has elaborate mechanisms to protect the individual from foreign pathogens. During development, the immune system is programmed and learns to distinguish self from non-self. The realization that a substantial number of potentially autoaggressive cells reside in healthy individuals has prompted the focus of attention on mechanisms of regulation that prevent autoimmune pathology. Despite the safeguards of the immune system, some self-reacting lymphocytes are not inactivated or killed and can cause one of several illnesses known as autoimmune diseases. Immunologists are well aware that if they understood all the mechanisms for tolerance induction, they might be able to prevent autoimmune diseases.

The immune system is capable of recognizing foreign proteins whose chemistry is only slightly different from its own molecules using three critical processes:

- Specific methods for recognition of foreign antigens involving antigen-specific receptors on B-lymphocytes and T-lymphocytes
- Exertion of control through the idiopathic network at both the T-cell and B-cell levels
- Processing and presentation of self-antigens, particularly as they relate to the induction of tolerance during ontogeny, are critical to the regulation of autoimmunity

A major focus of attention is on the three-party interaction of antigen plus the major histocompatibility complex plus T-cell receptor.

The failure of inherent mechanisms to prevent the recognition of "self" antigens by the immune system may result in an autoimmune response. Thus, *autoimmunity* refers to an immune reaction of the body against substances that are normally present in the body. To understand autoimmunity, it is important to first understand tolerance. *Tolerance* can best be defined as a state of antigen-induced immunological unresponsiveness. (Autoimmunity can be considered a failure of tolerance.) Tolerance is not synonymous with generalized immunosuppression; it is antigen-specific and causes no impairment of the immune response to antigens other than the ones used to induce tolerance.

Because tolerance is antigen-specific, it must involve the T lymphocyte, B lymphocyte, or both clone(s) specific to the antigen in question. At the clonal level, tolerance can only result from clonal deletion or clonal anergy. The clonal deletion theory states that T cell clones reactive to self-antigens are positively eliminated in the thymus during development. The clonal anergy theory states that self-reactive clones may exist but are in a long-term state of unresponsiveness. The suppressor T cell theory states that the peripheral auto reactive T cells are controlled by specially organized T cells.

Implantation is one of the most important aspects of pregnancy that immunological studies have targeted. Implantation represents a critical developmental process in that it requires the interaction of immunologically and genetically distinct tissues. The immune system may influence pregnancy success or failure during any of the critical steps of implantation. Blastocyst attachment occurs only during the "implantation window," the time period when the uterine epithelium is receptive. Failure of this synchronization precludes success, as demonstrated in human studies of implantation.

Implantation is the most important limiting factor in human reproduction. Only 25% of all fertilized ova will generate a live birth and 50% appear to fail at time of implantation. Studies of the karyotype on products of conception from early recurrent pregnancy loss have shown that chromosomal abnormalities alone do not account for this number of pregnancy losses, meaning that the majority of these losses are chromosomally normal human embryos, particularly in women under the age of 36 years.[1-3] Human preimplantation embryos express major histocompatibility antigens theoretically capable of inducing an immune response,[4] but the role of these antigens in pregnancy has not been delineated. It is possible that maternal immune responses play a role in the failure of implantation.[5]

Basic Immune Principles

Innate and Acquired Immune Defense Systems

The importance of effective defenses against foreign invaders has led to development of an *innate* (or natural) and an *acquired* (or specific) immune defense system. While these immune systems often cooperate in their defense against foreign invaders, their responses differ in intensity, timing, and specificity (Table 14.1).

Innate Immunity

The innate or natural defenses against pathogens consist of certain tissues, phagocytic and cytotoxic cells, and a

Table 14.1 Features of Innate and Acquired Immunity

Characteristic	Innate Immunity	Acquired Immunity
Specificity	Non-specific	Antigen-specific
Physicochemical barriers	Skin, mucous membranes	Peripheral and mucosal immune systems
Effector cells	NK cells, macrophages	B and T lymphocytes
Circulating molecules	Complement	Antibodies
Soluble mediators	Macrophage-derived cytokines (IFN-α, IFN-β, TNF-α)	Lymphocyte-derived cytokines (IFN-γ)
Response time	Rapid	Primary and secondary
Memory	None	Long-lived

variety of effector molecules. These defenses at the skin and mucous membranes may be perfectly adequate to clear potentially harmful environmental antigens. The initial entry of foreign organisms or cells is met by an initial inflammatory response which may be amplified by the activation of serum complement components resulting in the generation of enzymes and the deposition of their products on the surface of foreign antigens. The innate system can differentiate between a harmful invader, such as bacteria or viruses, by promoting an offensive environment. Other invaders, such as sperm or blastocyst, result in an inflammatory response that is accommodating to the guest. However, as a consequence of this first exposure to an antigen, and despite the intensity of the response, innate immune responses do not lead to any change in the type and magnitude of the response should the organism encounter this foreign antigen again. Thus, the innate response to unrelated or the same antigen seen more than once is exactly the same, there is no specificity to the defensive response and no adaptation to first exposure.

Acquired Immunity

The acquired immune response is characterized by an adaptation to the first exposure to the foreign antigen, known as the *primary response*. A subsequent exposure to the same antigen results in a *secondary response* that is quantitatively and qualitatively different than the primary response. The acquired immune response consists of antigen-specific cells and molecules that often interface with components of the innate immune defense systems. During *opsonization*, antibodies bind to bacterial surface antigens facilitating their *phagocytosis* by macrophages. In addition, macrophages may process and present protein antigens to specific T cells during a primary immune response.

Antigen-specific lymphocyte responses are characterized by their proliferation and further differentiation of function such as the production of soluble mediators including antibodies and cytokines and the development of antigen-specific memory. A typical primary response to antigen has a lag phase of about 5 days, has a slow rate of increase to a low plateau, with a low sensitivity to the antigen. The low affinity antibody produced is predominantly Immunoglobulin (Ig) M. In contrast, the secondary response (and all subsequent memory responses) to the same antigen are quantitatively and qualitatively different. The response occurs in 1 to 2 days, rises rapidly to a high plateau, and has a high degree of sensitivity to the antigen. The antibody affinity is high and may be predominated by IgG, IgA, or IgE depending on the location in which the immune response takes place.

The major features of the specific immune response are consistent with Burnett's *clonal selection theory*. This hypothesis states that (1) the antigen specificity of a single lymphocyte is established in the absence of antigen during its initial maturation, (2) each lymphocyte has a unique antigen specificity manifested by multiple copies of the same receptor, and (3) the proliferation and differentiation of the lymphocyte is induced by antigen binding to the specific receptors. The interaction of antigens and lymphocyte receptors is governed by the relative affinity of the receptor for the antigen. The clonal selection hypothesis explains that subsequent responses to the same antigen are more rapid, robust, and long lasting. The proliferation of specific lymphocytes in response to antigen results in the generation of antigen-responsive clones of cells.

As a consequence of antigen-induced proliferation, some of the proliferating lymphocytes may commit to long-term *memory cells* that persist for many years. Activated B cells may undergo *isotype switching* by changing from the production of IgM to IgG, IgE, or IgA thus allowing different physiological and biological properties. By these mechanisms, a maturing immune response maintains antigen specificity but establishes memory and functional diversity.

The development of adaptive immunity based on the clonal selection of lymphocytes bearing specific receptors is critical in allowing all pathogens, no matter how novel, to be recognized and in allowing the development of immunological memory.

Basic Effectors of Immune Responses

Cellular Effectors

Leukocytes are the cellular effectors of immunity and include the lymphocytes, monocytes, macrophages, dendritic cells, neutrophils, basophils, and eosinophils (Table 14.2). Lymphocytes can be further divided into subclasses based on function and upon cell surface markers, called "cluster of differentiation" or CD markers. Lymphocyte subclasses include T cells, B cells, and natural killer (NK) cells. Both B and T lymphocytes originate in the bone marrow and participate in antigen specific immune responses.

Thymus Derived (T) Cells

In humans, T cells circulate through the thymus, where they gain specific CD markers, antigen specificity and tolerance to self (discussed later in the chapter). T lymphocytes maturing in the thymus express both CD4 and CD8 cell surface receptors during early development, but those exiting the thymus express only one of these cell surface markers. CD4 positive T cells typically develop into helper T cells when they reach peripheral lymphoid tissues. Helper T cell effector functions include the secretion of soluble mediators of immune responses such as cytokines. These

Table 14.2 Characteristics of T, B, and NK Lymphocytes

Characteristic	T Cytotoxic T Suppressor	T Helper T Inducer	B Cells	NK Cells
Symbol	Tc/s	Th	B	NK
Surface antigen	CD8+	CD4+	CD19+	CD56+
MHC restricted	Class I	Class II	Class I and II	None
Target cells	Tumors, virally infected cells, allografts	B cells, Tc cells and precursors, macrophages		Tumor cells, virally infected autologous cells
Function	Kill foreign cells, down-regulate cells	Interleukin secretion	Immunoglobulin production	Immune surveillance, cytotoxic
Differentiation	Thymus	Thymus	Bone marrow	Bone marrow
Specificity	Antigen peptides	Antigen peptides	Native antigen epitopes	Unknown
Antigen receptor	T cell receptor	T cell receptor	Cell surface immunoglobulin	Fc portion of immunoglobulin

cytokines, in turn, modulate B cell, T cell, and macrophage responses. CD8 positive T cells leaving the thymus are destined to mature into either cytolytic T cells (CTLs), which function to lyse infected or otherwise altered target cells, or into suppressor T, currently called regulatory T (Treg) cells.[6,7] Less is understood about Treg cells than about T helper and cytolytic T cells.

Bone Marrow Derived (B) Cells

B cells appear to be educated in the bone marrow prior to exit into the peripheral immune system.[8] Like T lymphocytes, B lymphocytes are antigen specific. Unlike T lymphocytes, B cells function to secrete antibodies, which characterize humoral immune responses (discussed later in the chapter).

Natural Killer (NK) Cells

The third type of circulating lymphocyte is the natural killer cell. Natural killer cells have characteristic cell surface markers and receptors and their activities are typically not antigen specific, although this concept continues to evolve.[9] NK cells function in the recognition of cells lacking Major Histocompatibility Complex (MHC) Class I products (discussed later in the chapter) and in first–line defense against virally-infected or oncogenically-transformed target cells. NK cells also display cell surface receptors allowing recognition of antibody-coated target cells. Through these receptors, NK cells function as the major effector of antibody-dependant cellular cytotoxicity (ADCC).

Monocytes, Macrophage, and Dendritic Cells

Like other leukocytes, monocytes are derived from bone marrow stem cells. Monocytes circulate in the peripheral blood and populate nearly every tissue.[10] Within specific tissues, monocytes mature into macrophages. In some tissues, these macrophages have been given specific names (e.g., alveolar macrophages in the lung and Kupffer cells in the liver). Macrophages are phagocytic cells that participate in development of innate as well as antigen-specific immune responses. They can signal T and B cells via secretion of cytokines and via delivery of antigen in forms recognized by these cells (antigen presentation). They may also stimulate direct lysis of altered target cells. Dendritic cells are closely related to macrophages. Most derive from a similar cellular lineage in the bone marrow. Like macrophages, dendritic cells are phagocytic and they play an important role in antigen presentation.[11] Dendritic cells aid in the development of antigen-specific B cell responses.

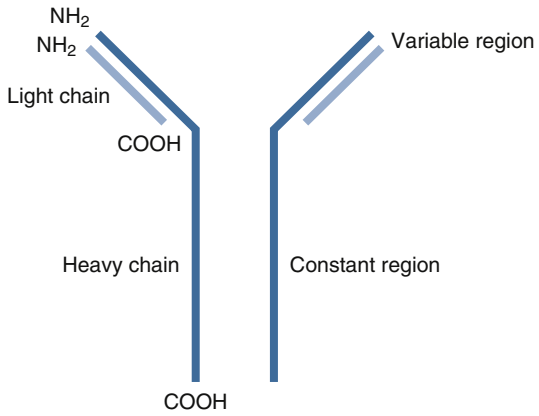


FIGURE 14.1 A typical immunoglobulin molecule. The constant region (carboxy [COOH] terminal) isotype of the heavy chain determines function. The combined variable regions (amino [NH_2] terminal) of the heavy and light chains determine antigen specificity. IgG, IgE, and IgD isotypes are typically monomers of this basic structure.

Other Effector Cells

Neutrophils, eosinophils, and basophils are effector cells with specific importance in innate defense against pathogens. Each has also been associated with specific immune-mediated disease states. Eosinophils, for instance, are classically associated with defense against parasites and have pathophysiological importance in asthma.

Soluble Components of Immune Responses

Immunoglobulins and Humoral Immunity

Immunoglobulin molecules are comprised of dimerized subunits of heavy and light chains (Fig. 14.1).[8] The N-terminal portions of each of the heavy and light chains are highly polymorphic and are termed their variable regions. The variable region of one heavy chain combines with the variable region of one light chain to give the immunoglobulin its antigen specificity. The C-terminal segments of the immunoglobulin heavy and light chains are called their constant segments and have minimal polymorphism. The constant portion of the immunoglobulin heavy chain interacts with other immune response components. Since these interaction

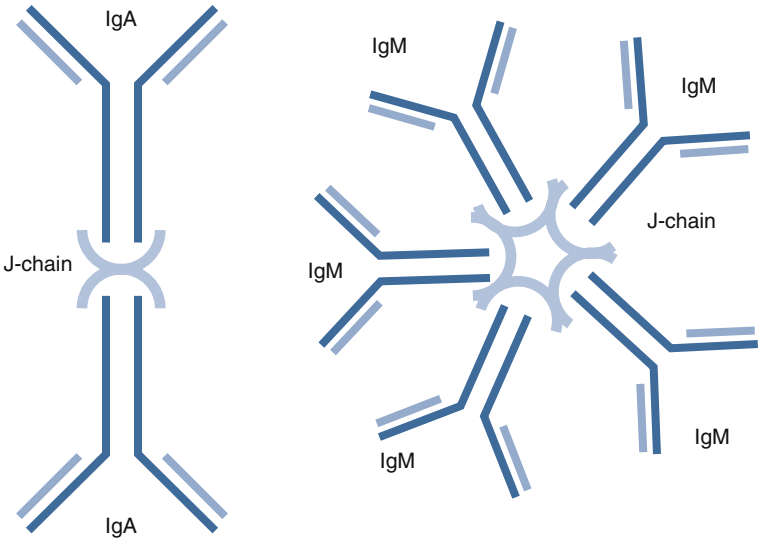


FIGURE 14.2 IgA dimers and IgM pentamers. Each structure is connected by a joining chain (j-chain).

are governed by the heavy chain constant region isotype, so too is immunoglobulin effector function. Ig molecule isotypes include IgA, IgD, IgE, IgG, and IgM. IgG, IgE, and IgD molecules are typically present as single Ig molecules (monomers). IgA molecules usually circulate as dimers and IgM molecules as pentamers (Fig. 14.2).

Each Ig isotype has distinctive functions. IgA dimers, connected by a joining or J chain, have been best described in the mucosa of the intestine and the female reproductive tract. Here, they are actively transported into the mucosal lumen via interaction of the J chain with a mucosally-derived secretory component. This allows exposure of IgA to antigens at the mucosal surface. IgE molecules interact with eosinophils in parasitic infections allowing eosinophil-mediated target lysis. IgE molecules are also implicated in delayed-type hypersensitivity via mast cell interactions. Membrane-bound IgD and IgM monomers are components of the antigen-recognizing B cell receptor on naïve B cells. Pentavalent IgM molecules activate the complement cascade. Secreted IgM multimers are also characteristic of the early antigen specific responses of naïve B cells.

Primary and Secondary Immune Responses

If the antigen recognized by the naïve B lymphocyte is not a peptide, T cell help will be absent. In turn, the initial response, albeit antigen-specific, will be of lowered affinity, will involve more IgM secretion than IgG, and will not generate immunological memory. In contrast, if the naïve B cell recognizes peptide antigen, the associated help from CD4+T cells will promote the generation of both primary and secondary humoral immune responses after repeated antigenic stimulus. As described earlier, the initial or primary immune response requires fairly significant antigenic stimulus and typically peaks approximately 5 to 10 days after antigen exposure. Primary responses usually involve more IgM secretion than IgG and the magnitude of the response is often lower than that after a second exposure to peptide antigen. Primary exposure to peptide antigen promotes the generation of memory B cells, which drive the characteristics of the secondary humoral immune response. Antigen exposure, in combination with T cell help, simultaneously activates B cells, leading to the antibody isotype switching that characterizes secondary responses. Upon re-exposure to the same peptide antigen, this secondary response ensues. Secondary responses require less antigenic exposure than primary responses, they peak more rapidly (2 to 5 days postexposure), and they are typically more robust than primary responses. As a result of isotype switching, IgG rather than IgM molecules represent the predominant immunoglobulin subtype in secondary immune responses.

These IgG molecules have multiple immune effector functions. They can cross the placenta, allowing immune transfer from mother to fetus. IgG can directly bind antigen via its variable region. This allows the free constant portion of the IgG heavy chain (Fc portion) to be recognized and internalized by phagocytic cells in a process termed opsonization. Similarly, IgG bound to cell-associated antigen can signal lytic attack by cytotoxic T cells, NK cells, or NKT cells promoting a process called antibody-dependant cellular cytotoxicity (ADCC). Finally, IgG molecules can activate the complement cascade.

Complement

The complement cascade is a vital part of innate immunity. It may be useful to compare the complement cascade to a more familiar clinical entity, the coagulation cascade. Both have components that circulate in inactive forms. Activation of each pathway can occur via two mechanisms. Reminiscent of the intrinsic and extrinsic coagulation pathways, the complement cascade can be activated via classical and alternative pathways (Fig. 14.3).[12] The classical pathway is activated when a complement component, C1, binds to antigen-antibody complexes (IgG or IgM). The alternative pathway is activated when complement component C3b binds to activating surfaces such as the cell wall of a bacterial pathogen. Each pathway sets off a series of activating enzymatic digestions of subsequent complement cascade components. Like the coagulation cascade, both of the activation pathways in the complement cascade intersect, joining as a final common effector pathway. Late components of the complement cascade can interact with specific complement receptors, resulting in pathogen phagocytosis, activation of the humoral immune system, or both. Alternatively, activation of the complement cascade can result in

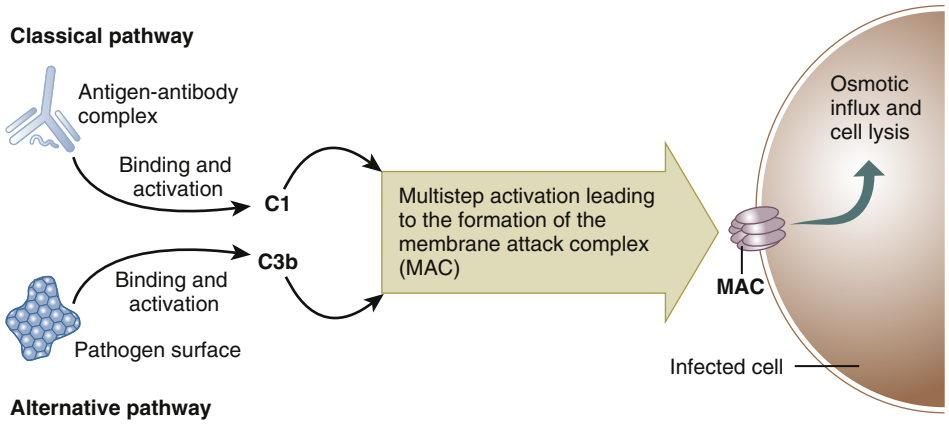


FIGURE 14.3 Activation pathways for complement-mediated cytolysis. The classical pathway of complement activation is initiated by the binding of antigen-antibody complexes to complement factor C1. The binding C3b to activating surfaces such as a bacterial cell wall activates the alternative pathway. Activation of either pathway results in a cascade of subsequent enzymatic activations. These pathways converge, forming the membrane attack complex (MAC). The MAC allows osmotic shifts that lyse the affected target cell. Complement-mediated activities can also include promotion of inflammatory responses, direct opsonization of pathogenic substances and clearance of unwanted immune complexes.

the formation of a membrane attack complex (MAC) that results in indirect osmotic lysis of a target cell via the formation of an ion permeable transmembrane pore in the target cell.

Cytokines

The rapidly enlarging and pleiomorphic family of cytokines joins the immunoglobulins and the components of the complement cascade to complete the list of important soluble mediators of immune responses. Cytokines are a family of secreted proteins, all of which are generated by immune cells. They include the interleukins, interferons, tumor necrosis factors, transforming growth factors, and the chemokines. The actions of cytokines may be autocrine, paracrine, or endocrine and effects are mediated by specific cytokine receptors. Most cytokines have short half-lives, so direct actions are typically of short duration. However, cytokines often stimulate additional immune cell activity with cascading cytokine secretion and effects. Members of this large family of soluble immune mediators often have complementary and/or redundant activities. In addition to myriad other roles in immunobiology, cytokines help to direct T helper cell differentiation. CD4+ T helper cells appear to circulate from the thymus to peripheral tissues in a functionally immature state (Th0[13]). The T helper cell phenotype/polarization model holds that Th0 cells differentiate into their mature phenotypes based largely upon the cytokine microenvironment in which they reside at the time when they first recognize antigen and on the cell surface coreceptors expressed on the Th0 cell (Fig. 14.4).[13] Certain cytokines appear to be of particular importance in the polarization of T helper cell responses. If antigen is recognized by Th0 cells in an environment dominated by the cytokines interleukin 12 (IL12), IL18, and interferon gamma (IFNγ), the resulting T helper effector phenotype is characterized by the secretion of inflammatory-type cytokines, including interferon gamma (IFNγ), interleukin 2 (IL2), and tumor necrosis factor alpha (TNFα). This type of response is called a T helper 1 (Th1) response. Alternatively, if Th0 cells recognize antigen in a microenvironment dominated

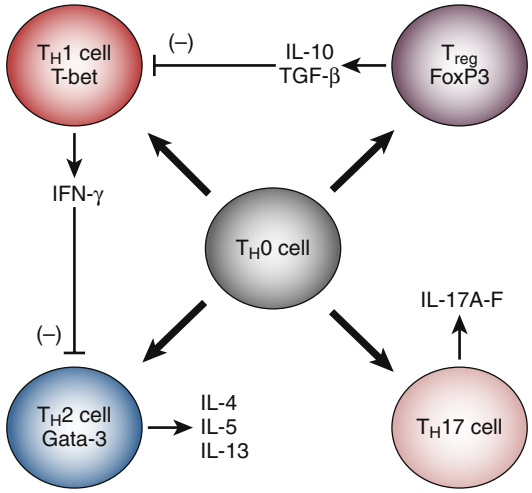


FIGURE 14.4 The TH1/TH2 polarization paradigm. The cytokine microenvironment in which antigen is presented by antigen-presenting cells to immature (TH0) T helper cells promotes development into TH1, TH2, TH17, and T_{reg} phenotypes. It appears that each T helper phenotype supports further development of itself while opposing developments of its counterparts.

by IL4, the predominant T helper response is of the Th2 type and is characterized by secretion of IL4, IL5, IL9, and IL13.[13,14] These Th2 cytokines, in turn, stimulate allergy-type responses, including mast cell and eosinophil activation and antibody production. Th1 responses enhance Th1 responses while inhibiting Th2 responses. Th2 responses initiate a positive feedback loop which promotes further Th2 activity while inhibiting Th1 effects. Several other T helper responses have also been distinguished. Th3 and Tr1 responses[14,15] are characterized by the secretion of transforming growth factor beta (TGFβ); T regulatory (Treg) responses are defined by the secretion of IL10; and Th17 responses by the secretion of IL-17.[16]

Altered T helper cell polarization may lead to immune-mediated diseases. Although the etiologies are often incompletely described, these disorders are characterized by a

dysregulation of cytokine secretion. For instance, patients with some forms of asthma appear to have inappropriate polarization of immune responses in their lungs towards the Th2 and Th17 phenotypes.[17,18] Those with Crohn disease polarize intestinal mucosal immune responses toward the Th1 phenotype. It has been hypothesized that some cases of spontaneous isolated or recurrent pregnancy loss may be the result of an insufficient Th2 polarization of maternal lymphocytes in the presence of placental antigens.

Basis of Immune Specificity and Immune Cell Education

While many of the salient attributes of the immune system can be addressed in a description of the cellular and soluble components of immune responses, there are two additional defining features of immune responses that require specific discussion: the specificity of immunological antigen recognition and T lymphocyte education.

Antigen Presentation

Most of the antigens recognized by the effector cells involved in acquired immune responses cannot be detected in isolation. Rather, antigens must be specifically presented to effector cells. This presentation process places antigens into a context in which the cell presenting the antigen can be recognized by the effector cell as "self-derived" and the antigen can be seen as foreign. This process is called antigen presentation and it is essential to antigen specific development of both cellular and humoral immune responses.[19]

The Major Histocompatibility Complex

The major histocompatibility complex region on the short arm of human chromosome 6 is a four million base pair deoxyribonucleic acid (DNA) segment that encodes many of the molecules involved in innate and acquired immune responses (Fig. 14.5**).** This highly polymorphic DNA region contains nearly 130 genes and approximately 100 pseudogenes. Not all of these genes are linked to immunity but two sets of genes within the region play central roles in antigen presentation. These genetic loci encode the MHC Class I and MHC Class II products (Fig. 14.6**)** and several other molecules involved in antigen presentation. MHC Class I molecules [e.g., human leukocyte antigen (HLA)-A, -B, and -C] are present on the surface of nearly every cell in the human body. They aid in defense against intracellular pathogens such as viruses by presenting virus-derived antigens at the surface of infected cells and in the detection of oncogenically-transformed cells by presenting abnormal intracellular cellular proteins that occur as a result of oncogenic transformation. Class I products also play a central role in transplant recognition and rejection. In each case, MHC Class I molecules act as important ligands for the T-cell receptor (TCR) on CD8+ cytotoxic-suppressor T cells. They may also act as important ligands for a variety of receptors on NK cells. MHC Class I-mediated intercellular interactions generally activate the cellular portion of the immune response and result in killing of the antigen-presenting (i.e., virally infected, cancerous, or transplanted) cell expressing the Class I molecule.

Unlike the globally-expressed MHC Class I molecules, MHC Class II molecules (human leukocyte antigen (HLA)-DR, HLA-DP, and HLA-DQ) are present on the surface of a limited number of "professional" antigen-presenting cells. These include dendritic cells, macrophages, monocytes, B cells, and tissue specific antigen presenters (e.g., Langerhan's cells in the skin). MHC Class II molecules are important in defense against extracellular pathogens, typically bacterial in origin. The T cell receptor on CD4+ T helper cells acts as the major ligand for MHC Class II products. Accordingly, MHC Class II-mediated interactions with these T helper cells typically modulate humoral immune responses.

The remarkable polymorphism of the classical MHC Class I and Class II genes allows for the recognition and presentation of an enormous diversity of peptides. In fact, the MHC genes are typically the most polymorphic of all loci in every species. Between the MHC Class I (intracellular pathogens-proteins) and Class II (extracellular pathogens-proteins) antigen presentation pathways, nearly every protein in the human body can be sampled. Those derived from foreign (non-self antigens) or altered self are recognized as such and trigger an immune response. In the absence of an autoimmune disorder, peptides derived from unaltered self do not trigger responses.

A large number of other immune related genes are located within the HLA locus. These include many of the complement component and cytokine genes (Fig. 14.5). Other MHC molecules are also encoded within the HLA region of chromosome 6. These include non-classical MHC Class I products (HLA-E, -F, and -G) and MHC Class I-like products such as MHC Class I polypeptide-related sequence A and B (MICA and MICB). These molecules tend to be less polymorphic than their classical counterparts and are thus predicted to have less important roles in antigen presentation. Non-classical and MHC Class I-related products have been implicated as central in immune responses at mucosal surfaces, including those within the reproductive tract (discussed later in the chapter).

Lymphocyte Education

It is essential that cells in the immune system be able to differentiate self from non-self. The education of B lymphocytes in this regard may involve only a single step process—one of negative selection. Those immature B cells that express receptors that recognize self antigen with high affinity do not survive to exit the bone marrow. This is negative selection against self-antigen and promotes B cell tolerance to recognition of self antigens. Self tolerance by B cells is independent of MHC molecules.

In contrast, T lymphocytes are educated in the thymus via a two step process.[20,21] Immature T cells entering the thymus express neither the T cell receptor (TCR) nor the CD4 or CD8 coreceptors. Upon entering the thymus, all three cell surface molecules come to be expressed. During a process called thymic education, CD4/CD8 double positive cells undergo both positive and negative selection events in which T cells "mature" from CD4/CD8 double positive cells to single positive (CD4+ or CD8+) cells. This two-step maturation positively selects for those T cells with T cell receptors that recognize self MHC products. Those recognizing self MHC Class I molecules are destined to become CD8 single positive T cytotoxic/suppressor cells. Those recognizing self MHC Class II molecules

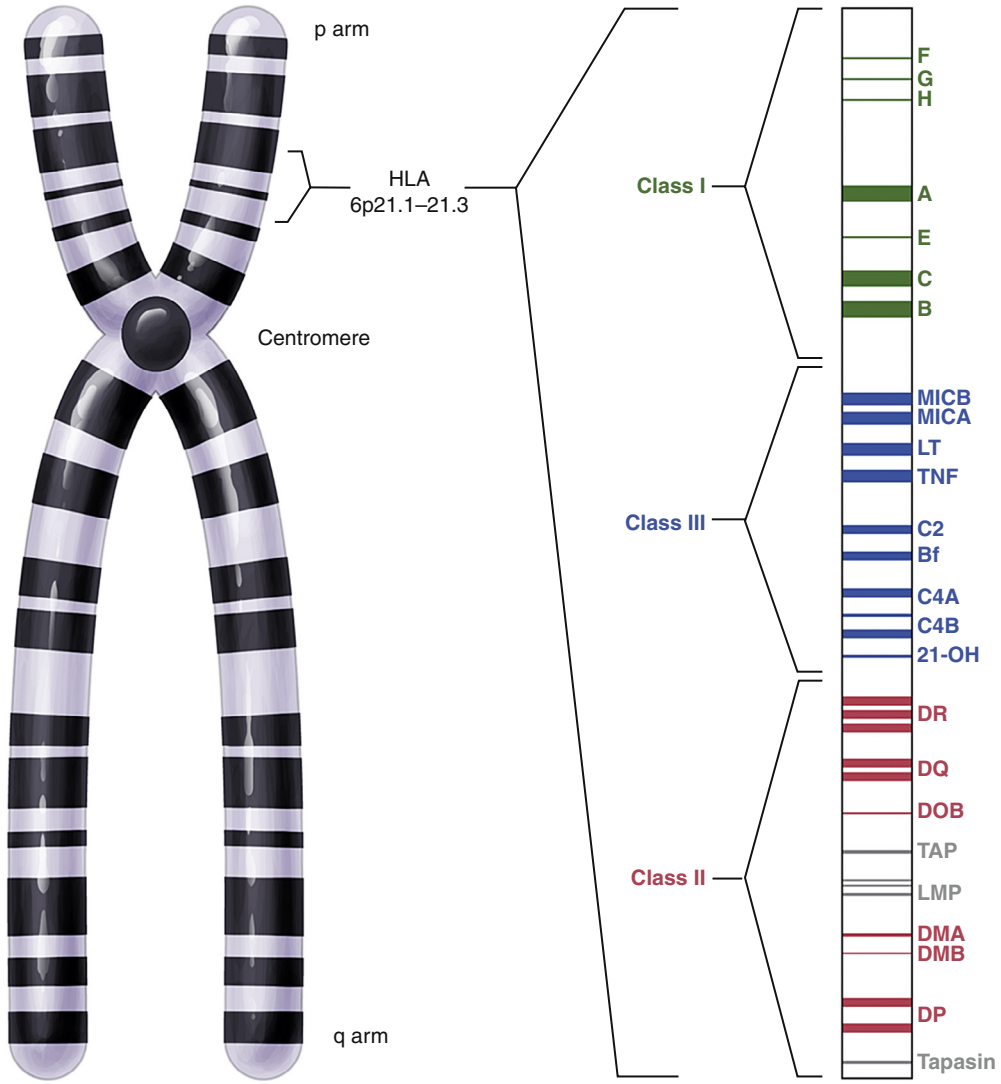


FIGURE 14.5 The Major Histocompatibility Complex. The major histocompatibility complex (MHC) is a region on the short arm (p arm) of human chromosome 6 that encodes the human leukocyte antigens and a variety of molecules related to antigen presentation and immune response. The MHC covers over four million base pairs (6p 21.1 to 21.3) and encodes well over 200 genes, many with an immense degree of polymorphism. The genes can be organized into MHC Class I, MHC Class II, and MHC Class III regions. The telomeric region encodes the MHC Class I molecule heavy chains, both those considered to be classical and highly polymorphic (HLA-A, -B and, with more limited polymorphism, HLA-C) and those which are considered non-classical (HLA-E, -F, -G, and -H). The centromeric region of the MHC encodes the MHC Class II heavy chains (HLA-DR, -DQ, and –DP) and several other molecules involved in antigen presentation. Among these are the transporter associated with antigen presentation (TAP) molecules (which move peptides from the cytoplasm into the endoplasmic reticulum to be loaded into the MHC Class I peptide-binding groove) and proteins involved in peptide loading of the MHC Class II molecule (DMA, DMB, DOB). Tapasin is a TAP-associated glycoprotein. The MHC Class III region is centrally located on the MHC and encodes for several components of the complement cascade (C2, factor B/Bf, C4A, and C4B), tumor necrosis factors alpha and beta (TNF-α and TNF-β), lymphotoxins (LT), and the MHC Class I like molecules, MICA and MICB.

are destined to mature into CD4 single positive T helper cells. The immature CD4/CD8 double positive thymocytes that do not recognize self MHC will die by apoptotic mechanisms. The second portion of the two-step intra-thymic T cell maturation process is one of negative selection. Negative selection of T cells within the thymus appears to depend on the strength of the recognition of MHC-peptide antigen complexes by the TCR of the developing T lymphocyte. If recognition is particularly strong, the cells undergo apoptosis. This functions to eliminate cells reactive against self-peptide antigens, which are likely to be fairly abundant within the antigen-binding clefts of the thymic antigen-presenting cells. T lymphocytes exiting the thymus should therefore recognize self MHC but ignore self antigen. Recent evidence indicates that NK cells are also educated,[22] possibly at the effector sites.

The Mucosal Immune System

The Common Mucosal Immune System

The large area of the body's mucosal surfaces is protected by innate humoral factors and cells and by specific antibodies that are selectively transported into external secretions. These antibodies are predominantly of the IgA isotype.

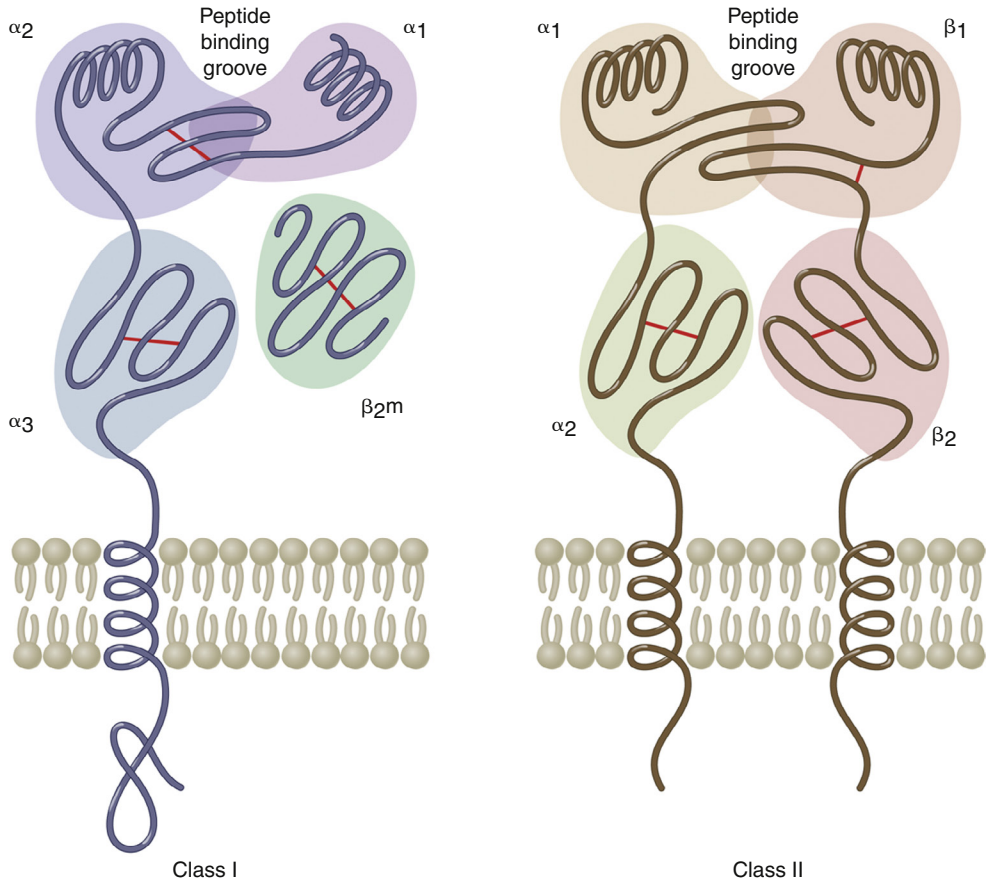


FIGURE 14.6 Molecular structure of the Class I and Class II molecules of the HLA system. To be stably expressed at the cell surface, the MHC Class I molecule must consist of a trimer of a heavy chain, a light chain (β_2 microglobulin; β_2 m) and an antigenic peptide bound within the peptide-binding groove. The MHC Class I heavy chain consists of three subunits. The α_1 subunit contains the heavy chain's amino terminus and, combined with the α_2 domain, creates the molecule's highly polymorphic peptide binding groove. These domains, along with the majority of the α_3 domain, lie in the extracellular space. The α_3 domain also contains the transmembrane segment and carboxy terminal portion of the Class I heavy chain. The carboxy terminal tail lies within the cytoplasm of the cell. Unlike most other MHC Class I molecules, the carboxy tail of HLA-G is truncated and contains only 6 amino acids. The MHC Class II molecule is a dimer of two heavy chains, α and β. The α_1 and β_1 domains of the heavy chains combine to create the Class II peptide-binding groove. The α_2 and β_2 domains contain the transmembrane segments and the intracellular carboxy-terminal tails.

Environmental antigens from ingested food, inhaled particles in air, and resident microorganisms at mucosal surfaces represent the most important sources of exogenous stimulation of the entire immune system. The surface area of the mucosal membranes includes several hundred square meters versus only approximately 1.8 square meters for skin. Large numbers of cells that process antigens, produce antibodies, and produce innate humoral factors such as lysozyme, lactoferrin, peroxidase, and digestive enzymes are present in mucosal tissues and contribute to the protection of mucosal surfaces.[23] The existence of a common mucosal immune system and the determination of the origin of mucosal IgA plasma cells in humans are based on a number of indirect studies. External secretions of glands anatomically remote from the site of antigen stimulation contain secretory-IgA antibodies that are specific for microbial and food antigens. Ingestion of bacteria and viruses induces a parallel appearance of specific secretory-IgA in saliva, milk, and tears, but not in serum. The best known example is that of specific immunity passed on from mother to infant through secretory-IgA contained in breast milk. Specific antibody-forming cells can be transiently detected among peripheral blood mononuclear cells collected 7 to 12 days after oral immunization, and their presence precedes the appearance of specific secretory-IgA antibodies in saliva and tears. Analyses of the properties of IgA from stimulated human peripheral blood mononuclear cells revealed that such cells produce predominately polymeric IgA and display an IgA subclass distribution reminiscent of IgA cells in mucosal tissues.[24]

The common mucosal immune system can be summarized as follows (Fig. 14.7). Various environmental antigens are taken up by specialized epithelial cells (M cells) covering Peyer patches, and enter the Peyer patches which contain populations of antigen-processing and antigen-presenting cells. Immature surface IgM+ B cells, presumably of bone marrow origin, become surface IgA+ under the influence of special T cells, Peyer patch dendritic cells, or T-cell -derived cytokines. Such isotype-committed and antigen-committed cells exit the Peyer patches and, after maturation in mesenteric lymph nodes, enter the blood circulation through the thoracic duct and lodge in the mucosal tissues and glands

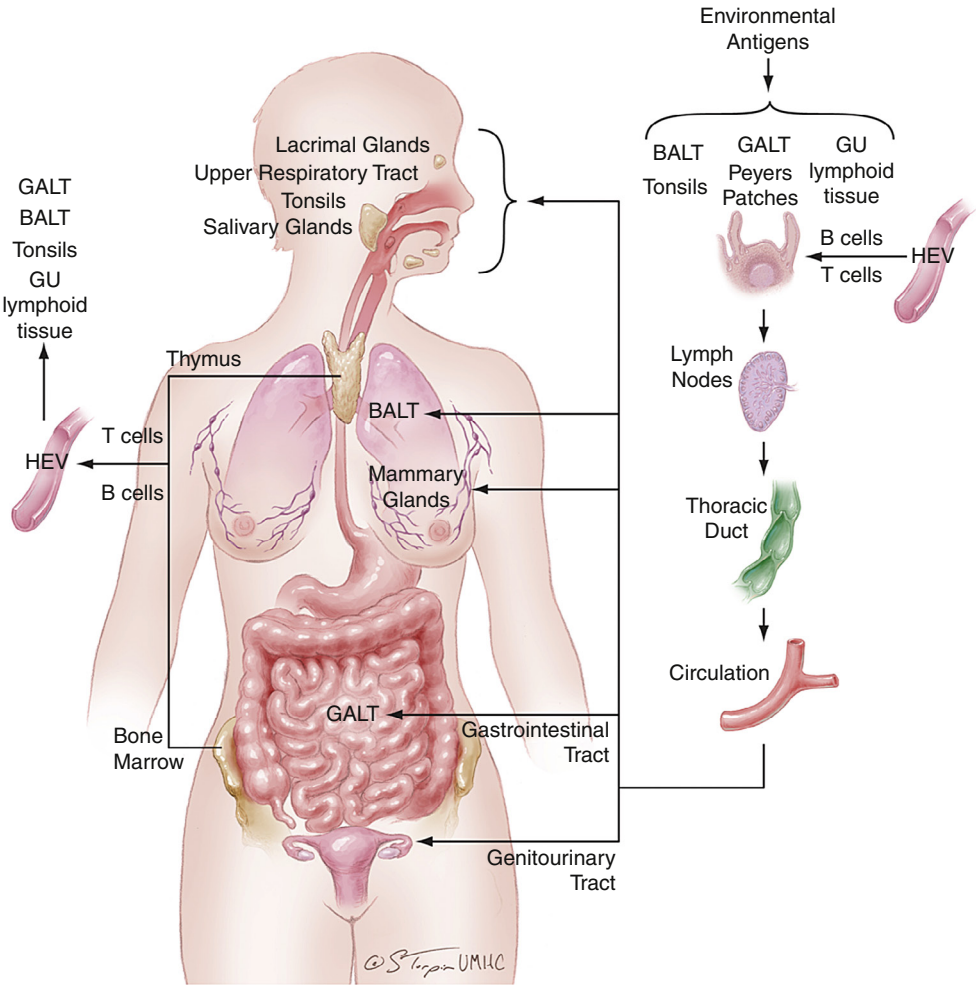
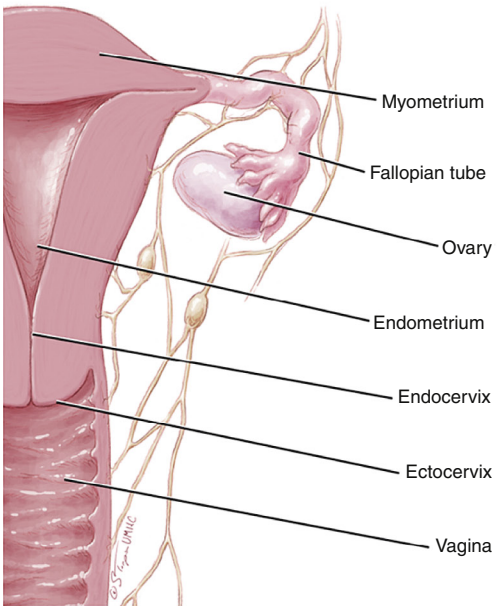


FIGURE 14.7 Model of the common mucosal immune system in humans. Human immune cells encounter environmental antigens largely across the immense area of mucosal surfaces that line the oral, gastrointestinal, respiratory, and urogenital tracts. Induction of antigenic responses at one of these sites often induces mucosal immune responses in the others. Although mucosal inductive sites are present at each of these mucosal surfaces, the best studied inductive site is in the gastrointestinal tract. Here, various environmental antigens are taken up by specialized epithelial cells covering an organized immune cell population called a Peyer patch. The classical immune cells populating the Peyer patch reach this site by traversing high endothelial venules (HEV) and include T cells, B cells, and dendritic cells. Antigen-processing and presentation at this site is robust and may also involve local mucosal epithelial cells. Isotype-committed and antigen-sensitized precursors of mucosal IgA plasma cells from mucosal inductive sites (largely gut-associated and bronchus-associated lymphoid tissues [GALT and BALT], but also tonsils and genitourinary mucosal tissues) pass through lymph nodes to enter the general circulation through the thoracic duct. The cells then home to and populate mucosal tissues and exocrine glands. B cells from the inductive sites will undergo terminal differentiation into IgA plasma cells. CD4+ and CD8+ T cells will also home to mucosal effector sites.

(primarily the intestine) where they terminally differentiate into IgA-producing plasma cells.

The Mucosal Immune System of the Female Reproductive Tract

Although the female reproductive tract is considered an integral component of the mucosal immune system, the genital immune system displays characteristic features that are distinct from those of other typical mucosal sites.[25] Most importantly, the mucosal immune system of the female reproductive tract is under a strong hormonal control that regulates the transport of immunoglobulins, the levels of cytokines, the distribution of various cell populations, and antigen presentation in the genital tissues during the reproductive cycle. In addition to protecting against potentially dangerous infectious agents, it must adapt to a spectrum of physiological events that include fertilization, implantation, pregnancy, and parturition. Throughout the menstrual cycle, an immunological balance is maintained to respond to the challenges of bacteria, yeast, and viruses without interfering with events that surround conception. During pregnancy, in addition to protecting against infection, the mucosal immune system undergoes further adaption to support a fetal-placental unit that is immunologically distinct. To meet these challenges, the reproductive tract has separate compartments consisting of the ovaries, fallopian tubes, uterus, cervix, and vagina that are precisely regulated by the endocrine system. Each of the sites displays aspects of the mucosal immune system that are designed to contribute to maternal and fetal survival. Unique to the genital tract and unlike other mucosal secretions in which secretory-IgA is the dominant isotype, IgG levels in the



	Humoral Immunity			Cellular Immunity				
	Ig-producing cells	J chain	Epithelium pIgR	T cells CD4 CD8	NK cells	Mphage/ monocytes	Dendritic cells	Neutrophils
Myometrium	–	–	–	– –	–	–	–	–
Fallopian tube	+	+	+	++ ++	+	+	+	+++
Ovary	–	–	–	+ +	–	+/–	+/–	+
Endometrium	+/–	–	++	++ ++	++	+	–	++
Endocervix	+++ IgA=IgG>IgM	+	+++	++ ++ aggregates	+	+	++	++
Ectocervix	++	+	+	+++ +++	+	+	++	++
Vagina	+	+	–	+++ +++	+	+	++	++

FIGURE 14.8 Components of the mucosal immune system in the female reproductive tract. –,negative; +/–, occasionally positive or questionable; +, weakly positive; ++, moderately positive; +++, strongly positive.

lower genital tract secretions equal or exceed the levels of secretory-IgA. Finally, local antigenic stimulation of the lower female genital tract appears to be less effective in the induction of immune responses because of the absence of classical mucosal lymphoepithelial inductive sites such as intestinal Peyer patches (Fig. 14.8).

Immunoglobulins in Genital Tract Secretions

Immunoglobulin concentrations in the genital tract are dependent on hormonal and local factors such as the presence of inflammation. Several studies concerning the origin of antigen-specific IgG in the female genital tract secretions suggest that locally produced, as well as plasma-derived IgG, contribute to the IgG pool in genital tract secretions. Regardless of the source, the route of passage of IgG into the lumen of the genital tract-passive, paracellular diffusion, or active, receptor-mediated transport has not been elucidated.

The IgA of cervical mucus is primarily secretory-IgA, with a small component of monomeric IgA. The IgA subclass distribution resembles that of the lower intestinal tract with IgA1 and IgA2 present in roughly equal proportions. The increased proportions of IgA2 may provide a functional advantage to certain specific antibodies because they are resistant to the IgA1 proteases of many pathogenic bacteria.[26]

Cytokines in Genital Tract Secretions

Unlike other mucosal tissues, the reproductive tract is characterized by progressive tissue growth and remodeling during each menstrual cycle. Cell proliferation, apoptosis, and cell migration are essential for reproductive tract renewal to occur. Growth in preparation for fertilization, implantation, and successful pregnancy is mediated by a changing pattern of cytokine and adhesion molecule expression that is regulated by the sex steroid hormones.[27] Multifunctional cytokines such as Il-1β, IL-6, and IL-10 that are produced throughout the reproductive tract have important roles in the maturation of B lymphocytes to immunoglobulin-producing plasma cells. Estrogen increases the expression of polymeric IgA receptor, thereby increasing the transport of IgA into the lumen when cultured human endometrial cells are incubated with Il-4 and IFN-γ. Regulatory cytokines, such as IL-10, IL-1β, and IL-6 are present in human cervical mucus and their levels correlate with the cyclic reproductive hormones estradiol and progesterone.[23] Cytokines and chemokines are differentially secreted from immortalized human endocervix-derived epithelial cell lines depending on the hormone milieu in which the cells are exposed.[28]

Cells of the Female Reproductive Tract

Estradiol and progesterone secreted during the menstrual cycle act both directly and indirectly on epithelial cells and other immune cells to modify immune function in a way that is unique to specific sites throughout the female reproductive tract.[29] The female reproductive tract contains a full spectrum of leukocytes with the potential to function in ways similar to those present in other mucosal tissues and in the peripheral blood. Macrophages, distributed primarily between the epithelial and muscle layers, are found both scattered throughout the fallopian tubes, cervix, vagina, and in small aggregates in the uterus. Uterine NK cells, which are CD56+/CD16− and therefore phenotypically distinct from peripheral blood CD56−/CD16+ NK cells, are present in the uterine endometrium and increase in number during the secretory phase of the menstrual cycle and during the first trimester of pregnancy.[30] Immune cells make up 6% to 20% of the total non-muscle cells in the fallopian tube, uterus, endocervix, ectocervix, and vagina and are not significantly influenced by the stage of the menstrual cycle.[31]

Table 14.3 Differences in the Properties of Human Serum and Secretory IgA Antibodies and in the Characteristics of IgA Cells in Non-Mucosal and Mucosal Compartments

Characteristic	Serum/Systemic	Secretory/Mucosal
Molecular form of IgA	Monomeric IgA	Polymeric IgA
Subclass of IgA	IgA1 > > IgA2	IgA1≥IgA2
Transport into secretions	No	Yes
Ontogeny	Matures late	Matures early
J-chain expression	Mostly negative	Mostly positive
Secretory component binding	Mostly negative	Mostly positive
Origin of precursors	Bone marrow	Peyer's patches

Approximately 50% of the leukocytes in the reproductive tract are CD3+T cells, with the remainder consisting of neutrophils (CD66b+), B cells (CD19+), and monocytes (CD14+). In particular, NK cells and CD3+ T lymphocytes, including the CD4+ T helper cells and CD8+ T cytotoxic-suppressor cells, are the two major leukocyte subsets present among endometrial leukocytes. Studies have demonstrated that cervical Class I MHC-restricted CD8+ and Class II MHC-restricted CD4+ cytotoxic T cells can lyse autologous target cells made to express HIV-1 and other viral proteins.

Discrete lymphoid aggregate (follicles) in the endometrium are composed primarily of a B cell core (CD19+) surrounded by T cells (CD3+, CD8+, CD4−) and an outer halo of macrophages (CD14+). Antigen presenting cells in the reproductive tract, which are responsible for initiating the immune response by presenting antigens to T lymphocytes, are derived proportionally from reproductive tract cells and peripheral blood mononuclear cells.

Independence of the Secretory and Systemic Immune Systems

Comparison of the molecular properties of IgA in serum and secretions and of the cells in the secretory tissues and bone marrow suggests that human serum and secretory IgA systems display a great degree of independence (Table 14.3). The molecular properties of IgA produced by the bone marrow (the primary source of serum IgA) and the secretory tissues and glands (the source of secretory IgA) are different in that serum IgA is predominantly monomeric, whereas secretory IgA consists of J-chain-containing polymers. Furthermore, the parallel distribution of IgA subclasses in serum and bone marrow cells, on the one hand, and external secretions and cells in the secretory tissues and glands, on the other, strongly suggest that the two systems are represented by populations of IgA molecules and cells with distinct properties. The ontogenies of the serum and secretory IgA systems display characteristically independent patterns of maturation. Adult levels of IgA are reached in external secretions as early as one month after birth but before 2 years of age, while adult levels of serum IgA are not achieved until adolescence.

Comparison of the levels and molecular properties of the immunoglobulins in blood and in the external secretions of the female genital tract indicate that in humans the systemic and mucosal compartment of the immune system display a considerable degree of independence.[23] In comparison to other components of the common mucosal immune system, the mucosal immune system of the female reproductive tract is characterized by a significant contribution of the systemic compartment with respect to the immunoglobulin isotype distribution, unique distribution and phenotypes of B and T cells, strong hormonal dependency, lack of typical lymphoepithelial inductive sites and relative weakness of immune responses to local infections or immunizations with certain antigens.

Alloimmunity and Reproduction

The Fetal Allograft

Maintenance of human pregnancy requires a tightly balanced and cooperative effort between the mother and fetus. Although the concept of the fetal allograft, first posited by Billingham and colleagues[32] nearly 6 decades ago, remains important, the immune interactions between mother and fetus are two-sided and even more complex than can be modeled by a simple allograft. The fetus, which carries maternal and paternal antigens, is more specifically called a semi-allograft but must still circumvent potentially catastrophic alloimmunity. It does this through alterations in antigen presentation and robust secretion of immunoregulatory pregnancy hormones. The mother, in turn, must balance tolerance to paternal alloantigens against a continued need for defense against pathogen invasion.[33] She does this via local alterations in decidual immune cell subpopulations combined with alterations in local and systemic immunoregulatory hormone and chemokine-cytokine secretion.

Cellular and Soluble Mediators of Immune Responses at the Maternal-Fetal Interface

Immune Cell Subtypes

As mentioned above, the immune cell repertoire of the human endometrium differs dramatically from that of the peripheral blood and is characterized by very few B lymphocytes but fairly abundant T cells, macrophages, NK-like cells, and a number of more specialized immune effector cell subtypes. The relative proportions of these resident cells demonstrate remarkable menstrual cyclicity. A few of these cellular populations deserve specific description, as their presence in both the female reproductive tract and in the peri-implantation decidua implicate a particularly important role for innate immune interactions during implantation and early pregnancy.

Peripheral immune cells have been described that have characteristics of both NK cells and T cells. These NKT cells and their ligands (e.g., CD1) have been detected in the endometrium and decidua of humans.[34] NKT cell interactions with the MHC-like antigen presenting molecule, CD1d, can result in proinflammatory or in tolerogenic effector functions,[35] suggesting that these interactions may be important in maintaining the delicate immune balance at the maternal-fetal interface. Other NK-like cells also populate the late luteal phase endometrium and decidua of women. These unusual cells have been variably called decidual granular lymphocytes, large granular lymphocytes (LGLs), and decidual natural killer cells (dNKs) and they exhibit phenotypic

differences in their cell surface CD expression from typical NK cells found in the periphery. Still, most investigators consider them to be NK cell variants. The proportion of NK cells in the decidua of early pregnancy rises to nearly 70% to 90% of the total endometrial lymphocyte population.[36] While NK cells can display activating and inhibitory receptors on their cell surfaces, the predominant interactions of those in the decidua involve killer inhibitory receptor (KIR) ligation with trophoblast MHC Class I products (discussed previously in the chapter)[36,37] and the outcome of these interactions is unlikely to be cytotoxic.[38] Rather, the cytokine and chemokine products of the decidual NK cell tend to be tolerogenic and to support trophoblast invasion and decidual spiral artery remodeling.[36,37] Very elegant investigations have shown that decidual NK cells are derived from a small population of endometrial NK cells that await pregnancy for robust expansion.[39] These cells do not appear to be recruited from the peripheral blood, as previously hypothesized. Thus, the measurement of *peripheral* NK cell number or activity as a predictor of pregnancy outcome is unlikely to reflect aberrancy in the function of *decidual* NK cells in pregnancy maintenance.

Animal and human data have also suggested roles for "suppressor" macrophages and TCR γδ+ T lymphocytes in the immunology of early pregnancy. Mimicking the subcategorization of T helper cells, macrophages have been classified based on their cytokine secretion patterns and the abundant macrophages of the uterine decidua typically exhibit an immunosuppressive phenotype or M2 polarization.[40] The M1 phenotype is proinflammatory. In contrast, M2 cells exert robust interleukin-10 (IL-10) secretion and indoleamine 2,3 dioxygenase activity. As mentioned above, TCRγδ+ T cells have presumed importance in innate immunity. Their presence within the human endometrium increases dramatically in early pregnancy, they preferentially secrete IL-10 and TGF-β and they support trophoblast invasion and inhibit trophoblast apoptosis.[41] All should promote pregnancy maintenance.

Soluble Immune Effectors

The human endometrium and decidua are replete with immune and inflammatory cells capable of cytokine and chemokine secretion, but the role of individual factors in pregnancy maintenance may be fairly minimal. Most cytokine genes have been individually deleted in animal models and few, if any, of these factors appear to be absolutely required in pregnancy maintenance. Still, two are frequently discussed as particularly integral. Leukemia inhibitory factor (LIF), and IL11, both IL6 family cytokines, have been demonstrated to be required for blastocyst implantation, but not for subsequent embryogenesis in mice,[42,43] indicating the defect induced by their deletion is in the maternal compartment. Further delineation of the mechanisms for these implantation abnormalities indicate that LIF functions at the blastocyst attachment/adhesion phase and IL-11 in the response to the implanted blastocyst.[44] The fact that essential roles for individual cytokines in pregnancy maintenance is so limited speaks to the likely redundancy in this system and spurs future studies addressing the integrated effects of multiple cytokines.

Application of the Th1/Th2 paradigm to pregnancy maintenance has led to the hypothesis that the intrauterine environment surrounding normal human pregnancies is Th2-dominant and *in vitro* data have shown that peripheral blood cells from women with normal pregnancy histories secrete Th2 type cytokines when exposed to placental antigens; some women with histories of recurrent pregnancy loss have Th1 responses in the same *in vitro* assay systems.[45,46] Inflammatory cytokines characteristic of Th1 responses (e.g., IFNγ) have been shown to be harmful to the implanting embryo.[47] While the Th1/Th2 paradigm has been useful as a stepping stone in our understanding of the immunology of pregnancy maintenance, rapid expansion in descriptions of cytokine/chemokine activities and in T helper cell subpopulations suggests that the Th1/Th2 paradigm must also be expanded to include Th17, Th3, T regulatory, and possibly even more cytokine-secreting immune cells at the maternal-fetal interface. Further, the antigen recognized at the site of implantation that might cause dysregulation of the local T helper response phenotype has yet to be identified *in vivo*. *In vitro* data have been based on the supposition that the antigen(s) is placental in origin, which is a reasonable but unverified hypothesis. Cytokine profiles in the microenvironment of an implanting fetus are also difficult to document and dysregulation in the presence of a failing pregnancy will always be obfuscated by the question of cause versus effect. The possibility that dysregulation of T helper response causes pregnancy loss remains intriguing but unproven.

Antigen Presentation in the Placenta

There is little controversy that the characteristics of antigen presentation in the placenta are unique. For many years, Medawar's paradox of the fetal allograft appeared consistent with the assumption that the placenta lacked MHC Class I and Class II transplantation antigens. This assumption, however, was only partially correct. It is true that MHC Class II molecules are not expressed on the surface of placental trophoblast cells, nor are the classical MHC Class I transplantation antigens HLA-A and -B. However, the classical MHC Class I product, HLA-C, and two non-classical products, HLA-G and HLA-E, are found on particular subpopulations of trophoblast cells.[48-50]

The human placenta, which is of the hemochorial type, is a complex structure that forms intimate association with maternal tissues primarily through two types of cells: (1) syncytiotrophoblast (SynT) cells, the fetally-derived trophoblast cell subset that coats the surface of the placenta villi, are directly bathed in maternal blood and (2) extravillous trophoblast (EvT), which invade deeply into the maternal decidua, interact directly with maternal decidual immune cells, and remodel the uterine spiral arteries.[51] Both of these trophoblast subsets are derived from an ill-defined, stem-cell like cytotrophoblast (cytoT) subpopulation (Fig. 14.9).[52,53] At the surface of placental villi, within the decidua and within decidual spiral arteries, fetally-derived trophoblast cells intimately contact maternal immune effector cells, thereby exposing the fetus to potential MHC-restricted recognition as non-self.

While the syncytiotrophoblast cells of the placental villi are devoid of MHC Class I and Class II molecules, those of the EvT and of the decidual endovascular trophoblast (EnT) are not. The unique MHC Class I expression pattern of the extravillous and endovascular cytotrophoblast

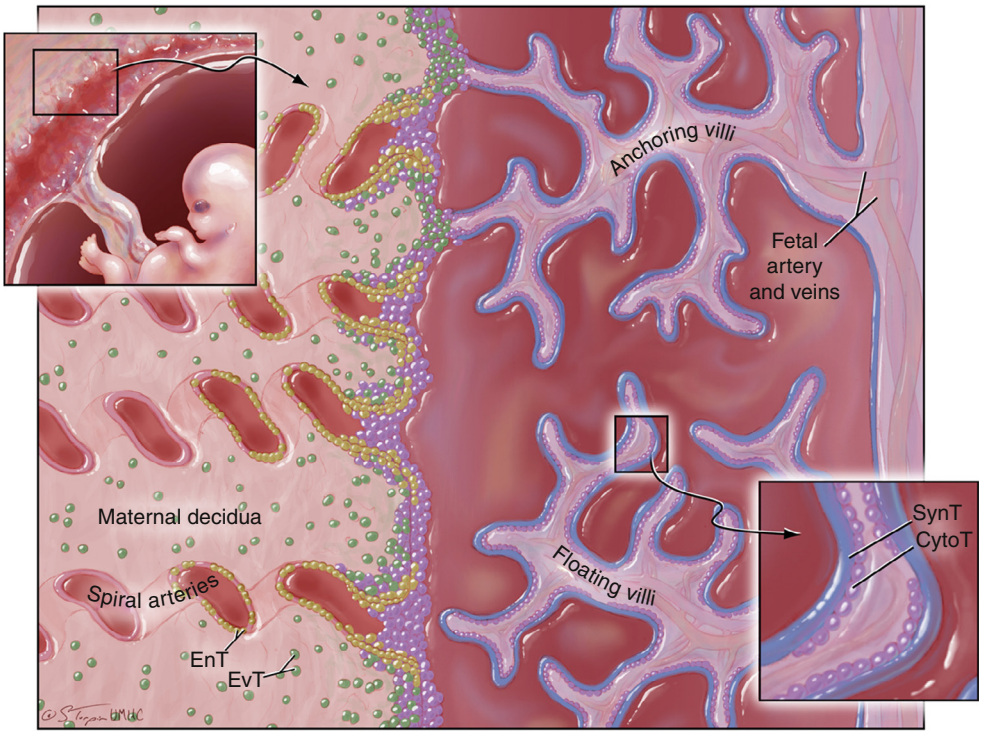


FIGURE 14.9 Trophoblast cells at the maternal-fetal interface. Only the trophoblast cells of the implanting blastocyst and developing fetus make direct contact with maternal tissues, including maternal immune cells. These cells include the syncytiotrophoblast (SynT) cells, the extravillous trophoblast (EvT) and the endovascular trophoblast (EnT) cells. SynT and EvT arise from stem cell-like cytotrophoblast cells. Over the entire surface of the floating and anchoring villi of the placenta, cytotrophoblast cells are covered by a multinucleated layer of SynT which comes into direct contact with maternal blood bathing the intervillous spaces. At the points of contact between the anchoring villi and the maternal decidua, a population of cytotrophoblast cells differentiates into extravillous trophoblast cells which have invasive properties. In humans (unlike mice) these cells can invade throughout the entire maternal decidua (where they contact decidual immune cells) and into the inner third of the myometrium. The endovascular trophoblast cells remodel and replace the typical cells in the walls of the maternal spiral arteries, making them relatively atonic conduits supplying maternal blood to the interstitial space. Here, the EnT are seen to be in direct contact with maternal blood. It is unclear whether EnV develop from EvT, directly from CytoT, or both. Here we depict a placenta with trophoblast plugging of the spiral arteries at their entry into the interstitial space. These plugs will be gone by about 12 to 13 weeks of gestation.

cell and its contact with the maternal immune system has spurred extensive hypotheses and investigations to explain why all placental cells down-regulate expression of the classical transplantation antigens (HLA-A and -B), while invasive extravillous and extravillous cytotrophoblast express HLA-C, -E, -G, and possibly -F[54,55] do not. These particular classical (HLA-C) and non-classical (HLA-E, -F, and -G) molecules are intriguing in that they seem to exhibit a fairly limited degree of polymorphism, making them less effective than their highly polymorphic classical MHC Class I molecule (HLA-A and -B) counterparts in antigen presentation. Most investigators now agree that trophoblast antigen-presenting molecules are central in innate immunity during pregnancy, particularly through interactions with the abundant, albeit somewhat enigmatic decidual NK cells (dNK; discussed later in the chapter). This novel approach, although radically shifting attention away from the Medawar allo-transplantation paradigm, does have attractive evolutionary support. For instance, since NK cells will recognize and kill cells that lack MHC Class I products, the expression of Class I products with limited polymorphism (HLA-G and -E) or limited surface expression half-life (HLA-C) may protect extravillous cytotrophoblast cells from NK-like dNK-mediated attack.[37,56] With deference to Medawar and colleagues,[32] down-regulation of more classical transplantation antigens (HLA-A and -B) on all trophoblast cells may protect from allogenic T cell attack. Trophoblast cells are, in fact, resistant to lysis by CD8+ T cells *in vitro* while fetal fibroblasts are not.[13] Alternatively, interactions of trophoblast-expressed HLA-C, -E, -F, or -G with surface receptors on maternal immune effector cells may modulate immune cell cytokine-chemokine expression profiles.[57,58] Interactions between trophoblast MHC molecules and activating and inhibitory receptors on decidual NK cells and macrophages have received particular attention.[37,58,59] These interactions are poor inducers of cytotoxicity,[38] but are important in promoting immune effector functions supporting pregnancy[58,60] as well as decidual and vascular trophoblast invasion, spiral artery remodeling and angiogenesis.[61-63] The effects of trophoblast MHC Class I products on maternal adaptation to implantation of the allogeneic pregnancy need not be direct. For instance, circulating soluble forms of MHC Class I products, such as HLA-B, have been shown to promote tolerance.[64] When secreted into the maternal circulation, similar soluble forms of trophoblast HLA-G appear to promote maternal tolerance to pregnancy-associated tissues.[57] Finally, isolated polymorphisms in trophoblast Class I molecules[54,65] and in its immune cell receptors[66] and alterations in the Class I/NK receptor repertoire[67,68] have been associated with adverse

pregnancy outcomes, particularly isolated and recurrent pregnancy loss. Interestingly, evidence in mice suggests that antigen presentation in pregnancy is severely restricted and only commences after the stage of endovascular invasion (after 9 to 12 weeks in humans).[69]

Immunomodulation and Pregnancy Maintenance

Most investigators now believe that during a normal pregnancy, the implanting fetus is usually recognized by the maternal immune system and immune responses, including proinflammatory-proangiogenic responses to fetal antigens, have been demonstrated.[62,70] These responses, however, occur in an environment characterized by dramatic systemic and local hormonal and metabolical alterations. Many of these alterations have potential immunomodulatory effects and any or all may be critical for pregnancy maintenance. While the pregnant state has long been considered a state of relative immunosuppression to protect the allogenic fetus, this appears to overly simplify the immunology of pregnancy. Pregnancy is not a state of total systemic immunosuppression but rather a state of dramatic immune modulation.

Some evidence does support the concept that a decrease in systemic maternal immune responses occurs in normal pregnancy and may be essential to pregnancy maintenance. For instance, failure to down-regulate maternal responses to recall antigens, such as tetanus toxoid and influenza, has been linked to poor pregnancy outcome among Recurrent Pregnancy Loss (RPL) patients.[71] T helper cell type 1 (Th1)-mediated autoimmune diseases, such as rheumatoid arthritis (RA) and multiple sclerosis (MS), often improve during pregnancy. T helper cell type 2 (Th2)-mediated disorders such as systemic lupus erythematosus (SLE), however, frequently worsen during pregnancy.[72] Further, some viral diseases and several parasitic diseases[73] are particularly aggressive when first encountered during pregnancy. Still, pregnant women are not more susceptible to the vast majority of infectious diseases, nor are they safeguarded from the effects of the majority of autoimmune disorders.

Whether pregnant or not, women are, in general, more susceptible to autoimmune diseases than men. This striking gender difference in immune resposivity[74] is thought to reflect effects of the reproductive hormones on peripheral cell mediated immunity. Reproductive steroid hormones (e.g., estrogen, progesterone, and testosterone) and protein hormones (e.g., prolactin) are all potent immune modulators.[75-77] The effects of reproductive hormones within the microenvironment of the maternal-fetal interface may be further enhanced by the fact that their concentrations are significantly higher than those in the maternal circulation.[78]

Circulating levels of maternal estrogens and progesterone are elevated early in pregnancy and this elevation continues throughout gestation (Fig. 14.10).[72] Many of the described immunomodulatory effects of these hormones can be considered to support pregnancy. Progesterone's immunosuppressive characteristics include an inhibition of mitogen-induced proliferation of CD8+ T cells and of cytokine secretion by these cells and a promotion of Th2-type responses with increased expression of LIF.[33,74,79] Both responses would be predicted to promote an immune environment favoring pregnancy maintenance. Interestingly, the effects of progesterone on immune cell function are not necessarily mediated by the classical cytoplasmic-nuclear receptor[80] but rather, can occur via direct cell membrane alterations[81] or via the membrane progesterone receptor. Estrogen-related immune alterations that would be predicted to promote pregnancy maintenance include the down regulation of delayed type hypersensitivity (DTH) reactions, the promotion of Th2-type immune responses and demonstrated protection against chronic allograft rejection.[79,82,83] Prolactin has stimulatory effects on both cell-mediated and humoral immune responsiveness.[77] The magnitude of these effects depends on posttranscriptional modifications of the prolactin molecule and may extend to innate immune effector cells such as γδ T cells.[84] Human chorionic gonadotropin has also been recently implicated in pregnancy immunomodulation through purported effects on recruitment of regulatory T cells to the site of implantation during early human pregnancy.[85]

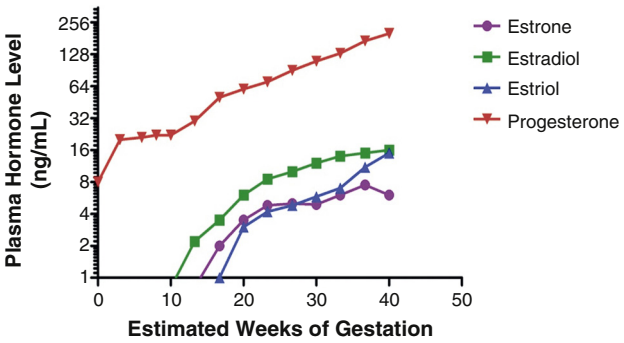


FIGURE 14.10 Plasma hormone levels during pregnancy. Levels of estrone, estradiol, estriol, and progesterone in ng/mL during human gestation to 40 weeks.

Metabolical alterations during pregnancy may also play a role in protective immunomodulation. Indoleamine 2,3 dioxygenase (IDO) is involved in the catabolism of the amino acid, tryptophan. Since T cells require tryptophan for activation and proliferation, local alterations in tryptophan supply and/or in IDO activity would be predicted to alter T cell mediated immune activity. IDO activity has recently been shown to support the suppressive function of T regulatory cells.[86] Alterations in tryptophan concentrations and in IDO activities have been linked to several adverse pregnancy outcomes, including pregnancy loss and preeclampsia.[52,87]

Alloimmunity and Recurrent Pregnancy Loss

Since the fetus is antigenically different from the mother, the mother's immune system might be predicted to identify the fetus as foreign and execute an immune response. The abundance of immune cells within the decidua and the production of numerous immunological cytokines at the maternal-fetal interface make the potential for an immune-associated pregnancy rejection plausible since, unless the appropriate mechanism(s) are in place to modify maternal immune processes, the semi-allogeneic fetus should be rejected. A variety of alloimmune factors have been postulated to cause recurrent pregnancy loss and innumerable investigations have been performed to address the potential role of alloimmune factors in RPL. Several approaches to the diagnosis of alloimmune RPL have been considered in almost painstaking detail, including antipaternal cytotoxic antibody testing, mixed lymphocyte culture reactivity

testing, embryotoxicity evaluations, immune cell phenotyping, peripheral cytokine phenotyping, and human leukocyte antigen (HLA) profiling. While none of this testing has proven to be clinically useful in its present forms, many of these diagnostic approaches are currently employed in clinical practice. They are outlined briefly here because they have historical and mechanistic interest and not as an endorsement of their clinical utility.

Embryotoxicity assays have been proposed as a means to detect abnormal peripheral cellular immune responses to human trophoblast-derived antigens.[88] The embryotoxic factor (ETF) assay measures the development of an inflammatory reaction to the presence of placental antigens by maternal peripheral immune cells that may predict similar *in vivo* responses during a subsequent pregnancy. While the theoretical basis for the use of the ETF assay may have some merit, the commercially available test has proven to be neither consistent nor predictive of pregnancy outcome.

Immune phenotyping using flow cytometric analysis of peripheral blood cells, especially CD56+ natural killer (NK) cells, has been extensively evaluated in recurrent pregnancy loss; a comprehensive review in 2011 reports over 780 publications, of which only 12 were deemed of sufficient quality for inclusion in an analysis.[89] The authors of this review found no consistent evidence that peripheral NK cell testing predicted subsequent pregnancy outcome in women with RPL. Flow cytometric analyses have recently confirmed in non-pregnant women with RPL that peripheral NK cell phenotypes are quite different from endometrial NK phenotypes,[65] further suggesting that peripheral phenotyping is unlikely to be useful in improving our understanding of immunology at the maternal-fetal interface. In summary, the current clinical literature supports neither testing for peripheral NK cell activity nor immunomodulatory treatments based on the results of these assays.[90]

Antipaternal cytotoxic antibody testing and antileukocyte antibody assays are likewise of little value in the assessment of couples with recurrent pregnancy wastage. These antibodies are usually not detected before 28 weeks gestation and are often not detectable in non-pregnant women. This makes them of little clinical utility as screening tools since they cannot prospectively and predictably direct interventions. Testing for the development of "blocking antibodies" using mixed lymphocyte culture (MLC) reactivity assays does not aid in the prediction of pregnancy outcome, but rather, reflects the number and duration of prior pregnancies.

Peripheral cytokine profiling studies are plagued by concerns about cytokine dysregulation being a cause rather than an effect of the pregnancy demise. Further, since the measurement of peripheral cytokine levels does not appear to reflect changes occurring locally at the maternal-fetal interface[91] and the specific cytokines or cytokine patterns of etiological importance in human pregnancy loss remain unclear, such testing among women with recurrent pregnancy loss remains experimental and should be offered only under an IRB-approved protocol and at no cost to the patient or third party insurer.

The use of HLA typing in the diagnostic work-up of recurrent pregnancy loss patients has also been specifically investigated. The best investigation of the hypothesis that maternal and paternal MHC haplotype similarities might result in pregnancy loss involved an isolated religious sect in South Dakota: the Hutterites. From this circumscribed, stable, and inbred population came initial data refuting a role for HLA matching in adverse pregnancy outcome.[92] However, a more recent and expanded investigation of the same population reported that maternal and paternal HLA matching can be associated with adverse pregnancy outcomes.[93] However, as discussed in this manuscript, exact HLA matching is virtually never seen in outbred populations and therefore HLA typing is not recommended in patients with recurrent pregnancy loss. Recent large studies have also shown no association between HLA-DQα homozygosity and RPL.

To summarize a long and voluminous literature, no testing for alloimmune-associated pregnancy wastage is currently indicated in the evaluation of RPL and no treatment interventions based on this testing should be instituted except as part of an IRB-approved research study. A multicentered, prospective, randomized, placebo-controlled trial comparing intravenous immunoglobulin to saline for the treatment of idiopathic secondary recurrent miscarriage concluded that there was no benefit in treatment of patients with potential alloimmune factors.[94]

Autoimmunity and Reproduction

Numerous studies have attempted to identify autoantibodies associated with pregnancy failure. The most commonly studied antibodies in women with pregnancy loss and infertility include antiphospholipid and antithyroid antibodies. Antiovarian antibodies have been associated with polyglandular autoimmune syndromes including ovarian insufficiency and ovarian failure. Other immunological factors of potential interest to reproductive endocrinologists include antigliadin antibodies, antinuclear antibodies, antisperm antibodies, and endometriosis-associated immune responses.

When autoantibodies are found in association with pregnancy failure there are several possible explanations:

- A direct stimulus generates autoantibodies which may impact the health of the pregnancy (e.g., autoimmune hemolytic immunity)
- A remote disease process leads to tissue damage that may result in antibody formation indirectly affecting the pregnancy (e.g., thyroiditis)
- A factor is present that simultaneously negatively impacts the pregnancy and induces autoantibodies (e.g., a virus)

Autoantibodies may be *organ-specific*, as in the case of Hashimoto's thyroiditis. In this disorder, there is a specific lesion in the thyroid involving infiltration by mononuclear cells and destruction of follicular cells; this is followed by the production of antibodies with absolute specificity to thyroglobulin or thyroid peroxidase. On the other hand, autoantibodies may be *non-organ specific*. For example, in patients with systemic lupus erythematosus, antibodies are formed but lesions and autoantibodies are not confined to any one organ. Although antibodies may be detected for many years after they are initially identified, their presence does not always indicate a disease process. There may be considerable overlap in the specificity of the autoantibodies; this is more common for non-organ specific antibodies.

An increased familial incidence of autoimmunity has been determined to be genetic based on studies of identical and non-identical twins. Familial autoimmunity more frequently is organ-specific. Strong associations have been established between the major histocompatibility complex and autoimmune disease.[95] Linkage of human leukocyte antigen (MHC Class II) haplotypes predispose individuals to Hashimoto's thyroiditis (DR4 and DR3), Celiac disease (DQ2 and DQ8), and Type 1 diabetes (DR3, DR4, and DQβ1).

Antiphospholipid Antibodies (aPL)

Antiphospholipid antibodies (aPL) are acquired antibodies (IgG, IgM, and/or IgA isotypes) directed against negatively charged phospholipids, a group of inner and outer cell membrane antigens found in mammals. Obstetrical antiphospholipid antibody syndrome (APS) is diagnosed in the presence of certain clinical features in conjunction with positive laboratory findings. Although obstetrical APS was originally reported in association with slow progressive thrombosis and infarction in the placenta, it is most often associated with poor obstetrical outcomes. In fact, obstetrical APS is one of the most commonly identified causes of recurrent pregnancy loss. Thus, obstetrical APS is distinguished from APS in other organ systems where the most common manifestation is thrombosis. Present in an estimated 2% to 5% of the general population, aPL are commonly associated with other autoimmune diseases (secondary APS) such as systemic lupus erythematosus, but can also present in isolation in the form of primary antiphospholipid syndrome.

Antiphospholipid syndrome was first described in 1983 in patients with systemic lupus erythematosus, the presence of anticardiolipin antibodies and thrombosis. Since this initial presentation the criteria for diagnosis have been revised.[96] In order for a patient to be diagnosed with antiphospholipid syndrome at least one of the clinical criteria and one of the laboratory criteria must be met (Box 14.1).

Box 14.1 Research Criteria for the Diagnosis of Antiphospholipid Antibody Syndrome

CLINICAL CRITERIA

1) Vascular Thrombosis: One or more episodes of arterial, venous, or small vessel thrombosis in any tissue or organ.
2) Morbidity in Pregnancy
   - One or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation.
   - One or more premature births of a morphologically normal neonate prior to the 34th week of gestation secondary to eclampsia or severe preeclampsia or recognized features of placental insufficiency.
   - Three or more unexplained consecutive spontaneous miscarriages before the 10th week of gestation.

LABORATORY CRITERIA: (MUST BE PRESENT ON TWO OR MORE OCCASIONS AT LEAST 12 WEEKS APART):

- Lupus anticoagulant present.
- Anticardiolipin Antibody; medium or high titer (>40 micrograms of IgG or IgM phospholipids or >99th percentile) of IgG or IgM isotype.
- Anti-β2-glycoprotein; medium or high titer (>40 micrograms of IgG or IgM phospholipids or >99th percentile) of IgG or IgM isotype.

Recurrent Pregnancy Loss and Antiphospholipid Antibodies

Recurrent pregnancy loss affects up to 5% of reproductive aged women. Evaluation classically begins after three consecutive miscarriages of less than 10 weeks gestation but may be warranted earlier if a prior miscarriage was found to be euploid, or if there is concomitant infertility or advanced maternal age.[97] An identifiable cause of RPL will be detected in approximately 50% of couples after an investigation of possible genetic, anatomical, immunological, and endocrine causes (Table 14.4).[98]

Antiphospholipid antibodies (aPL) will be identified in a significant number of women with RPL after a careful evaluation. aPL interact with the maternal-fetal interface in multiple ways and are associated with RPL.[25] The involvement of aPL in adverse pregnancy outcomes is thought to be more as an autoimmune factor than a thrombophilic factor; histological studies of placentas obtained following spontaneous miscarriage have found defective endovascular trophoblastic invasion rather than intervillous blood clots.[99] Studies have linked aPL with decreased release of hCG from human

Table 14.4 Standard Evaluation and Management of Recurrent Pregnancy Loss

Etiology	Diagnostic Evaluation	Abnormal Result	Therapy
Structural genetic	Cytogenetic analysis of both partners	3%–5%	Genetic counseling, donor gametes, preimplantation genetic screening
Anatomical	Hysterosalpingogram, hysteroscopy, or sonohysterography	15%–20%	Hysteroscopic septum resection, myomectomy, lysis of adhesions
Endocrinological	Midluteal progesterone	8%–12%	Progesterone
	Thyrotropin		Levothyroxine
	Prolactin		Bromocriptine, cabergoline
	Hemoglobin A1c		Metformin
Immunological	Lupus anticoagulant Antiphospholipid antibodies β2 Glycoprotein 1	15%–20%	Heparin + aspirin
Lifestyle issues	Tobacco use	15%–20%	Eliminate consumption
	Excessive alcohol		Reduce consumption
	Exposure to toxins		Eliminate exposure
	Obesity		Lower body mass

placental explants, prevention of *in vitro* trophoblast migration and invasion, inhibition of trophoblast cell adhesion molecules and activation of complement on the trophoblast surface inducing an inflammatory response.[100] In women referred to reproductive endocrinologists with APS and histories of early loss, the prevalence of prior thromboembolic events is low. However, these patients are still at risk for thromboembolic events. Estrogen-containing oral contraceptive pills (OCPs) should be avoided in women with repeated positive aPL.

Antiphospholipid antibodies have been found in 15% of patients with recurrent first trimester loss. The original clinical trial on treatment of obstetrical APS that reported the superiority of unfractionated heparin and low-dose aspirin[101] has been reaffirmed by a recent systematic literature review and meta-analysis. Multiple independent investigators found that patients with aPL-associated recurrent pregnancy loss who were treated with unfractionated heparin and low-dose aspirin had improved live birth rates.[102] However, studies proposing anticoagulation for the treatment of unexplained RPL in women without aPL have not shown a benefit of treatment.[103,104]

Controversy still exists concerning testing for antibodies against other phospholipids, but an increasing number of studies suggest that antibodies to phosphatidyl serine are also associated with pregnancy loss. The College of American Pathologists added antibodies to phosphatidyl serine to its proficiency test profile in 2012. In women who have a diagnosis of APS by meeting both clinical and laboratory criteria, the chance for successful pregnancy is reduced. In these cases, treatment appears to be a clear option particularly in the case of those with prior thromboembolic events. Current clinical guidelines support the use of subcutaneous heparin and aspirin. This treatment should begin with a positive pregnancy test, continue throughout pregnancy, and extend postpartum.

Infertility and aPL

Antiphospholipid antibodies have been reported with increased frequency in women with unexplained infertility and in women with recurrent implantation failure after *in vitro* fertilization.[105] It is plausible that aPL contained in the serum could have a significant impact on ovulation, fertilization, or early embryonic development. As aPL have been shown to interact at the maternal-fetal interface resulting in a defective endovascular trophoblastic invasion, it is scientifically plausible that aPL may induce infertility via their negative effects on implantation.

One method of assessing the effects on aPL on fertility and implantation is to evaluate the effects of aPL on the success of IVF procedures in women who have not received aPL-related anticoagulation therapy. More specifically, one can compare the IVF outcomes among aPL positive subjects to those who are aPL negative. This analysis was performed by the American Society for Reproductive Medicine who concluded that although the frequency of aPL is increased in infertile patients undergoing IVF, the cumulative evidence does not demonstrate a clear negative impact of aPL on IVF success.[106] Therefore, since infertility outcomes, including IVF have not been demonstrated to be significantly impacted by the presence of aPL, these patients should not be routinely screened for aPL.

Antithyroid Antibodies (ATA)

Antithyroid antibodies (ATA) directed against thyroglobulin and thyroid peroxidase are commonly found in patients with Graves disease, postpartum thyroiditis, and Hashimoto's thyroiditis. However, ATA have been reported in healthy individuals and are observed more frequently in women during their reproductive years. The prevalence of ATA has been reported in 15% to 20% of normal pregnant women compared to 20% to 25% in women with recurrent miscarriage[107] and 46% of pregnant women with a diagnosis of hypothyroidism.[108]

Pregnancy Loss and ATA

Multiple investigations into the role of thyroid autoimmunity in RPL have been reported, but the interpretation of the evidence as a whole is difficult secondary to numerous variations in study design. Older studies have shown a significantly increased relative risk for thyroid autoimmunity in women with RPL who were euthyroid, but most of these studies used TSH values of 4.5 or 5.0 mIU/mL to define the upper limits of normal. The American Endocrine Society defines hypothyroidism as present when TSH values are greater than 2.5 mIU/mL and defines normal TSH levels between 1.0 and 2.5 mIU/mL during the first trimester of pregnancy. It is likely that many of the studies included women with ATA who had TSH levels greater than 2.5 mIU/mL but less than 5.0 mIU/mL and that clinical hypothyroidism, not the ATA, accounted for the increased prevalence of RPL.[109]

The most likely explanation is that hypothyroidism (TSH > 2.5 mIU/mL) resulting from ATA may be responsible for the increased incidence of RPL in this population. ATA have been reported in increased frequency among women with RPL and may indicate thyroid dysfunction. However, if the patient is euthyroid, the presence of antithyroid antibodies does not affect pregnancy outcome.[110,111] Women who have positive antithyroid antibodies and are euthyroid are at an increased risk for hypothyroidism during and after pregnancy. These women should have their thyrotropin levels tested during each trimester and postpartum for thyroiditis.

Studies on the treatment of subclinical hypothyroidism are variable and current guidelines on screening patients with RPL for thyroid dysfunction and autoimmunity are conflicted. Currently, it is advised to screen all women with RPL using an ultrasensitive assay for TSH. When TSH values are between 1.0 and 2.5 mIU/mL, no further action is indicated. If TSH values are above 2.5 mIU/mL, some clinicians will check for ATA and treat patients with levothyroxine to obtain TSH values between 1.0 and 2.5 mIU/mL. During pregnancy the requirement for replacement usually increases by about 25% so the clinician can increase replacement doses or monitor TSH levels closely during pregnancy. Because of the risk of postpartum thyroiditis, patients with thyroid dysfunction should be monitored and dosages of replacement adjusted as indicated.

Infertility and ATA

Clinical hypothyroidism that results from primary thyroid disease commonly leads to anovulation and infertility. The overall incidence of clinical hypothyroidism in anovulatory women is 4% to 7%.[108,112] The mechanism of anovulation in

these patients is an alteration of gonadotropin-releasing hormone (GnRH) pulsatility mediated through an increase in TRH and its effects on the production of prolactin. Therefore, any patient with both infertility and irregular menses should be screened with a serum TSH. The effects of subclinical hypothyroidism on fertility are unknown. However, individuals with subclinical hypothyroidism who conceive need to be re-evaluated during pregnancy as both their need for thyroid hormone will increase with pregnancy, and their "new normal" TSH levels will drop secondary to the stimulatory effects of hCG on thyroid hormone production.

Unlike the clear effects of hypothyroidism on anovulation and subsequent infertility, the effects of ATA on fertility are much less clear. Previous studies of infertile women undergoing IVF have not demonstrated either an increased rate of ATA in this population of patients nor a negative impact of ATA on IVF outcomes.[112] Despite the data against a relationship between the presence of ATA and adverse IVF outcomes, some investigators have evaluated the effects of immune-directed therapy in euthyroid women with elevated ATA levels on infertility treatment.[113] The relationship between ATA and fertility remains a subject that has not been extensively studied, and to date, there is clearly no indication for routinely testing infertile women for ATA.

Autoimmune Ovarian Insufficiency (AOI)

Increasingly ovarian autoimmunity is being implicated in the etiology of ovarian insufficiency, previously known as premature ovarian failure or premature menopause.[114] The diagnosis of autoimmune-mediated ovarian insufficiency is hampered because of the lack of specific and sensitive markers of disease. Antiovarian antibodies include antibodies against a heterogeneous group of antigens, including molecular targets in the zona pellucida, theca interna, granulosa cells, ooplasm and heat shock protein 90-β.[115] The best characterized cases of antiovarian antibodies has been in conjunction with polyglandular autoimmune syndromes.

When the dysfunction of two or more endocrine glands occurs in association with circulating organ-specific antibodies directed against the involved glands, the term polyglandular autoimmune syndrome (PAS) is applied.[116] The two major subtypes of PAS, types I and II, are distinguished according to age of presentation, characteristic patterns of endocrine deficiencies present, and inheritance pattern (Table 14.5). The principle endocrine components are adrenal insufficiency, thyroid hyperfunction or hypofunction, insulin-dependent diabetes mellitus, and premature gonadal failure. The basic underlying abnormality associated with polyglandular autoimmunity is a defect in the T-suppressor cell function and in PAS type I is caused by mutations in the autoimmune regulatory gene on chromosome 21.[117] There is increasing evidence that aberrant expression of HLA alleles B8 and DR3 located on chromosome 6 play important roles in the pathogenesis of PAS type II. Because of the key role of HLA-DR antigens in the processes of antigen presentation and T and B cell activation, it is thought that alleles of HLA DR3 and DR$ determine the targeting of specific tissues by autoreactive T cells, which leads to organ-specific autoimmunity as a result of a loss of tolerance.[118]

Table 14.5 Characteristics of Polyglandular Autoimmune Syndromes

Characteristic	PAS Type I (Blizzard Syndrome)	PAS Type II (Schmidt Syndrome)
Age of onset	Childhood	Adulthood
Incidence	<1:100,000	1:10,000
Genetics	Monogenic (*AIRE* gene)	Polygenic
Inheritance	Autosomal recessive	Autosomal dominant, linked to specific HLA haplotypes
Autoimmune Diseases		
Hypoparathyroidism	85%	100%
Adrenal insufficiency	65%	15%
Gonadal failure	45%	60%
Type I diabetes	10%	55%
Thyroid disease	10%	70%
Chronic mucocutaneous candidiasis	75%	None

Tissue damage and dysfunction occurring as a consequence of immune activation can develop through several mechanisms. Tissue damage may result from complement fixation and activation, from immune complex formation with complement activation, or via facilitation of natural killer cell binding. Polyglandular autoimmune syndromes include a spectrum of autoimmune endocrine disorders and can be divided into a rare juvenile type (PAS I) and a relatively common adult type with (PAS II) or without adrenal failure (PAS III).

Polyglandular Autoimmune Syndrome Type I

This syndrome is diagnosed in the presence of any two of the following three conditions: hypoparathyroidism, adrenal insufficiency, or chronic mucocutaneous candidiasis. More than 70% of individuals will have a combination of hypoparathyroidism and chronic mucocutaneous candidiasis, neither of which occurs in the other forms of polyglandular immunity. The onset of this disorder is during childhood with the first manifestations always developing before the age of 15 years. The average age of appearance of candidiasis, hypoparathyroidism, or both is 5 years of age and typically precedes the appearance of adrenal insufficiency, which generally occurs by age 13. Primary gonadal failure occurs in approximately half of these individuals.

The genetic mutation responsible for PAS I has been mapped to the autoimmune regulator gene on chromosome 21 (*AIRE* gene). Mutations in this gene result in the loss of central tolerance, the process by which developing T cells with potential reactivity for self-antigens are eliminated during early differentiation in the thymus. PAS type I has an autosomal recessive mode of inheritance. Females are affected about as frequently as males.

Circulating organ-specific antibodies are frequently present in patients with PAS type I. Patients with Addison's disease have circulating autoantibodies that have exclusive specificity to the adrenal cortex. Steroid cell antibodies cross-react with other steroidogenic tissues such as placental syncytiotrophoblast, Leydig cells of the testis, and the theca interna-granulosa layers of ovarian follicles.[119]

Early diagnosis and treatment of the hypoparathyroidism and adrenal insufficiency result in a favorable prognosis. Frequent clinical and laboratory surveillance of patients at risk of developing Addison's disease is important to enable diagnosis and initiation of replacement therapy before the development of acute adrenal crisis. Routine biochemical screening should be conducted at least annually but with increased frequency if clinical signs or symptoms suggestive of adrenal insufficiency develop. Early signals include complaints of weakness and easy fatigability with or without salt craving and, in children, a decrease in linear growth rate. In the past, biochemical monitoring of adrenal status was routinely accomplished with the ACTH stimulation test. More recently the adrenal-antibody test has been shown to have a comparable positive predictive value and a higher negative predictive value than the ACTH test, thus the adrenal-antibody test represents a more efficient tool in the clinical setting.[120]

Polyglandular Autoimmune Syndrome Type II (Schmidt Syndrome)

Polyglandular autoimmune syndrome type II (PAS II) is defined as the association of primary adrenal insufficiency with autoimmune thyroid disease or insulin-dependent diabetes. Individuals with PAS II present at a later age than PAS I with a peak incidence at ages 20 to 60 years, mostly in the third and fourth decades. There is often family clustering so it may be important to consider as affected those relatives of patients with a complete syndrome who themselves have one or more of the principle disease components but may still have normal adrenal function. In less than half of the cases, the initial manifestation is adrenal insufficiency with subsequent components of the disease appearing up to 20 years later. In approximately 30% of the cases, Addison's disease may follow the appearance of other endocrine disorders (Table 14.5). Primary hypogonadism is reported in approximately 10% of PAS II patients and may present as the only extra adrenal manifestation of this disorder.

The inheritance pattern of PAS II is complex. Autoimmune disease susceptibility appears to be inherited in an autosomal dominant fashion associated with specific HLA haplotypes. There is less than 100% concordance in monozygotic twins so environmental factors may be involved in disease pathogenesis contributing to a loss of self-tolerance. The alleles HLA B8 and HLA DR3 are found together more frequently than would be predicted from their frequency in the general population (linkage disequilibrium). The allele DR3 is most frequently associated with autoimmune endocrine disorders. Thus, PAS II is considered a polygenic disease with autosomal dominant inheritance and incomplete penetrance.

Immune Etiology of Ovarian Insufficiency

Premature ovarian failure has been defined as persistent amenorrhea between puberty and age 40 years associated with a hypergonadotropic state. The clinical manifestations are related to estrogen loss and include secondary amenorrhea, vasomotor instability, psychological symptoms, atrophy of the vagina and urethra, osteoporosis, and infertility. There are subcategories of women with regular menstrual cycles and elevated FSH who have incipient ovarian failure while other patients with transitional ovarian failure may present with oligomenorrhea and elevated FSH. Ultimately this heterogeneous condition of ovarian insufficiency develops through two major mechanisms, follicle dysfunction and follicle depletion.[121] Anti-müllerian hormone levels and antral follicle counts may be more accurate measures of follicular pool depletion in young women presenting with elevated FSH levels.[122]

Substantial evidence indicates that ovarian insufficiency can result from autoimmune processes directed against the steroidogenic cells of the ovarian follicle.[123] Ovarian histopathological studies in steroid cell antibody positive patients with premature ovarian failure consistently show lymphocyte oophoritis. CD4+ and CD8+ T lymphocytes and plasma cells make up most of the infiltrate.[124] This is reminiscent of other destructive autoimmune endocrinopathies such as Addison's disease. Microsomal enzymes catalyzing key steps of steroid hormone synthesis are the tissue antigen targets detected by immunofluorescent techniques. These enzyme targets include cytochrome P450 21-hydroxylase, an adrenal specific antigen, cytochrome P450 17-hydrolylase and cytochrome P450 side-chain cleavage enzyme, which are present in the adrenal cortex, the gonads, and the placenta. The presence of steroid cell antibodies may serve as a marker of ovarian insufficiency in women with Addison's disease; however, few women with premature ovarian failure will have ovarian antibodies in the absence of Addison's disease.[125]

Investigations into the causes of premature ovarian failure will infrequently provide answers for patients who are distressed with this diagnosis. The patient history will uncover cases of chemotherapy and/or pelvic radiotherapy while the karyotype will reveal Turner's syndrome and mosaic causes of ovarian failure. The family history should be explored for other similar cases as well as any history of autoimmune disorders. The initial hormonal evaluation of FSH, LH, estradiol, and TSH should include anti-müllerian hormone. Most clinicians include an initial test of serum levels of calcium and phosphorus with a complete blood count. Ultrasound evaluation of the ovaries for antral follicles has also been shown to be a useful prognosticator. Autoimmune testing for adrenal antibodies, cytochrome P450 microsomal antibodies, and thyroid antibodies are becoming increasingly more common. The value of periodic screening is unclear; however the suspicion of associated endocrinopathies should be high in physicians caring for patients with premature ovarian insufficiency. Spontaneous resumption of ovarian function has been reported in about 25% of young women with premature ovarian failure and with antral follicles documented by ultrasound, and subsequent live births have occurred in some cases.[126] Because intermittent ovarian activity is common in women diagnosed with premature ovarian failure, therapies utilizing corticosteroids for immunosuppression should be avoided. Periodic monitoring of ovarian reserve may be of benefit in assisting patients who may ultimately decide to use ovum donation as a means to having children.

Other Autoimmune Factors in Reproduction

Antisperm Antibodies (ASA)

Spermatozoa contain antigens that are foreign to both male and female immune systems. Sperm antibodies that are bound to spermatozoa are found in over half of men's

seminal fluid or serum after vasectomy as during this procedure, the blood-testis barrier is broken and sperm antigens are presented to the immune system. In this instance, the sperm-bound ASA can be found bound to the head, midpiece, or tail of the spermatozoa. ASA persist after vas reversal, and some of these individuals can subsequently father children, thus ASA generated in this fashion do not necessarily result in failure of fertilization in all cases.

The etiology of sperm-directed immunity in women is unknown; however, the possible mechanisms include cross-reactivity with microbial antigens, breach or sensitization at intestinal mucosal surfaces, and interferon gamma-mediated potentiating of the antisperm immune response in women whose male partners have sperm autoantibodies in their semen.[127] It has been suggested that ASA in women are a cause of unexplained infertility. Sperm immobilization, inhibition of cervical mucus penetration, and interference with events that lead to sperm-oocyte binding are some of the mechanisms by which antisperm antibodies impede fertilization. ASA have been postulated to interfere with successful pregnancy through inhibition of implantation of the early embryo. A few retrospective studies have suggested that sperm-bound antisperm antibodies decrease embryo quality and implantation rates. However, these data have not been replicated in large prospective studies.

Review of numerous retrospective and prospective analyses of pregnancy rates for couples with circulating ASA leads one to question the prognostic value of ASA screening for diagnosis of infertility. The inconclusive and inconsistent results of these studies may be attributed to the presence of heterogeneous populations of ASA, the varying antibody titers, the various populations studied, the source of the specimen (serum, cervical mucus, semen), and the method of testing.[128] Identification of several sperm antigens against which antibodies may be directed may help explain why ASA studies using samples from numerous infertile patients have not produced a clear explanation of a specific antibody's role in fertility impairment. It is now recognized that not all ASA interfere with fertilization or impair fertilization at the same level. A more practical approach to this question would be to first identify antibodies that bind to specific antigens that impair specific processes such as the acrosomal reaction. Until more specific testing can be developed, the routine testing of men and women for ASA cannot be recommended in infertile couples or in those with recurrent pregnancy loss. However, in men with asthenospermia suspected to be secondary to ASA, one should consider intracytoplasmic sperm injection (ICSI) in these patients as intrauterine insemination (IUI) does not preclude the effects of these antibodies in the uterus or fallopian tubes.

Antigliadin Antibodies (AGA)

Celiac disease is an immune-mediated enteropathy that develops in susceptible individuals upon ingestion of a gluten-containing diet.[129] The definition of celiac disease or gluten-sensitive enteropathy includes the following three features: (1) villous atrophy; (2) symptoms of malabsorption such as steatorrhea, weight loss, or other signs of nutrient or vitamin deficiency; and (3) resolution of the mucosal lesions and symptoms upon withdrawal of gluten-containing foods, usually within a few weeks to months. Patients with celiac disease possess antibodies against gliadin (AGA), tissue transglutaminase (IgA-tTG), or anti-endomysial antibodies (EMA-IgA).[130] In addition to the presence of one or more of these antibodies, the diagnosis of celiac disease includes the presence of intraepithelial lymphocytes within the enterocytes diagnosed from endoscopic-guided duodenal biopsies. Celiac disease is closely associated with other autoimmune endocrine disorders. In prospective studies, the prevalence of celiac disease has been reported to be between 1% to 19% in patients with type 1 diabetes, 2% to 5% in autoimmune thyroid disorders and 3% to 7% in primary biliary cirrhosis. The pathogenesis of the coexistent between celiac disease and other autoimmune diseases is not known.

Untreated celiac disease has been associated with pregnancy loss. The relationship between untreated celiac disease and pregnancy loss may be related to the immune system, since the lamina propria of the intestinal tract is the largest immune organ in the body. Patients with celiac disease have immune responses to gliadin fractions, primarily in the upper small intestine, characterized by inflammatory cells and villous atrophy in the lamina propria and epithelium. This association has been reported as resulting in an increased risk of miscarriage. Treatment of celiac disease with a gluten-free diet (reduction of wheat, barley, and rye from the diet) appears to prevent the problem of miscarriage. There is currently no clear linkage between celiac disease, associated autoantibodies, and infertility.

Although routine screening of women with either infertility or RPL for antigliadin, antitransaminase, or antiendomysial antibodies is not recommended, patients who present with symptoms of malabsorption such as steatorrhea, weight loss, or other signs of malnutrition should be screened for celiac disease. Women who have been diagnosed with celiac disease should delay pregnancy until they are stabilized on a gluten-free diet.

Antinuclear Antibodies (ANA)

Antinuclear antibodies (ANA) are a group of antibodies that target nuclear and cytoplasmic antigens. These antigens are essential to cell function through playing a role in transcription, translation, and cell cycle regulation. Low titer ANA (<1:80) are found in 20% to 30% of reproductive-aged women and are of no known consequence. However, higher positive ANA titers are associated with multiple autoimmune disorders such as systemic lupus erythematosus.[131]

In patients with SLE, these antibodies, particularly anti-Ro/SSA, can have a significant impact on their offspring. More specifically, the prevalence of congenital heart block, defined as an atrioventricular block diagnosed *in utero*, at birth, or within the neonatal period (0 to 27 days after birth) in the offspring of an anti-Ro/SSA-positive woman is 1% to 2%. Furthermore, 10% to 20% of these infants have a neonatal lupus rash.[132] However, in the absence of clinical disease, such as SLE, what impact a positive ANA has on either fertility or RPL is unknown. A prospective randomized trial evaluated the effect of prednisone and aspirin in women with ANA and RPL but concluded there was no benefit to therapy.[133] In the same study, women with RPL who were treated with prednisone during pregnancy had increased frequencies of gestational diabetes and hypertension and the babies were born earlier with lower weights.

Based on these data, screening asymptomatic women with RPL or who are infertile for ANA is not recommended. Patients who have a history of an autoimmune disease such as systemic lupus erythematosus, rheumatoid arthritis, or Reynaud's syndrome should be screened for ANA particularly for the effects that these antibodies may have on their neonates.

Endometriosis-Associated Immune Responses

Endometriosis is a benign gynecological disorder that affects 5% to 10% of reproductive-aged women. The disease is characterized by the presence of ectopic endometrial tissue that is commonly found adherent to the peritoneal surface of the posterior cul-de-sac and on the surface of the ovary. As endometriosis is a familial disease, it has the potential to carry a familial autoimmune pathogenic response. The disease is also associated with both a chronic inflammatory pelvic response and adhesion formation in many women. Endometriosis is clearly associated with both infertility and pelvic pain.

Despite decades of intensive investigations, little is known about the cellular pathogenesis of endometriosis. Although endometriosis is likely to be a multifactorial disease, the most prevalent theory of the pathogenesis of the disease is reflux menstruation. The pathogenic theory of reflux menstruation includes the proposition that women who are destined to develop endometriosis may have either a deficient immune system that cannot effectively clear endometrial tissue from their peritoneal surfaces or an immune system that develops a tolerance to the monthly flux of endometrium into the peritoneal cavity. Abnormal levels of immune cells such as macrophages, dendritic and natural killer cells have been found in the peritoneal cavity of women with endometriosis.[134] However these cells seem to be unable to detect and eliminate ectopic endometrial cells. In this regard, several studies have shown that peritoneal immune cells in women with endometriosis are dysfunctional and may contribute to the development of endometriosis. Still, the role of immune-surveillance in the pathogenesis of the disease is unclear.[135] Both IgG and IgA antibodies as well as lymphocytes have been found in increased levels within the normal endometrium of women with endometriosis. Whether an immunological abnormality predisposes an individual to the development of endometriosis, or whether the retrograde deposition of endometrium simply induces an inflammatory response is unknown. Although treatment with non-steroidal anti-inflammatory drugs (NSAIDs) can help reduce the pain associated with endometriosis, to date, no immune-directed therapy has been shown to improve either the infertility or the pelvic pain associated with this disease. Furthermore, no antibody or immune cell testing is recommended in women with endometriosis other than for the purposes of research.

Conclusions

For a pregnancy to succeed, two immunologically and genetically distinct tissues must coexist. During implantation, local and systemic immune factors, cytokines, and growth factors may interact with adhesion molecules and other matrix-associated proteins, glycoproteins, and peptides.[136] Several autoimmune factors have been investigated as possible influences on reproductive success and failure. Autoantibodies may persist for many years in the circulation as a marker of a prior autoimmune attack, but their presence alone does not necessarily indicate a current disease process. We are just beginning to understand the complex interactions between the endocrine and immune system that support the human fetal semi-allograft.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 15

Menopause and Aging

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Epidemiology

Menopause is defined by the last menstrual period. Because cessation of menses is variable and many of the symptoms thought to be related to menopause may occur prior to cessation of menses, there is seldom a precise timing of this event. Other terms used are *perimenopause*, which refers to a variable time beginning a few years before and continuing after the event of menopause, and *climacteric*, which merely refers to the time after the cessation of reproductive function. While the terms *menopausal* and *postmenopausal* are used interchangeably, the former term is less correct because "menopausal" should only relate to the time around the cessation of menses.

As life expectancy increases beyond the eighth decade worldwide, particularly in developed countries, an increasing proportion of the female population is postmenopausal. With the average age of menopause being at 51 years old, more than a third of a woman's life is now spent after menopause. Here symptoms and signs of estrogen deficiency merge with issues encountered with natural aging. As the world population increases and a larger proportion of this population is made up of individuals over 50, medical care specifically directed at postmenopausal women becomes an important aspect of modern medicine. Between the years 2000 and 2005, the world population older than 60 years old is expected to double, from 590 million to 1 billion. In the United States, the number of women entering menopause will almost double in the 30 years between 1990 and 2020 (Table 15.1).[1]

Age of menopause, which is a genetically programmed event, is subject to some variability. The age of menopause in western countries (between 51 and 52 years old),[2,3] is thought to correlate with general health status.[4-6] Socioeconomic status is associated with an earlier age of menopause.[2] Higher parity, on the other hand, has been found to be associated with a later menopause.[7] Smoking has consistently been found to be associated with menopause onset taking place 1 to 2 years earlier.[3,8] While body mass has been thought to be related to age of menopause (greater mass with later menopause),[9] the data have not been consistent. Malnourishment and vegetarianism have both been found to be associated with earlier onset of menopause.[10,11] However, physical and athletic activity has not been found consistently to influence the age of menopause.

There also appear to be ethnic differences in the onset of menopause. In the United States, African American and Hispanic women have been found to have menopause approximately 2 years earlier than white women.[12,13] Although parity is generally greater around the world than in the United States, the age of menopause appears to be somewhat earlier. Malay women have menopause at approximately age 45, Thai women at age 49.5, and Filipina women between ages 47 and 48.[14-16] Countries at higher altitude (Himalayas or Andes) have been shown to have menopause 1 to 1.5 years earlier.[17,18] Because the average age of menopause in the United States is 51 to 53 years with an age distribution weighted toward white women, menopause prior to age 40 is considered premature. Conversely, by age 58, 97% of women will have gone through menopause.

The primary determinate of age of menopause is genetic. Based on family studies, heritability for age of menopause averaged 0.87—suggesting that genetics explain up to 87% of the variance in menopausal age.[19] Multiple genes have been identified which relate to the age of menopause.[19a] These genes have been implicated in DNA repair and immune function.[19a]

Premature Ovarian Failure

Premature ovarian failure (POF) is defined as hypergonadotropic ovarian failure occurring prior to age 40. Recently it has been suggested to refer to POF as premature ovarian insufficiency (POI) to more effectively reflect its heterogeneous nature.[19b] In practice, both these terms are used interchangeably. POF has occurred in 5% to 10% of women

Table 15.1 U.S. Population Entering the Postmenopausal Years, Ages 55 to 64

Year	Population
1990	10.8 million
2000	12.1 million
2010	17.1 million
2020	19.3 million

Adapted from U.S. Bureau of the Census. Current population reports: projections of the population of the United States 1977 to 2050. Washington, D.C., U.S. Government Printing Office, 1993.

who are evaluated for amenorrhea,[20,21] thus the incidence varies according to the prevalence of amenorrhea in various populations. Estimates of the overall prevalence of POF in the general population range between 0.3% to 0.9% of women.[22,23]

Throughout life, there is an ongoing rate of atresia of oocytes. Because this process is accelerated with various forms of gonadal dysgenesis due to defective X chromosomes, one possible etiology of POF is an increased rate of atresia that has yet to be explained. A decreased germ cell endowment or an increased rate of germ cell destruction can also explain POF.[20] Nevertheless, some 1000 (of the original 2 million) primarily follicles may remain.[24,25] While most of these oocytes are likely to be functionally deficient, occasionally spontaneous pregnancies occur in young women in the first few years after the diagnosis of POF.

There are several possible etiologies of POF (Table 15.2). Defects in the X chromosome may result in various types of gonadal dysgenesis with varied times of expression of ovarian failure. Even patients with classical gonadal dysgenesis (e.g., 45,XO) may undergo a normal puberty, and occasionally a pregnancy may ensue[26-29] as a result of genetic mosaicism. Very small defects in the X chromosome may be sufficient to cause POF.[30] Familial forms of POF may be related to either autosomal-dominant or sex-linked modes of inheritance.[31-33]

Mutations in the gene encoding the follicle-stimulating hormone (FSH)-receptor (e.g., mutation in exon 7 in the gene on chromosome 2p) have been described,[34] but these are extremely rare outside of the Finnish population in which these mutations were originally described. An expansion of a trinucleotide repeat sequence in the first exon on the *FMR1* gene (Xq 27.3) leads to fragile X syndrome, a major cause of developmental disabilities in males.[35] The permutation in fragile X syndrome has been shown to be associated with POF.[36-37] Type 1 blepharophi-mosis/ptosis/epicanthus inversus (BPES) syndrome, an autosomal-dominant disorder due to mutations in the forkhead transcription factor FOXL2, includes POF.[38] Triple X syndrome has been associated with POF and dystrophic myotonia has also been linked to POF, although the mechanism underlying this relationship is unclear.[39]

Under the category of enzymatic defects, galactosemia is a major cause of POF that is related to the toxic buildup of galactose in women who are unable to metabolize the sugar. Even in women with fairly well controlled galactose-free diets, POF tends to occur.[40,41] Another enzymatic defect linked to POF is 17α-hydroxylase deficiency. This rare condition manifests differently from the other causes discussed here because the defect in the production of sex steroids leads to sexual infantilism and hypertension.[42-44]

Because of the prevalence of autoimmune disorders in women, the degree to which autoimmunity may be responsible for POF is unclear. One study has suggested an association in 17.5% of cases.[45] Virtually all autoimmune disorders have been found to be associated with POF, including autoimmune polyendocrinopathies like autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy *(APECED)*, which is caused by mutations in the autoimmune *(AIRE)* gene on 21q22.3.[46] The presence of the thymus gland appears to be required for normal ovarian function as POF has been associated with hypoplasia of the thymus.[47] In patients who have undergone ovarian biopsy as part of their evaluation, lymphocytic infiltration surrounding follicles has been described, as well as resumption of menses after immunosuppression.[48-51]

Immunoassays utilizing antibodies directed at ovarian antigens have been developed and have demonstrated positive findings in some patients with POF,[52] although the relevance of these findings remains unsettled. Ovarian autoantibodies could also conceivably be a secondary phenomenon to a primary cell-mediated form of immunity. Specific enzymes such as 3β-HSD may also be the target of ovarian autoimmunity.[53] From a practical standpoint, screening for the common autoimmune disorders is appropriate in women found to have POF. Although relatively rare, as many as 2% to 4% of women with POF have anti-adrenal antibodies,[54] and may be at risk for adrenal failure. Commercial antibodies to 21-hydroxylase are available and agree with other assays for adrenal cortex antibodies.[55] It has been suggested that screening women using dehydroepiandrosterone sulfate [DHEA(S)] may also be useful to detect signs of adrenal insufficiency.

More from a theoretical standpoint, abnormalities in the structure of gonadotropins, in their receptors, or in receptor binding could be associated with POF. While abnormal urinary forms of gonadotropins have been reported in women with POF,[56] these data have not been replicated. Abnormalities of FSH-receptor binding, as mediated by a serum inhibitor, have been described.[57] A genetic defect that may lead to alterations in FSH receptor structure was mentioned previously.[34]

Table 15.2 Techniques for the Detection of Osteopenia

Technique	Anatomical Site of Interest	Precision *in Vivo* (%)	Examination and Analysis Time (min)	Estimated Effective Dose Equivalent (μSv)
Conventional radiography	Spine, hip	NA	<5	2000
Radiogrammetry	Hand	1-3	5-10	<1
Radiographic absorptiometry	Hand	1-2	5-10	<1
Single x-ray absorptiometry	Forearm, heel	1-2	5-10	<1
Dual x-ray absorptiometry	Spine, hip, forearm, total body	1-3	5-20	1-10
Quantitative computed tomography	Spine, forearm, hip	2-4	10-15	50-100
Quantitative ultrasound	Heel, hand, lower leg	1-3	5-10	None

NA, not applicable.

Adapted from van Kuijk C, Genant HK: Detection of osteopenia. *In* Lobo RA (ed). Treatment of the Postmenopausal Woman: Basic and Clinical Aspects, 2nd ed. Philadelphia, Lippincott Williams & Wilkins, 1999, pp 287-292.

Under the category of ovarian insults, POF may be induced by ionizing radiation, chemotherapy, or overly aggressive ovarian surgery. Although not well documented, viral infections have been suggested to play a role, particularly mumps.[58] A dose of 400 to 500 rads is known to cause ovarian failure 50% of the time,[59,60] and older women are more vulnerable to experiencing permanent failure. A dose of approximately 800 rads is associated with failure in all women.[60,61] Ovarian failure (transient or permanent) may be induced by chemotherapeutic agents, although younger women receiving this insult have a better prognosis.[61-63] Alkalyzing agents, particularly cyclophosphamide, appear to be most toxic.[64]

By exclusion, the majority of women are considered to have idiopathic POF because no demonstrable cause can be pinpointed. Among these women, small mutations in genes lying on the X chromosome or yet to be identified autosomal genes may be the cause.

Management of Primary Ovarian Failure/Insufficiency

Evaluation of women under age 30 who have POF should include screening for autoimmune disorders and a karyotype; detailed recommendations for screening of such women are available.[65] In addition, vaginal ultrasound may be useful for assessing the size of the ovaries and the degree of follicular development, which, if present, may signify an immunological defect.

Treatment usually consists of estrogen replacement. If fertility is a concern, the most efficacious treatment is oocyte donation. Various attempts at ovarian stimulation are usually unsuccessful, and the sporadic pregnancies that may occur are just as likely to occur spontaneously (−5%) as with any intervention.[66,67] These spontaneous pregnancies are frequently encountered while receiving estrogen replacement. Noting irregular or unscheduled bleeding (while receiving hormonal treatment) is important as a sign of endogenous sex steroid production. Serum Anti-Mullerian Hormone (AMH) has been used to characterize women with ovarian insufficiency to determine where they may be in the spectrum of ovarian failure.[67a] However, AMH levels cannot help predict the chance of a spontaneous pregnancy. It has been shown in a large cohort of women (n = 358) followed for POF, that spontaneous ovarian function may ensue in 24% of women, usually within a year of the diagnosis. In this longitudinal follow-up, 4.4% of women had spontaneous pregnancies.[67b]

It has been well established that POF as well as bilateral oophorectomy prior to the usual age of menopause is associated with an increased risk[68] of cardiovascular disease (CVD) as well as increased mortality (Fig. 15.1). CVD mortality specifically is increased twofold to fourfold, and observational studies have suggested that using hormonal treatment (HT) reduces this risk. Accordingly, unless there are contraindications, estrogen should be considered in all young women with POF, at least until the age of natural menopause.

The Menopausal Transition (Perimenopause)

A workshop was convened in 2001 to build a consensus on describing various stages of the menopausal transition.[69] Ten years later another workshop was convened to update the staging system with more updated data.[69a] The Stages of Reproductive Aging Workshop + 10 (STRAW + 10) simplified bleeding criteria for the early and late menopausal transition and modified criteria for the late reproductive stage (stage −3) and the early postmenopausal stage (stage +1) (Fig. 15.2). Then the late reproductive stages were expanded to include information on decreasing levels of AHM and inhibin B to reflect recent longitudinal data on fluctuating hormonal levels at the time of menopause, which will be described in more detail below.

The ovary changes markedly from birth to the onset of menopause (Fig. 15.3).[70] The greatest number of primordial follicles is present *in utero* at 20 weeks' gestation and undergoes a regular rate of atresia until around the age of 37. After this time, the decline in primordial follicles appears to

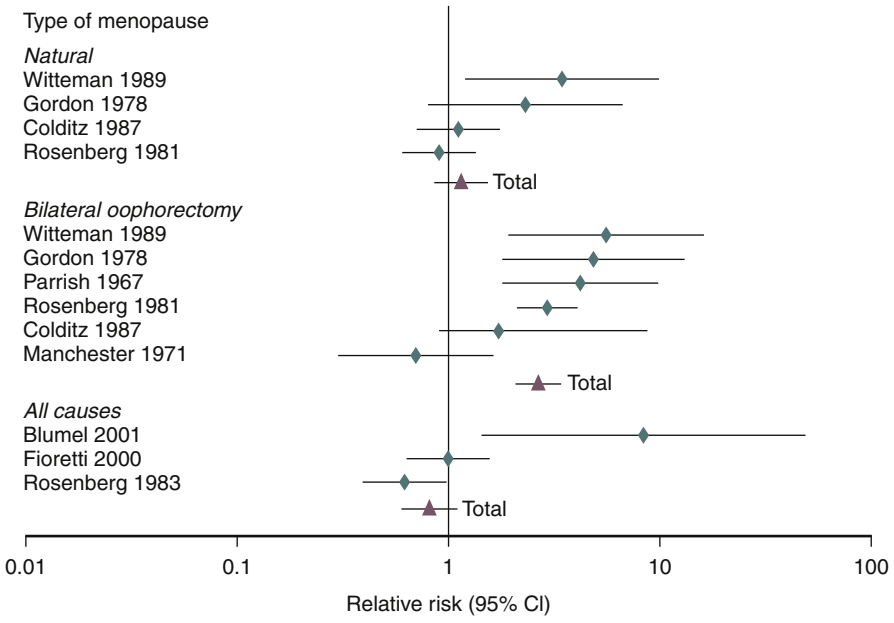
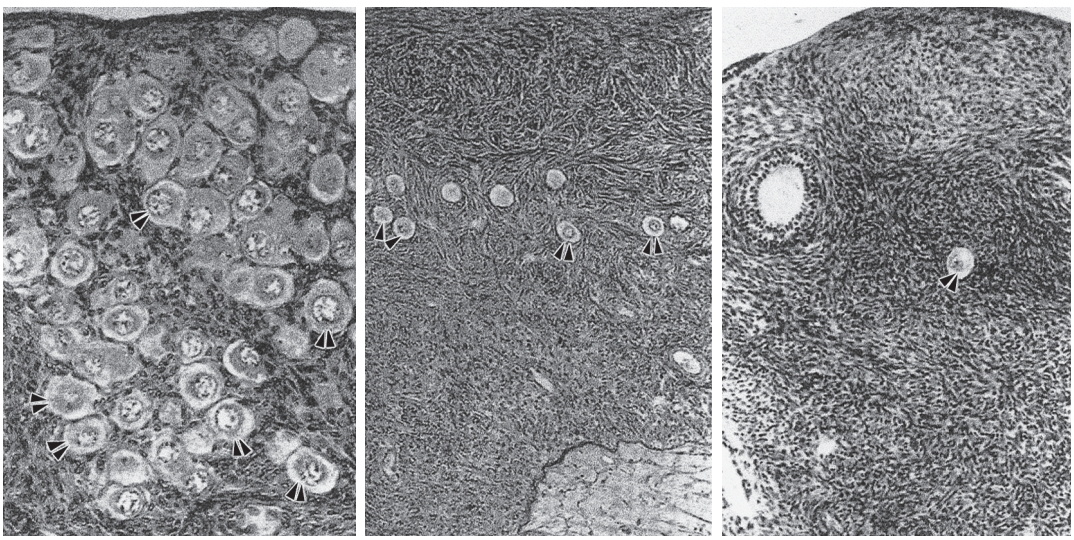


FIGURE 15.1 Effect of type of "early" menopause on cardiovascular disease. Data taken from a meta-analysis. *(From Atsma F, Bartelink ML, Grobbee DE, et al. Postmenopausal status and early menopause as independent risk factors for cardiovascular disease: a meta-analysis.* Menopause *13[2]:265–279, 2006 [review]. See reference 68 for data sources.)*

	Menarche					FMP (0)				
Stage	–5	–4	–3b	–3a	–2	–1	+1a	+1b	+1c	+2
Terminology	REPRODUCTIVE				MENOPAUSAL TRANSITION		POSTMENOPAUSE			
	Early	Peak	Late		Early	Late	Early			Late
					Perimenopause					
Duration	*variable*				*variable*	1–3 years	2 years (1+1)		3–6 years	*Remaining lifespan*
PRINCIPAL CRITERIA										
Menstrual Cycle	Variable to regular	Regular	Regular	Subtle changes in Flow/ Length	*Variable Length* Persistent ≥7-day difference in length of consecutive cycles	Interval of amenorrhea of ≥60 days				
SUPPORTIVE CRITERIA										
Endocrine FSH AMH Inhibin B			Low Low	Variable* Low Low	↑ Variable* Low Low	↑ >25 IU/L** Low Low	↑ Variable Low Low		Stabilizes Very Low Very Low	
Antral Follicle Count			Low	Low	Low	Low	Very Low		Very Low	
DESCRIPTIVE CHARACTERISTICS										
Symptoms						Vasomotor symptoms *Likely*	Vasomotor symptoms *Most Likely*			*Increasing* symptoms of urogenital atrophy

* Blood draw on cycle days 2–5 ↑ = elevated.
**Approximate expected level based on assays using current international pituitary standard. [67–69]

FIGURE 15.2 The Stages of Reproductive Aging Workshop + 10 staging system for reproductive aging in women. *(Adapted from Harlow SD, et al. Executive summary of the stages of reproductive aging workshop + 10: addressing the unfinished agenda of staging reproductive aging.* J Clin Endocrinol Metab *97[4]:1159–1168, 2012.)*



Birth 25 years old 50 years old

FIGURE 15.3 Photomicrographs of the cortex of human ovaries from birth to 50 years of age. Small nongrowing primordial follicles (*arrows*) have a single layer of squamous granulosa cells. *(Adapted from Erickson CF. An analysis of follicle development and ovum maturation.* Semin Reprod Endocrinol *4:233–254, 1986.)*

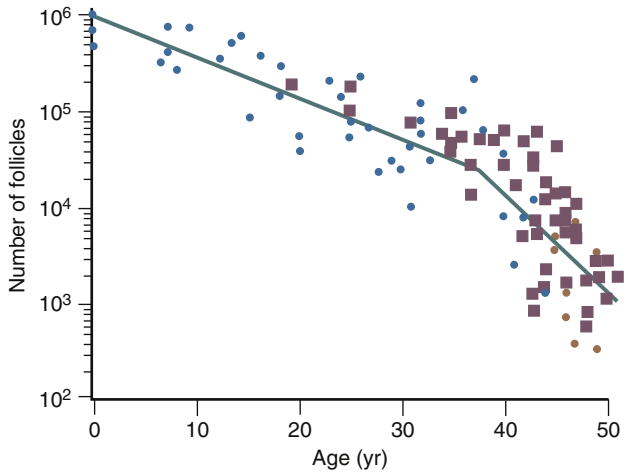


FIGURE 15.4 The age-related decrease in the total number of primordial follicles (PFs) within both human ovaries from birth to menopause. As a result of recruitment (initiation of PF growth), the number of PFs decreases progressively from about 1 million at birth to 25,000 at 37 years. At 37 years, the rate of recruitment increases sharply, and the number of PFs declines to 1000 at menopause (about 51 years of age). Note: The different colors and shapes in the graph represent different studies. *(Adapted from Faddy MJ, Gosden RJ, Gougeon A, et al. Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause.* Hum Reprod *7:1342–1346, 1992.)*

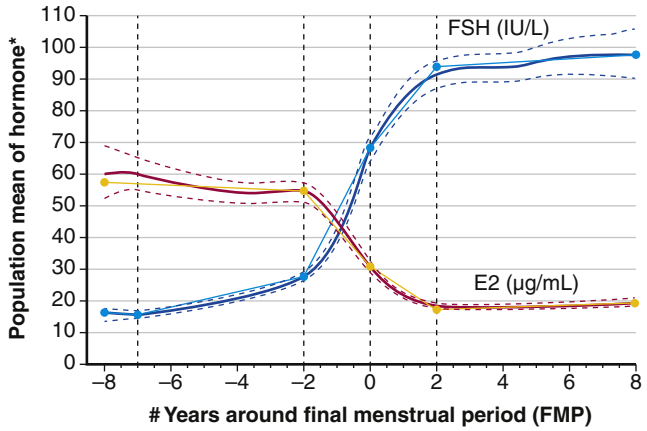


FIGURE 15.5 Adjusted population means (95% CI) for segmented mean profiles of follicle-stimulating hormone and estradiol across the final menstrual period in the Study of Women's Health Across the Nation (N = 1215). *(Adapted from Randolf J. Change in follicle-stimulating hormone and estradiol across the menopausal transition: effect of age at the final menstrual period.* J Clin Endocrinol Metab *96:746–754, 2011.)*

become more rapid between age 37 and menopause (Fig. 15.4) when no more than a thousand follicles remain.[71] These remaining follicles are primarily atresic in nature.

Types of Ovarian Changes

Although perimenopausal changes are generally thought to be endocrine in nature and result in menstrual changes, a marked diminution of reproductive capacity precedes this period by several years. This decline may be referred to as *gametogenic ovarian failure*. The concept of dissociation in ovarian function is appropriate. Gametogenic failure is signified by reduced early follicular phase inhibin secretion, rising serum FSH levels, and a marked reduction in fecundity. These changes may occur with normal menstrual function and no obvious endocrine deficiency, however, and they may occur in some women as early as age 35 (10 or more years before endocrine deficiency ensues).

Recent longitudinal data obtained from SWAN (study of women across the nation) have shown that estrogen levels begin to decline 2 years before the last menstrual period[71a] (Fig. 15.5). Older data had shown that this only occurred in the last 6 months.[72] The rise in FSH levels occurs several years before menopause but increases substantially in the last 2 years, and then stablilizes to steady state levels 2 years after menopause.[71a] These study levels then decrease in the late menopause (7th decade).[71a] There is also a very slow decline in androgen status (i.e., androstenedione and testosterone), which cannot be adequately detected at the time of the perimenopause.

Products of the granulosa cell are most important for the feedback control of FSH. As the functional capacity of the follicular units decrease, secretion of substances that suppress FSH also decrease. Most notably, inhibin B levels are lower in the early follicular phase in women in their late 30s (Fig. 15.6).[73-74] Indeed, FSH levels are higher throughout the cycle in older ovulatory women than in younger women (Fig. 15.7).[75]

The functional capacity of the ovary is also diminished as women enter into the perimenopause. With gonadotropin stimulation, while estradiol (E_2) levels are not very different between younger and older women, total inhibin production by granulosa cells is decreased in women over age 35.[76] From a clinical perspective, subtle increases in FSH on day 3 of the cycle, or increases in the clomiphene challenge test, correlate with decreased ovarian responses to stimulation and decreased fecundability.[77,78]

Although there is a general decline in oocyte number with age, an accelerated atresia occurs around age 37 or 38 (Fig. 15.4).[71] While the reason for this acceleration is not clear, one possible theory relates to activin secretion. Because granulosa cell–derived activin is important for stimulating FSH-receptor expression,[79] the rise in FSH levels could result in more activin production, which in turn enhances FSH action. A profile of elevated activin with lower inhibin B has been found in older women (Fig. 15.8).[80] This autocrine action of activin, involving enhanced FSH action, might be expected to lead to accelerated growth and differentiation of granulosa cells. Further, activin has been shown to increase the size of the pool of preantral follicles in the rat. At the same time, these follicles become more atretic.[81]

Clinical management of women in the perimenopause should address three general areas of concern:

1. Irregular bleeding
2. Symptoms of early menopause, such as hot flushes
3. The inability to conceive

Treatment of irregular bleeding is complicated by the fluctuating hormonal status. Estrogen levels may be higher than normal in the early follicular phase[72] and progesterone secretion may be normal, although not all cycles are ovulatory. For these reasons, short-term use of an oral contraceptive (usually 20 μg ethinyl estradiol) may be an option for otherwise healthy women who do not smoke to help them cope with irregular bleeding.

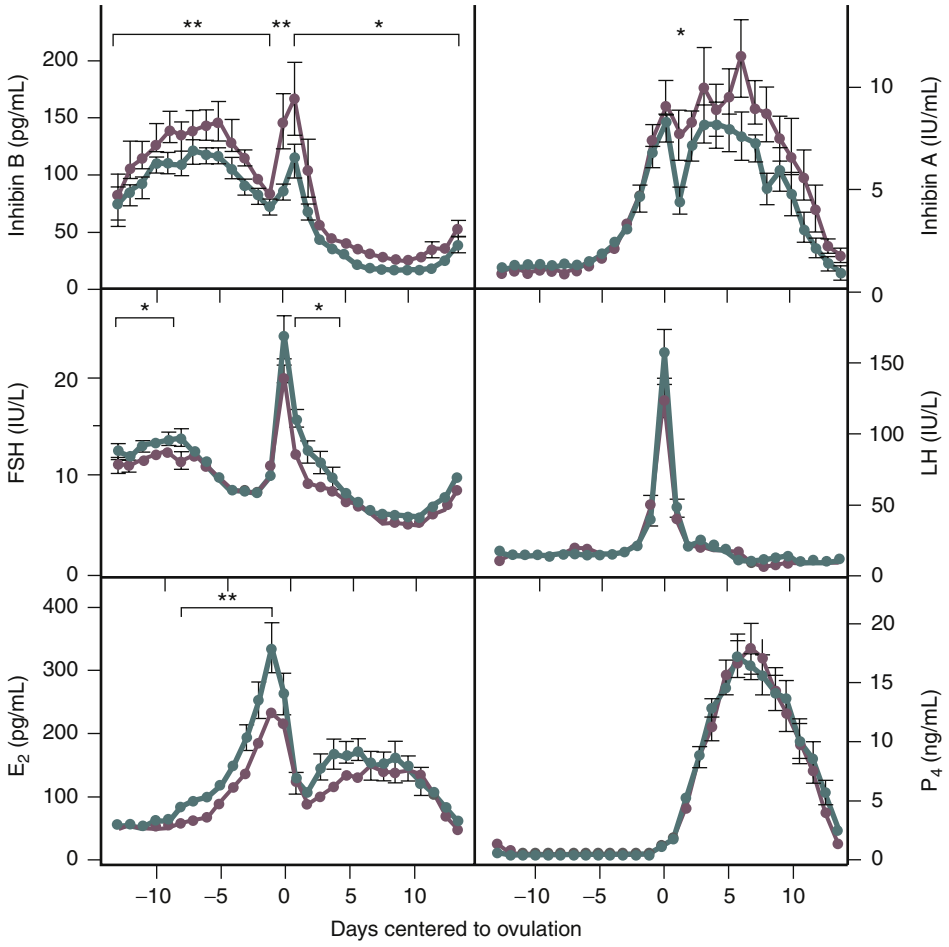


FIGURE 15.6 Mean inhibin B, follicle-stimulating hormone (FSH), estradiol (E_2), inhibin A, and progesterone (P4) levels in cycling women 20 to 34 years old *(purple)* and 35 to 46 years old *(green)*. Hormone levels are depicted as centered to the day of ovulation (*, $P < 0.04$;**, $P < 0.02$) when comparing the two age groups. *(Adapted from Welt CK, McNicholl DJ, Taylor AE, Hall JE. Female reproductive aging is marked by decreased secretion of dimeric inhibin.* J Clin Endocrinol Metab *84:105–111, 1999.)*

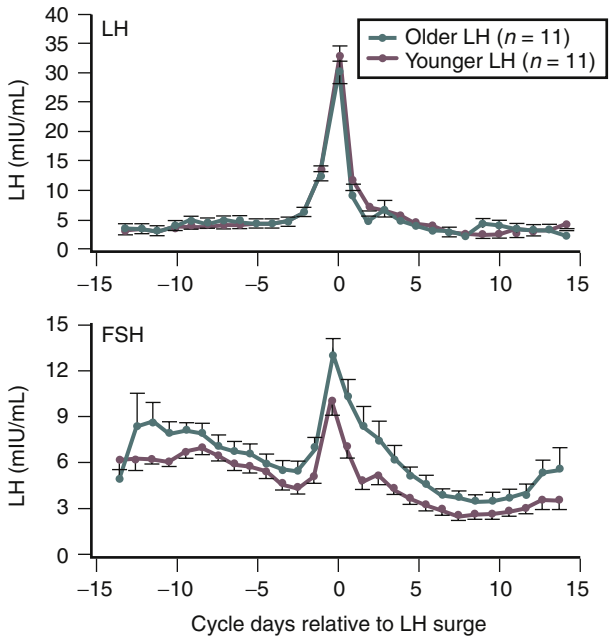


FIGURE 15.7 The daily serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels throughout the menstrual cycle of two groups (older and younger) of 11 women each (mean ± SE). The gonadotropin secretion pattern in normal women of advanced reproductive age is shown in relation to the monotropic FSH rise. *(Adapted from Klein NA, Battaglia DE, Clifton DK, et al. The gonadotropin secretion pattern in normal women of advanced reproductive age in relation to the monotropic FSH rise.* J Soc Cynecol Investig *3:27–32, 1996.)*

Early symptoms of menopause, particularly vasomotor changes, may occur as the result of fluctuating hormonal levels. In this setting, an oral contraceptive again may be an option if symptoms warrant therapy. Alternatively, lower doses of estrogen used alone may be another option.

Reproductive concerns often require more aggressive treatment because of decreased cycle fecundity. Once day 3 FSH levels increase and AMH levels are lower than established normative data (usually less than 0.4 ng/mL), the prognosis for pregnancy is markedly reduced. A more detailed discussion of AMH levels may be found in the next section.

Hormonal Changes with Established Menopause

Depicted in Figure 15.9 are the typical hormonal levels of postmenopausal women compared with those of ovulatory women in the early follicular phase.[82] The most significant findings are the marked reductions in E_2 and estrone (E_1). Serum E_2 is reduced to a greater extent than E_1. Serum E_1, on the other hand, is produced primarily by peripheral aromatization from androgens, which decline more slowly, principally as a function of age. Levels of E_2 average 15 pg/mL and range from 10 to 25 pg/mL, but are 10 pg/mL or less in women who have undergone oophorectomy. Serum E_1 values average 30 pg/mL but may be higher in obese women because aromatization increases as a function of the mass of adipose tissue.

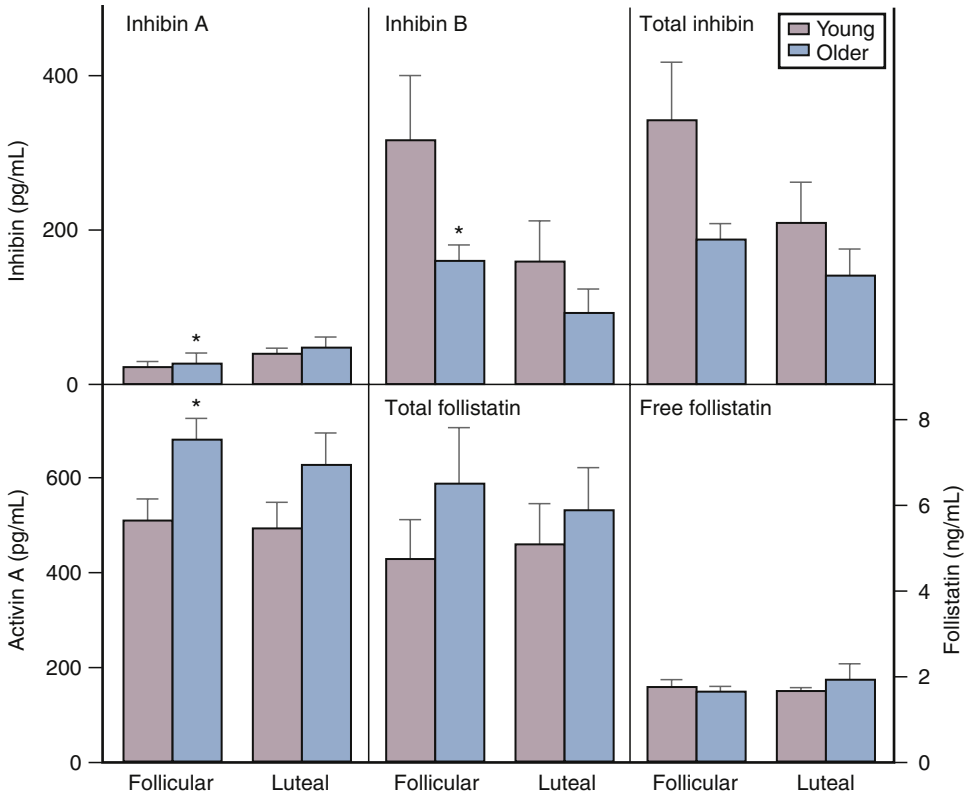


FIGURE 15.8 Mean concentrations of gonadal proteins from the same subjects. Total inhibin is a derived number from the sum of inhibin A and inhibin B. *, Group differences; $P < 0.05$. Net increase in stimulatory input resulting from a decrease in inhibin B and an increase in activin A may contribute in part to the rise in follicular phase follicle-stimulating hormone of aging cyclic women. *(Adapted from Reame NE, Wyman TL, Phillips DJ, et al. Net increase in stimulatory input resulting from a decrease in inhibin B and an increase in activin A may contribute in part to the rise in follicular phase follicle-stimulating of hormone of aging cyclic women.* J Clin Endocrinol Metab *83:3302–3307, 1998.)*

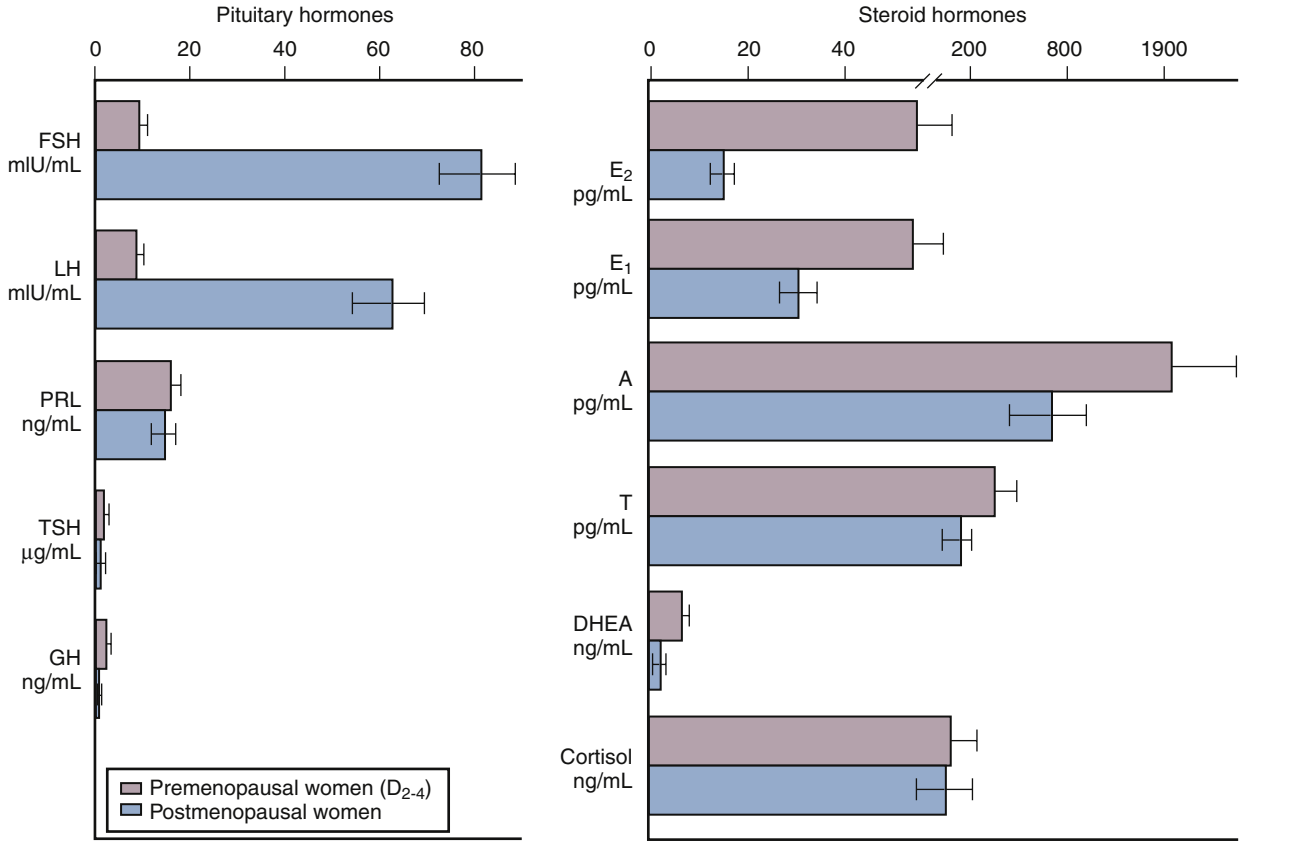


FIGURE 15.9 Circulating levels of pituitary and steroid hormones in postmenopausal women compared with levels in premenopausal women studied during the first week (days 2 to 4 [D_{2-4}]) of the menstrual cycle. A, androstenedione; DHEA, dehydroepiandrosterone; E_1, estrogen; E_2, estradiol; FSH, follicle-stimulating hormone; GH, growth hormone; LH, luteinizing hormone; PRL, prolactin; T, testosterone; TSH, thyroid-stimulating hormone. *(Adapted from Yen SSC. The biology of menopause.* J Reprod Med *18:287, 1977.)*

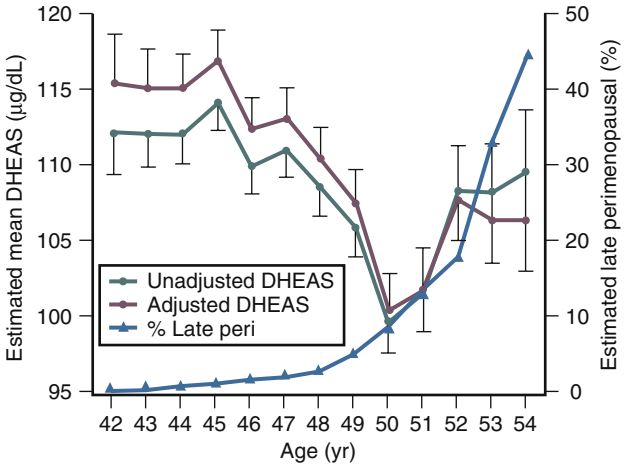


FIGURE 15.10 Mean (±SE) circulating dehydroepiandrosterone sulfate (DHEAS) at each year of age of the entire study population before and after adjustment for age, current smoking, menopausal status, log body mass index (BMI), ethnicity, site, and the interaction between ethnicity and log BMI. Also shown is the percentage of women at each year of age who have transitioned to late perimenopausal status. *(Adapted from Lasley BL, Santoro N, Randolf JF, et al. The relationship of circulating dehydroepiandrosterone, testosterone, and estradiol to stages of the menopausal transition and ethnicity.* J Clin Endocrinol Metab *87:3760–3767, 2002.)*

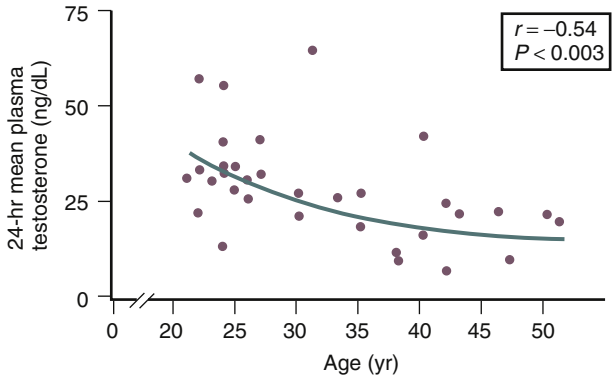


FIGURE 15.11 The 24-hour mean plasma total testosterone (T) level compared with age in normal women. The regression equation was T (nmol/L) = 37.8 × age (years)$^{-1.12}$ (r = –0.54; P < 0.003). *(Adapted from Zumoff B, Strain GW, Miller LK, et al. Twenty-four hour mean plasma testosterone concentration declines with age in normal premenopausal women.* J Clin Endocrinol Metab *80:1429–1430, 1995.)*

Estrone sulfate (E_1S) is an estrogen conjugate that serves as a stable circulating reservoir of estrogen, and levels of E_1S are the highest among estrogens in postmenopausal women. In premenopausal women, values are usually above 1000 pg/mL; in postmenopausal women, levels average 350 pg/mL.

Apart from elevations in FSH and luteinizing hormone (LH), other pituitary hormones are not affected. The rise in FSH, beginning in stage −3 as early as age 38 (Fig. 15.2), fluctuates considerably until approximately 4 years after menopause when values are consistently greater than 20 mIU/mL. Specifically, growth hormone, thyroid-stimulating hormone, and adrenocorticotropic hormone (ACTH) levels are normal. Serum prolactin levels may be very slightly decreased because prolactin levels are influenced by estrogen status.

Both the postmenopausal ovary and the adrenal gland continue to produce androgen. The ovary continues to produce androstenedione and testosterone but not E_2,[83,84] and this production has been shown to be at least partially dependent on LH. Androstenedione and testosterone levels are lower in women who have experienced bilateral oophorectomy, with values averaging 0.8 ng/mL and 0.1 ng/mL, respectively. The adrenal gland also continues to produce androstenedione, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (DHEAS); primarily as a function of aging, these values decrease somewhat (adrenopause), although cortisol secretion remains unaffected. Some data suggest that much "ovarian" testosterone production may actually arise from the adrenal.[85] Most likely, this production is by indirect mechanisms due to the adrenal supplying precursor substrate (DHEA and androstenedione).

Although DHEAS levels decrease with age (approximately 2% per year),[86] recent data have suggested that levels transiently rise in the perimenopause before the continuous decline thereafter (Fig. 15.10)[87] This interesting finding from the Study of Women Across the Nation (SWAN) also suggested that DHEAS levels are highest in Chinese women and lowest in African American women.[7]

Testosterone levels also decline as a function of age, which is best demonstrated by the reduction in 24-hour mean levels (Fig. 15.11).[88] Because of the role of the adrenal in determining levels of testosterone after menopause,[83] adrenalectomy or dexamethasone treatment results in undetectable levels of serum testosterone. Compared with total testosterone, the measurement of bioavailable or "free" testosterone is more useful in postmenopausal women. After menopause, sex hormone—binding globulin (SHBG) levels decrease, resulting in relatively higher levels of bioavailable testosterone or a higher free androgen index (Fig. 15.12).[89] In women receiving oral estrogen, bioavailable testosterone levels are extremely low because SHBG levels are increased. How this relates to the decision to begin androgen therapy in postmenopausal women will be discussed later in this chapter.

Elevated gonadotropin (FSH-LH) levels arise from reduced secretion of E_2 and inhibin as described earlier. Estrogen is important in controlling the production of GnRH mRNA in type 1 neurons.[90,91] In addition, the increase in gonadotropins observed at menopause appears to be enhanced by substance P[92] as well as by tachykinins produced in hypertrophied neurons,[93,94] which result from the decrease in E_2.

Unlike the rodent, where there is evidence for a hypothalamic factor involved in ovarian senescence,[95-97] no such clear evidence exists for women. The hypothesis proposed by Wise suggested that the effects of aging in the brain affect neurotransmitter systems that regulate GnRH, disrupting ovarian folliculogenesis and ultimately promoting senescence. Thus, the accelerated follicular loss that is apparent in the late 30s is postulated to be due to age-related desynchronization in the rhythmicity of GnRH secretion.[97]

Although some aging effects of the brain are likely to exist, there is abundant human evidence for an ovarian-induced menopause. While there is slowing of LH pulsatility with aging in rodents, LH pulse frequency and amplitude increase with age as menopause approaches in women (Fig. 15.13).[98] A sleep-entrained alteration in GnRH pulse dynamics has been observed in postmenopausal women, namely the inability to increase GnRH pulse amplitude at night.[99] There is some evidence that the high frequency and

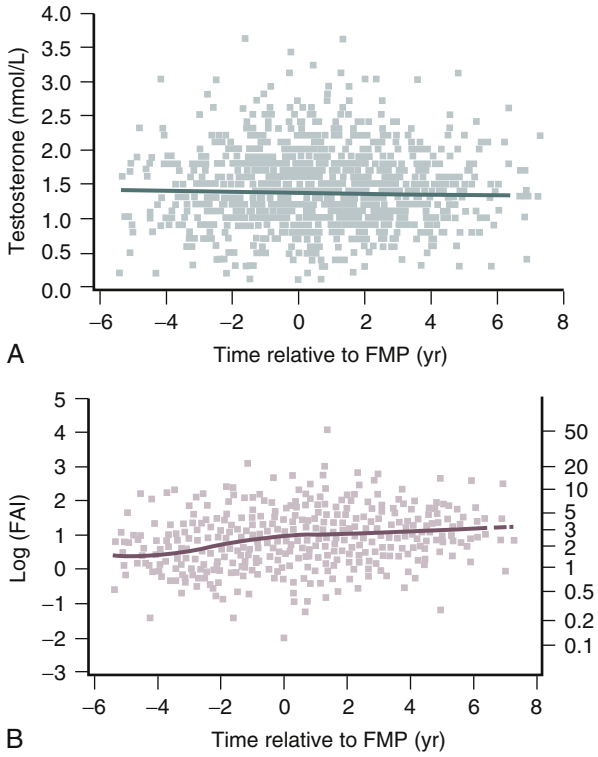


FIGURE 15.12 **A,** Linear regression model: observed testosterone and fitted levels of mean T across the menopausal transition. **B,** Double logistic model: observed free androgen index (FAI) and fitted levels of mean FAI across the menopausal transition. The left and right axes show FAI levels on the log and antilog scales, respectively. The horizontal axis represents time (years) with respect to first menstrual period (FMP); negative (positive) numbers indicate time before (after) FMP. *(From Burger HG, Dudley EC, Cui J, et al. A prospective longitudinal study of serum testosterone, dehydroepiandrosterone sulfate, and sex hormone-binding globulin levels through the menopause transition.* J Clin Endocrinol Metab *85:2832–2838, 2000.)*

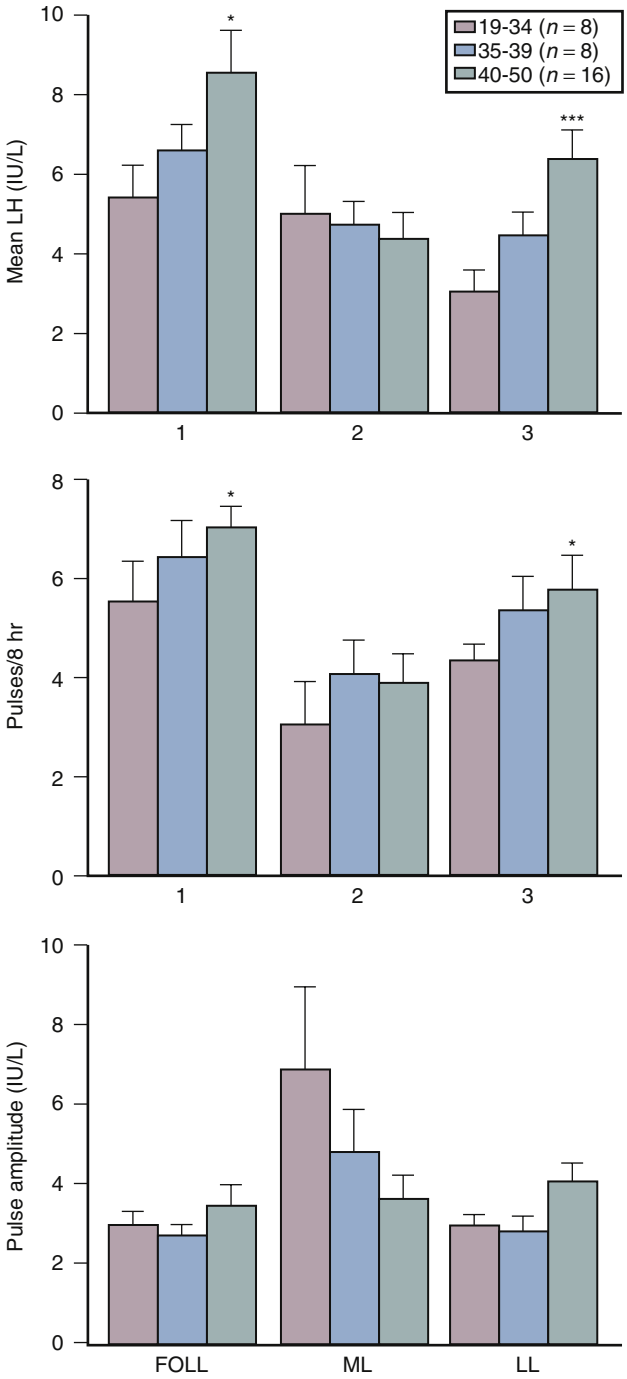


FIGURE 15.13 Effects of age on pulsatile luteinizing hormone (LH) secretory characteristics (age groups are in years). All subjects were studied across the same menstrual cycle. FOLL, early follicular phase; ML, midluteal phase; LL, late luteal phase. *, $P < 0.05$; ***, $P < 0.001$. *(Adapted from Reame NE, Kelche RP, Beitins IZ, et al. Age effects on follicle-stimulating hormone and pulsatile LH secretion across the menstrual cycle of pre-menopausal women.* J Clin Endocrinol Metab *81:1512–1518, 1996.)*

amplitude pulses of LH observed in the first few years in postmenopausal women slows down in late menopause;[100] this latter effect is clearly related to aging per se.

Ovarian aging is a programmed event, and the return of atresia, accelerating at around age 37.5, until the natural age of menopause, has now been shown to occur in almost the exact way in the chimpanzee.[101] Ovarian aging from a hormonal standpoint is best characterized by small elevations in serum FSH occurring at the beginning of the menstrual cycle (days 2 to 3), reductions in inhibin B, as well as steep declines in serum müllerian inhibiting substance (MIS) or AMH was introduced previously[102] (Fig. 15-14). It has been confirmed that once a level of inhibin B or AMH becomes undetectable, menopause will ensue in 4 to 5 years (Fig. 15.14A). AMH is a very useful and practical determinant in that it tends to undergo less cycle to cycle variation compared to FSH or inhibin B, and can be measured during any phase of the cycle.[103]

Effects on Various Organ Systems

Central Nervous System

The brain is an active site for estrogen action as well as estrogen formation.[104] Estrogen activity in the brain is mediated via ERα and ERβ receptors. Whether or not a novel membrane receptor (non-ERα/ERβ) exists is still being debated.[105] However, both genomic and nongenomic mechanisms of estrogen action clearly exist in the brain. Figure 15.15 illustrates the predominance of ERβ in the cortex (frontal and parietal) and the cerebellum, based on work in the rat.[106,107]

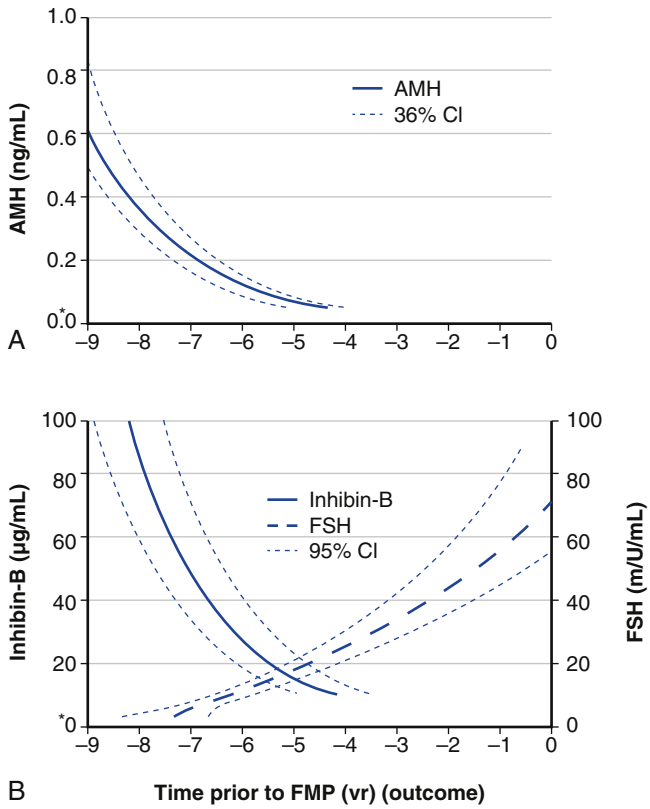


FIGURE 15.14 A, Anti-müllerian Hormone (AMH) decreases to undetectable levels (0.05 ng/mL) 5 years before the final menstrual period, and inhibin B (10 pg/mL) does so 4 years before the last menstrual period. **B,** Rapid decline in levels of AMH/MIS with aging. Levels are undetectable prior to menopause. *(Adapted From Sowers MR, Eyvazzadeth AD, McConnell D, et al. Anti-müllerian hormone and inhibin in the definition of ovarian aging and the menopause transition.* J Clin Endocrinol Metab *93[9]:L34768–L34783.)*

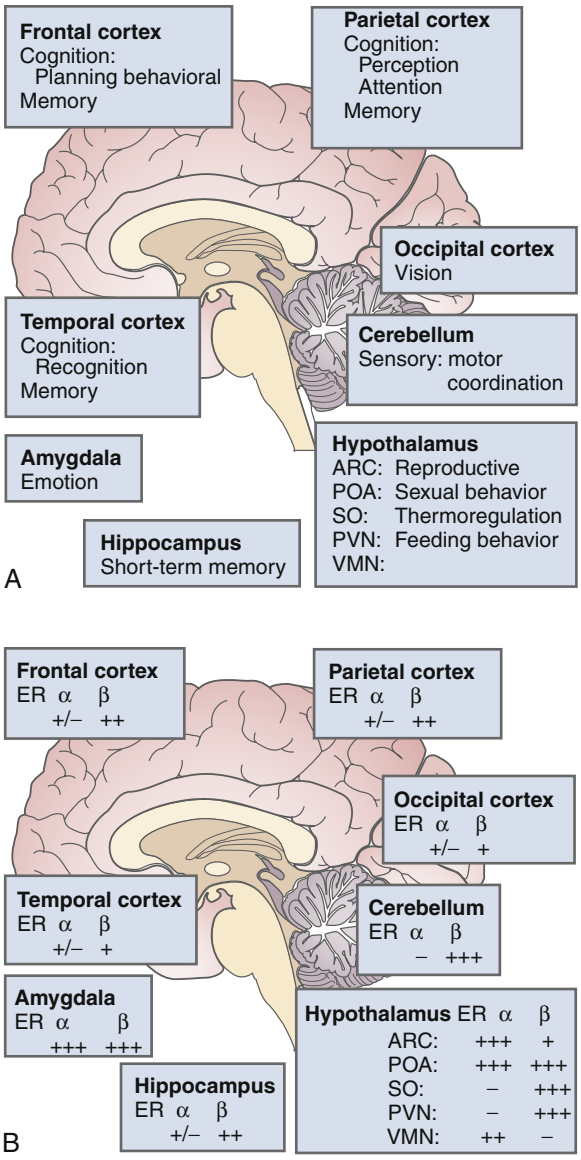


FIGURE 15-15 A, Each region of the brain has an important role in specific brain functions. Optimal brain activity is maintained by means of the integration of different areas by neural tracts. **B,** Distribution of estrogen receptors ERα and ERβ mRNA in the rat brain. ARC, arcuate nucleus; POA, preoptic area; PVN, paraventricular nucleus; SO, supraoptic nucleus; VMN, ventromedial nucleus. (**B,** *Adapted from Cela V, Naftolin F. Clinical effects of sex steroids on the brain. In Lobo RA [ed].* The Treatment of Postmenopausal Woman: Basic and Clinical Aspects, *2nd ed. Philadelphia, Lippincott Williams & Wilkins, 1999, pp 247–262.)*

While 17β-E_2 is a specific ligand for both receptors, certain synthetic estrogens have a greater affinity for ERβ.

There are multiple actions of estrogen on the brain as reviewed by Henderson (Box 15.1)[108] thus there are important functions linked to estrogen that contribute to well-being in general and, more specifically, to cognition and mood. The hallmark feature of declining estrogen status in the brain is the hot flush, which is more generically referred to as a vasomotor episode.

The *hot flash* usually refers to the acute sensation of heat, while the *flush* or vasomotor episode includes changes in the early perception of this event and other skin changes (including diaphoresis). Hot flushes usually occur for 2 years after the onset of estrogen deficiency, but can persist for 10 or more years.[109,110] In 10% to 15% of women these symptoms are severe and disabling.[109,111] In the United States the incidence of these episodes varies in different ethnic groups. Symptoms are greatest in Hispanic and African American women, intermediate in white women, and lowest among Asian women (Fig. 15.16).[112]

The fall in estrogen levels precipitate the vasomotor symptoms. Although the proximate cause of the flush remains elusive, the episodes result from a hypothalamic response (probably mediated by catecholamines) to the change in estrogen status. The flush has been well characterized physiologically. It results in heat dissipation as witnessed by an increase in peripheral temperature (finger, toe); a decrease in skin resistance, associated with diaphoresis; and a reduction in core body temperature (Fig. 15.17).[113] There are hormonal correlates of flush activity, such as an increase in serum LH and in plasma levels of pro-opiomelanocortin peptides (ACTH, β-endorphin) at the time of the flush,[114] but these occurrences are thought to be epiphenomena that result as a consequence of the flush and are not related to its etiology. Data from Freedman has suggested that the major physiological finding in women with and without hot flushes

is a narrowing of the temperature threshold for sweating and shivering in symptomatic women (Fig. 15.18).[115]

One of the primary complaints of women with hot flushes is sleep disruption. They may awaken several times during the night, and require a change of bedding and clothes because of diaphoresis. Nocturnal sleep disruption in postmenopausal women with hot flushes has been well documented by electroencephalographic (EEG) recordings.[116] Sleep efficiency is lower, and the latency to rapid eye movement (REM) sleep is longer in women with hot flushes compared with asymptomatic women.[117,118] This disturbed sleep often leads to fatigue and irritability during the day. The frequency of awakenings and of hot flushes are reduced appreciably with estrogen treatment (Fig. 15.19).[116,119,120] Sleep may be disrupted even if the woman is not conscious of being awakened from sleep. In this setting, EEG monitoring has indicated sleep disruption in concert with physiological measures of vasomotor episodes.

In postmenopausal women, estrogen has been found to improve depressed mood regardless of whether or not this is a specific complaint[121-127] (critics of some of this work point out that mood is affected by the symptomatology and by sleep deprivation). Blinded studies carried out in asymptomatic women have also shown benefit.[126] In an estrogen-deficient state such as occurs after the menopause, a higher incidence of depression (clinical or subclinical) is often manifest. However, menopause per se does *not* cause depression, and while estrogen does generally improve depressive mood, it should *not* be used for psychiatric disorders. Nevertheless, very high pharmacological doses of estrogen have been used to treat certain types of psychiatric depression in the past.[128-130] Progestogens as a class generally attenuate the beneficial effects of estrogen on mood, although this effect is highly variable.[131,132]

Cognitive decline in postmenopausal women is related to aging as well as to estrogen deficiency. The literature is somewhat mixed in showing whether there are benefits of estrogen in terms of cognition. In more recent studies, verbal memory appears to be enhanced with estrogen[133-136] and has been found to correlate with acute changes in brain imaging signifying brain activation.[135-137]

Dementia increases as women age, and the most common form of dementia is Alzheimer disease (AD). Listed in Box 15.1 are several neurotrophic and neuroprotective factors that relate to how estrogen deficiency may be expected to result in the loss of protection against the development of AD. In addition, estrogen has a positive role in enhancing neurotransmitter function, which is deficient in women with AD. This function of estrogen has particular

Box 15.1 Effects of Estrogen on Brain Function

ORGANIZATIONAL ACTIONS
- Effects on neuronal number, morphology, and connections occurring during critical stage of development

NEUROTROPHIC ACTIONS
- Neuronal differentiation
- Neurite extension
- Synapse formation
- Interactions with neurotrophins

NEUROPROTECTIVE ACTIONS
- Protection against apoptosis
- Antioxidant properties
- Anti-inflammatory properties
- Augmentation of cerebral blood flow
- Enhancement of glucose transport into the brain
- Blunting of corticosteroid response to behavioral stress
- Interactions with neurotrophins

EFFECTS ON NEUROTRANSMITTERS
- Acetylcholine
- Noradrenaline
- Serotonin
- Dopamine
- Glutamate
- γ-Aminobutyric acid
- Neuropeptides

EFFECTS ON GLIAL CELLS

EFFECTS ON PROTEINS INVOLVED IN ALZHEIMER DISEASE
- Amyloid precursor protein
- Tau protein
- Apolipoprotein E

Adapted from Henderson VW: Estrogen, cognition, and a woman's risk of Alzheimer's disease. *Am J Med* 103(suppl 3A):11–18, 1997.

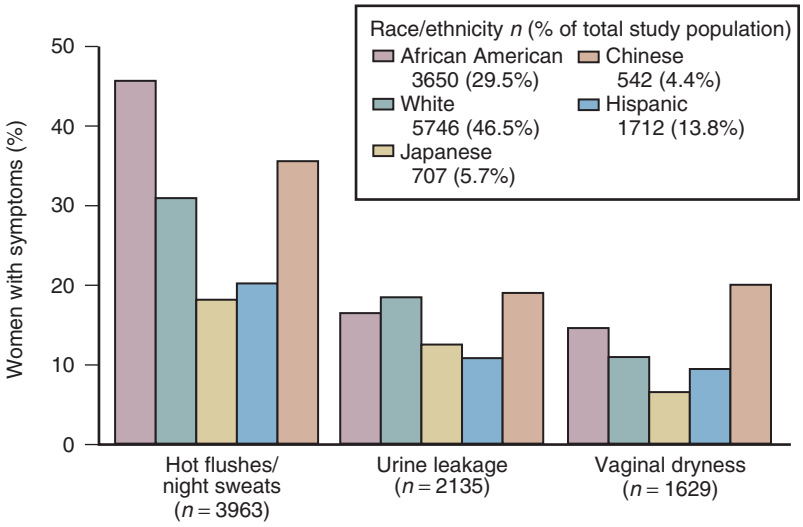


FIGURE 15.16 Study of Women's Health Across the Nation (SWAN): Symptom severity. *(Adapted from Gold EB, Sternfeld B, Kelsey JL, et al. Relation of demographic and lifestyle factors to symptoms in a multiracial/ethnic population of women 40-55 years of age.* Am J Epidemiol *152:463, 2000.)*

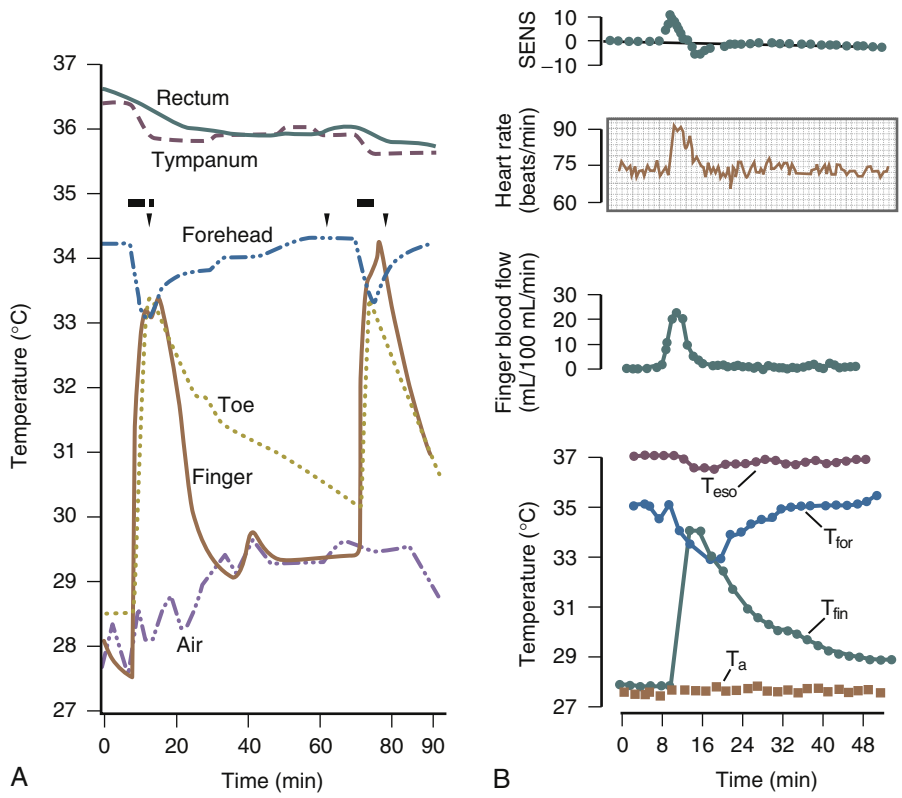


FIGURE 15.17 Temperature responses to two spontaneous flashes and evoked flash. *Down arrow* indicates finger stab for blood sample. *Black bars* indicate time of hot flush. SENS *(Data adapted from Molnar GW. Body temperature during menopausal hot flashes.* J Appl Physiol *38:499–503, 1975.)*

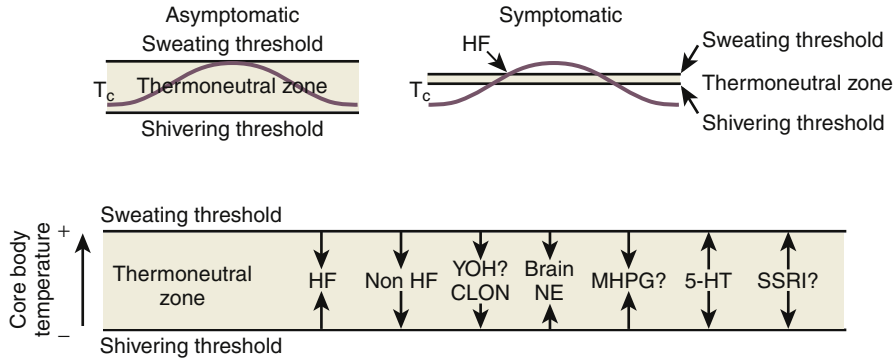


FIGURE 15.18 Narrowing of the thermoregulatory zone in symptomatic women. HF, hot flush. *(Data from Freedman RR. Menopausal hot flashes. In Lobo RA (ed). Treatment of the Postmenopausal Woman, 3rd ed.* New York, Academic Press, *2007, pp 187–198.)*

importance and relevance for the cholinergic system that is affected in AD.[138,139]

Estrogen use after menopause has been shown in observational studies to decrease the likelihood of developing or delays the onset of AD.[140-157] (Fig. 15.20). However, there are no randomized prospective studies as yet on this issue. It is clear, however, that if estrogen has a beneficial role, it is only in those women who receive estrogen at the onset of menopause; in older women it may be detrimental.[157a] Once a woman is affected by AD, estrogen is unlikely to provide any benefit.[158]

Collagen

Estrogen has a positive effect on *collagen*, which is an important component of bone and skin and serves as a major support tissue for the structures of the pelvis and urinary system. Both estrogen and androgen receptors have been identified in skin fibroblasts. Nearly 30% of skin collagen is lost within the first 5 years after menopause, and collagen decreases approximately 2% per year for the first 10 years after menopause.[159] This statistic, which is similar to that of bone loss after menopause, strongly suggests a link between skin thickness, bone loss, and the risk of osteoporosis.[160]

Although the literature is not entirely consistent, estrogen therapy generally improves collagen content after menopause and improves skin thickness substantially after about 2 years of treatment.[159,161-165] There is a possible biomodal effect with high doses of estrogen causing a reduction in skin thickness.[159-166] The supportive effect of estrogen on collagen has important implications for bone homeostasis and for the pelvis after menopause. Here, reductions in collagen support and atrophy of the vaginal and urethral mucosa have been implicated in a variety of symptoms, including prolapse and urinary incontinence.[167,168]

Symptoms of urinary incontinence and irritative bladder symptoms occur in 20% to 40% of perimenopausal and postmenopausal women.[169-171] Uterine prolapse and other gynecological symptoms related to poor collagen support, as well as urinary complaints, may improve with estrogen

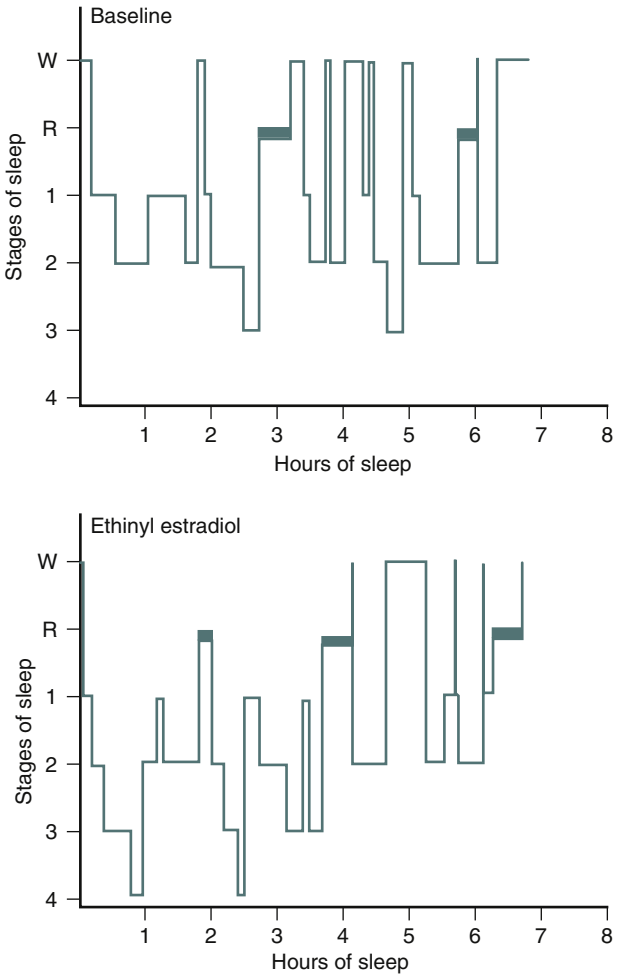


FIGURE 15.19 Sleepgrams measured in symptomatic patient before and after 30 days' administration of ethinyl estradiol, 50 μg four times daily. *(Adapted from Erlik Y, Tataryn IV, Meldrum DR, et al. Association of waking episodes with menopausal hot flushes.* JAMA *245:1741–1744, 1981.)*

therapy.[112,172-174] While estrogen generally improves symptoms, urodynamic changes have not been shown to be altered.[175,176] Estrogen has also been shown to decrease the incidence of recurrence of urinary tract infections.[177] These data relate to the use of vaginal estrogen, rather than the use of estrogen systemically. Somewhat paradoxically systemic estrogen may increase stress urinary incontinence, while local vaginal therapy may improve urge incontinence.[177a]

Restoration of bladder control in older women with estrogen has been shown to decrease the need for admission to nursing homes in Sweden.[178] Estrogen may also have an important role in normal wound healing. In this setting, estrogen enhances the effects of growth factors such as transforming growth factor-β (TGFββ).[179,180]

Genital Atrophy

Vulvovaginal complaints are often associated with estrogen deficiency. In the perimenopause, symptoms of dryness and atrophic changes occur in 21% and 15% of women, respectively. However, these findings increase with time, and by 4 years these incidences are 47% and 55%, respectively.[181,182] With this change, an increase in sexual complaints also occurs, with an incidence of dyspareunia of 41% in sexually active 60-year-old women.[183] Estrogen deficiency results in a thin and more pale vaginal mucosa. The moisture content is low, the pH increases (usually greater than 5), and the mucosa may exhibit inflammation and small petechiae.

With estrogen treatment, vaginal cytology changes have been documented, transforming from a cellular pattern of predominantly parabasal cells to one with an increased number of superficial cells. Along with this change, the vaginal pH decreases, vaginal blood flow increases, and the electropotential difference across the vaginal mucosa increases to that found in premenopausal women.[184] Recent studies have suggested that intravaginal DHEA (0.25% to 1%) is efficacious for altering vaginal cytology and symptoms of atrophy, presumably by allowing for local conversion to other androgens and estrogen.[184a] More studies, however, are needed.

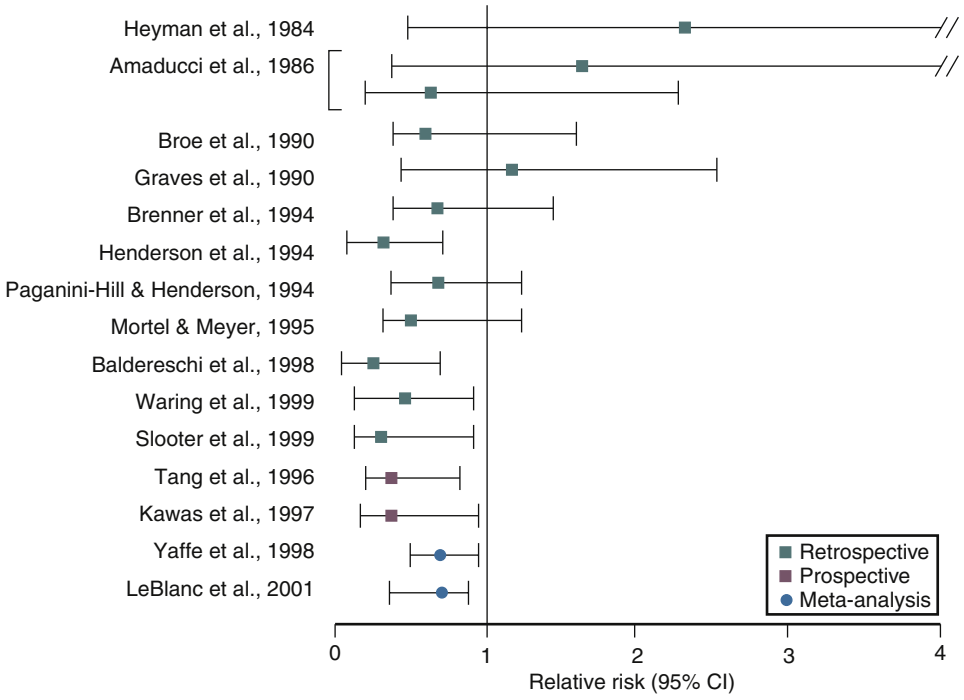


FIGURE 15.20 Estrogen and hormone replacement therapy use, and Alzheimer disease risk. *(Adapted from LeBlanc ES, Janoowsky J, Chan BK, Nelson HD. Hormone replacement therapy and cognition: systematic review and meta-analysis.* JAMA *285:1489–1499, 2001.)*

Bone Loss

Estrogen deficiency has been well established as a cause of bone loss. This loss can be noted for the first time when menstrual cycles become irregular in the perimenopause. From 1.5 years before the menopause to 1.5 years after menopause, spine bone mineral density has been shown to decrease by 2.5% per year, compared with a premenopausal loss rate of 0.13% per year.[185,186] Loss of trabecula bone (spine) is greater with estrogen deficiency than is loss of cortical bone.

Postmenopausal bone loss leading to osteoporosis is a substantial healthcare problem. In white women, 35% of all postmenopausal women have been estimated to have osteoporosis based on bone mineral density.[187] Further, the lifetime fracture risk for these women is 40%.[188] The morbidity and economic burden of osteoporosis is well documented.[189] Interestingly, there are data to suggest that up to 19% of caucasian men also have osteoporosis.

Bone mass is substantially affected by sex steroids through classic mechanisms to be described later in this chapter. Attainment of peak bone mass in the late second decade (Fig. 15.21)[190] is key to ensuring that the subsequent loss of bone mass with aging and estrogen deficiency does not lead to early osteoporosis. Estradiol, together with Growth Hormone (GH) and insulin-like growth factor-1, act to double bone mass at the time of puberty[191] beginning the process of attaining peak bone mass. Postpubertal estrogen deficiency (amenorrhea from various causes) substantially jeopardizes peak bone mass. Adequate nutrition and calcium intake are also key determinants. While estrogen is of predominant importance for bone mass in both women and men, testosterone is important in stimulating periosteal apposition; as a result, cortical bone in men is larger and thicker.[192,193]

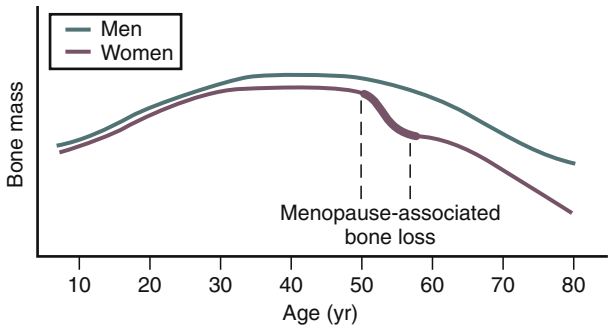


FIGURE 15.21 Bone mass by age and sex. *(Adapted from Finkelstein JS. In Cecil RL, Goldman L, Bennett JC [eds]. Cecil Textbook of Medicine, 21st ed. Philadelphia, Saunders, 1999, pp 1366-1373; and Riggs BL, Melton LJ III. Involutional osteoporosis.* N Engl J Med *314:1676–1686, 1986.)*

Estrogen receptors (ERs) are present in osteoblasts,[194,195] osteoclasts,[196,197] and osteocytes.[198,199] Both ERα and ERβ are present in cortical bone, while ERβ predominates in cancellous or trabecular bone.[200] However, the more important actions of estradiol are believed to be mediated via ERα.

Estrogens suppress bone turnover and maintain a certain rate of bone formation.[201] Bone is remodeled in functional units, called *bone multicenter units* (BMUs), where resorption and formation should be in balance.[202] Multiple sites of bone go through this turnover process over time. Estrogen decreases osteoclasts by increasing apoptosis and thus reduces their lifespan.[203] The effect on the osteoblast is less consistent, but E_2 antagonizes gluco-corticoid-induced osteoblast apoptosis.[201,204] Estrogen deficiency increases the activities of remodeling units, prolongs resorption, and shortens the phase of bone formation[201]; it also increases osteoclast recruitment in BMUs, thus resorption outstrips formation.

The molecular mechanisms of estrogen action on bone involve the inhibition of production of pro-inflammatory cytokines including interleukin-1, interleukin-6, and tumor necrosis factor, which inhibits bone resorption. Receptor-activation of NFKB ligand (RANKL) is responsible for osteoclast differentiation and action.[205,206] A scheme for how all these factors interact has been proposed by Riggs (Fig. 15.22).[207]

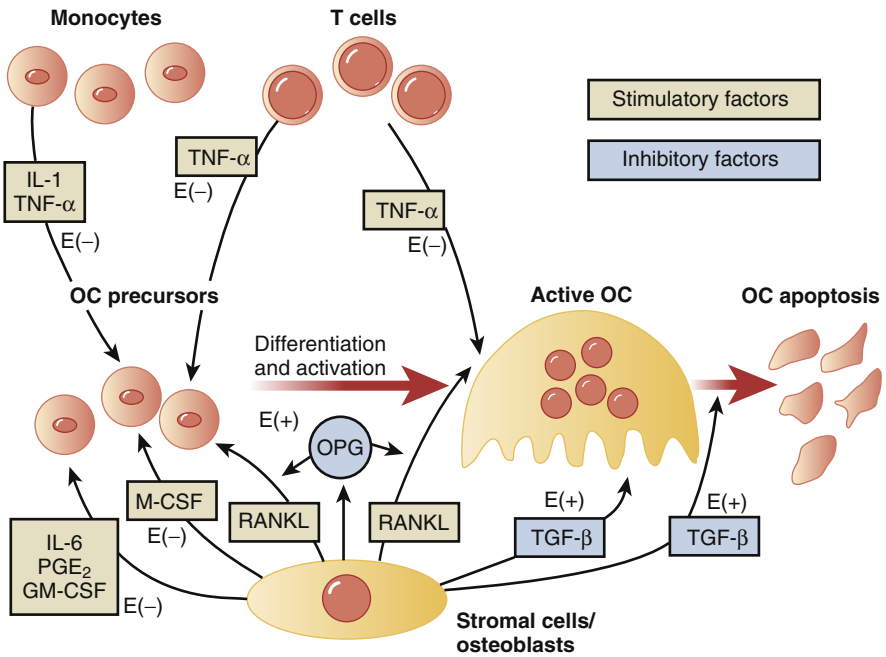


FIGURE 15.22 Model for mediation of effects of estrogen (E) on osteoclast formation and function by cytokines in bone marrow microenvironment. Stimulatory factors are shown in orange and inhibitory factors are shown in blue. Positive (+) and negative (–) effects of E on these regulatory factors are shown in red. The model assumes that regulation is accomplished by multiple cytokines working together in concert. *(Adapted from Riggs BL. The mechanisms of estrogen regulation of bone resorption.* J Clin Invest *106:1203–1204, 2000.)*

In women, Riggs has suggested that bone loss occurs in two phases. With estrogen levels declining at the onset of menopause—leading to an accelerated phase of bone loss—predominantly cancellous bone loss occurs. Here 20% to 30% of cancellous bone and 5% to 10% of cortical bone can be lost in a span of 4 to 8 years.[208] Thereafter a slower phase of loss (1% to 2% per year) ensues where more cortical bone is lost. This phase is thought to be induced primarily by secondary hyperparathyroidism.[209] The first phase is also accentuated by the decreased influence of stretching or mechanical factors, which generally promote bone homeostasis, as a result of estrogen deficiency.[210-212]

Genetic influences on bone mass are more important for attainment of peak bone mass (heritable component, 50% to 70%) than for bone loss. Polymorphisms of the vitamin D receptor gene, TGFβ gene, and the Spl-binding site in the collagen type 1 AI gene have all been implicated[213] as being important for bone mass.

While testosterone is important for bone formation and stimulation of bone mass, even in men estrogen action is of paramount importance.[214-216] Bone mass was shown to increase in an aromatase-deficient man upon estrogen administration.[217]

Bone mass can be detected by a variety of radiographic methods (Table 15.2).[218] *Dual energy x-ray absorptiometry (DEXA) scans* have become the standard of care for detection of osteopenia and osteoporosis. By convention, the *T score* is used to reflect the number of standard deviations of bone loss from the peak bone mass of a young adult. Osteopenia is defined by a T score of −1 to −2.5 standard deviations; osteoporosis is defined as greater than 2.5 standard deviations. Since bone mass does not completely reflect bone strength, which is really what matters in terms of fracture risk, several approaches have been made to assess bone strength. An assessment of biochemical bone turnover (discussed later in the chapter) in addition to bone mass is deemed important. One newer approach is to assess bone microarchitecture, a so-called virtual bone biopsy, by using a high resolution peripheral (p) QCT (Fig. 15.23).[219] This technique is only available as a research tool at present.

Various biochemical assays are also available to assess bone resorption and formation in both blood and urine (Table 15.3).[220] At present, serum markers appear to be most useful for assessing changes with antiresorptive therapy.

There are now many agents that can prevent osteoporosis. The use of estrogen will depend on whether or not there are other indications for estrogen treatment and whether there are contraindications. Estrogen has been shown to reduce the risk of osteoporosis as well as to reduce osteoporotic fractures. In the Women's Health Initiative study (WHI), hip fractures as well as all fractures were reduced with conjugated equine estrogens (CEE) and medroxyprogesterone acetate (MPA) and CEE alone, and this occurred in a non-osteoporotic population. Indeed, in a cohort of women who were followed after stopping hormones when the results of the WHI were published, there was increased risk of hip fracture and lower bone density compared to those women who continued therapy.[220a]

A dose equivalent to 0.625 mg of conjugated equine estrogens (CEE) was once thought to prevent osteoporosis, but we now know that lower doses (0.3 mg of CEE or its equivalent) in combination with progestogens are able to prevent bone loss[221,222] although there are no data on fractures. Whether or not the addition of progestogens, by stimulating bone formation, increases bone mass over that of estrogen alone is unclear. The androgenic activity of certain progestogens such as northindrone acetate also has been suggested to play a role.[223]

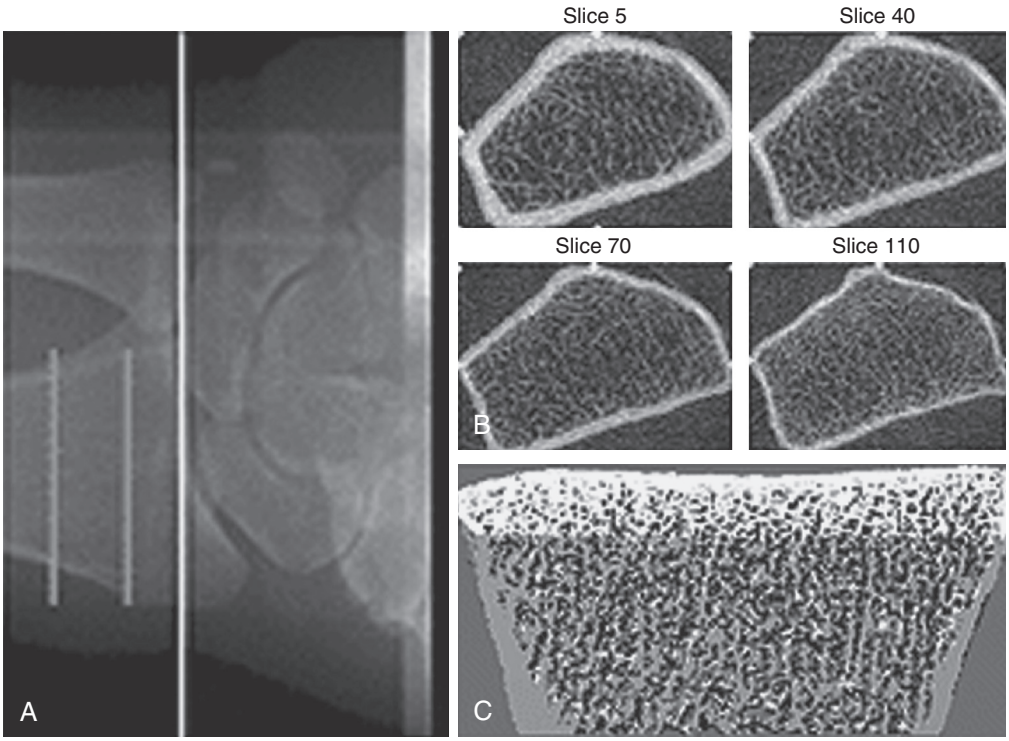


FIGURE 15.23 High-resolution peripheral quantitative computed tomography (pQCT). **A,** Radiograph of distal radius; *lines* indicate the section analyzed. **B,** Representative cross-sectional images from computed tomography slices. **C,** Representative three-dimensional image. *(Adapted from Khosla S, Riggs BL, Atkinson EJ, et al. Effects of sex and age on bone microstructure at the ultradistal radius: a population-based noninvasive* in vivo *assessment.* J Bone Miner Res; *21:124–131, 2006.)*

Selective estrogen receptor modulators (SERMs) such as raloxifene, droloxifene, and tamoxifen have all been shown to decrease bone resorption. Raloxifene has been shown to decrease vertebral fractures in a large prospective trial.[224] Tibolone has also been shown to be an effective treatment for osteoporosis. Tibolone (not marketed in the United States) has SERM-like properties, but it is not specifically a SERM because it has mixed estrogenic, antiestrogenic, androgenic, and progestogenic properties due to its metabolites. The drug does not cause uterine or breast cell proliferation but is beneficial for vasomotor symptoms. It prevents osteoporosis and has been shown to be beneficial in treatment of osteoporosis as well.[225,226]

Bisphosphonates have been shown to have a significant effect on the prevention and treatment of osteoporosis. With this class of agents (etidronate, alendronate, residronate, ibandronate), and Zoledronic acid incorporation of the bisphosphonate with hydroxyapetite in bone increases bone mass. The skeletal half-life of bisphosphonates in bone can be as long as 10 years.[227] Most data have been derived with alendronate, which, at a dosage of 5 mg daily (35 mg weekly) prevents bone loss; at 10 mg daily (70 mg weekly), alendronate is an effective treatment for osteoporosis, with evidence available that this treatment reduces vertebral and hip fractures.[228] Ibandronate is available as a monthly pill and by injection. It has primary efficiency for vertebral fracture protection. Zoledronic acid, 5 mg, OK is available as an intravenous therapy. Calcitonin, 50 IU OK subcutaneous injections daily, or 200 IU intranasally has been shown to inhibit bone resorption. Vertebral fractures have been shown to decrease[229,230] with calcitonin therapy. Long-term effects, however have not been established.

Fluoride has been used for women with osteoporosis because it increases bone density.[231] Currently, a lower dose (50 μg daily) of slow-release sodium fluoride does not seem to cause adverse effects (gastritis) and has efficacy in preventing vertebral fractures.[232,233] Intermittent parathyroid hormone (PTH 1-34, Teriparatide) is an agent that increases bone mass in women with osteoporosis. In a randomized trial lasting 3 years, average bone density increased in the hip and spine with fewer fractures observed.[234] This therapy is now available in the United States, but is expensive and is reserved for women who are difficult to treat and have a history of fractures.[234a]

Adjunctive measures for prevention of osteoporosis are calcium, vitamin D, and exercise. Calcium with vitamin D treatment has been shown to increase bone in only older individuals.[235] These modalities alone are not thought to be effective for the treatment of osteoporosis. A woman's total intake of elemental calcium should be 1500 mg daily if no agents are being used to inhibit resorption, and 400 to 800 IU OK of vitamin D should also be ingested. Levels of serum 25 hyroxy (OH) vitamin D have been found to be abnormally low (<20 ng/mL) in a large population of women, particularly in geographic regions of less sunlight exposure. Exercise has been shown to be beneficial for building muscle and bone mass and for reducing falls.[236,237] Guidelines regarding management of osteoporosis were published by the National Osteoporosis Foundation in February 2008 (www.nof.org). An update analysis has suggested that 37% of women are candidates for treatment to prevent fractures.[237a] Also, World Health Organization (WHO) has produced guidelines for assessing an individual's risk of osteoporosis based on history, anthropometry, and bone mineral density (BMD). This new paradigm, called Fracture Risk Assessment Tool, may be obtained at www.shef.ac.uk/FRAX.

Table 15.3 Bone Turnover Markers

Marker	Specimen
Bone Resorption Markers	
Cross-linked N-telopeptide of type 1 collagen (NTX)	Urine, serum
Cross-linked C-telopeptide of type 1 collagen (CTX)	Urine (αα and ββ forms) Serum (ββ form)
MMP-generated telopeptide of type 1 collagen (ICTP or CTX-MMP)	Serum
Deoxypyridinoline, free and peptide bound (fDPD, DPD)	Urine, serum
Pyridinoline, free and peptide bound (fPYD, PYD)	Urine, serum
Hydroxyproline (OHP)	Urine
Glycosyl hydroxylysine (GylHyl)	Urine, serum
Helical peptide (HelP)	Urine
Tartrate-resistant acid phospharase 5b Isoform specific for osteoclasts (TRACP 5b)	Serum, plasma
Cathepsin K (Cath K)	Urine, serum
Osteocalcin fragments (uOC)	Urine
Bone Formation Markers	
Osteocalcin (OC)	Serum
Procollagen type 1 C-terminal propeptide (PICP)	Serum
Procollagen type 1 N-terminal propeptide (PINP)	Serum
Bone-specific alkaline phosphatase (bone ALP)	Serum

Eastell R, Hannon RA: Biochemical markers of bone turnover. In: Lobo RA, ed. Treatment of the Postmenopausal Woman Basic and Clinical Aspects, ed. 3, St. Louis: Elsevier Academic Press; 2007, p. 337-350.

Cardiovascular Effects

Clearly after menopause, the risk of cardiovascular disease in women is increased. Data from the Framingham study[238] have shown that the incidence is three times lower in women before menopause than in men (3.1 per 1000 per year in women aged 45 to 49). The incidence is approximately equal in men and women aged 75 to 79 (53 and 50.4 per 1000 per year, respectively). This trend also pertains to gender differences in mortality due to cardiovascular disease. Coronary artery disease is the leading cause of death in women, and the lifetime risk of death is 31% in postmenopausal women versus a 3% risk of dying of breast cancer.[239]

Although cardiovascular disease becomes more prevalent only in the later years following a natural menopause, premature cessation of ovarian function (before the average age of menopause) constitutes a significant risk. Premature menopause, occurring before age 35, has been shown to increase the risk of myocardial infarction twofold to threefold, and oophorectomy before age 35 increases the risk severalfold.[240]

An analysis of several studies on this issue has been conducted and reviewed[241] with the data depicted in Figure 15.24, on the effect of early menopause on the prevalence

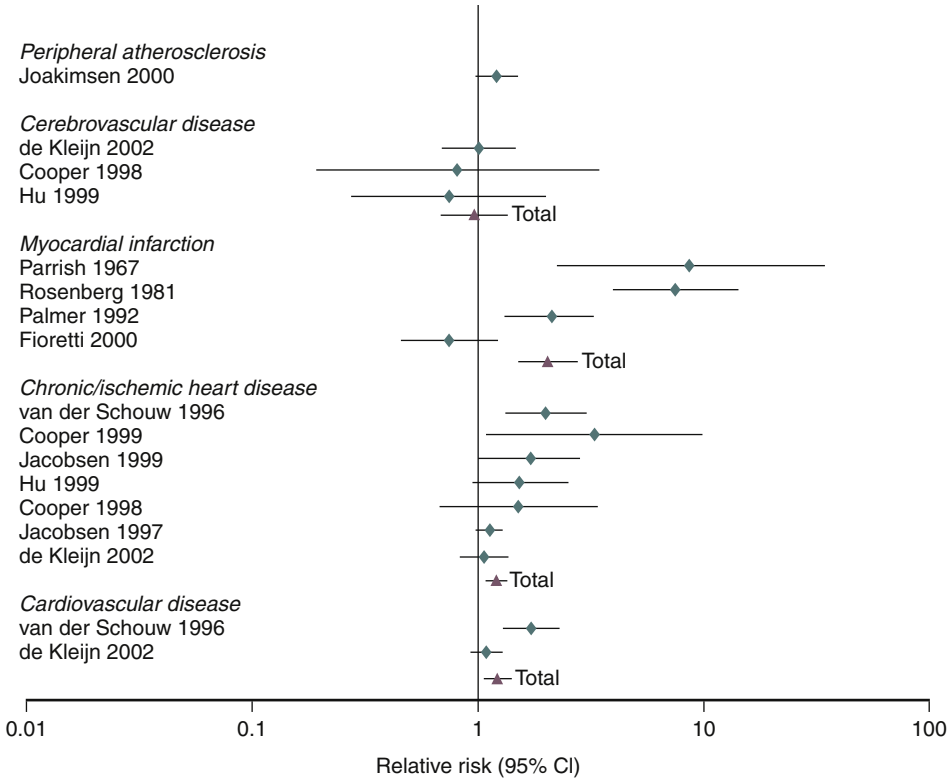


FIGURE 15.24 Effect of "early" menopause on types of cardiovascular disease. *(From Atsma F, Bartelink ML, Grobbee DE, et al. Postmenopausal status and early menopause as independent risk factors for cardiovascular disease: a meta-analysis.* Menopause *13[2]:265–279, 2006 [review]. See reference 68 for data sources.)*

and type of CVD. It has been suggested that total mortality is increased if bilateral oophorectomy occurs even after the natural menopause, until around age 60. This change in total mortality is due to an excess in coronary disease, suggesting a protective effect of the ovary even beyond the normal age of menopause.[242]

When the possible reasons for the increase in cardiovascular disease are examined, the most prevalent finding is that of the accelerated rise in total cholesterol in postmenopausal women. The changes of weight, blood pressure, and blood glucose with aging, while important, are not thought to be as important as the rate of rise in total cholesterol, which is substantially different in women versus men. This increase in total cholesterol is explained by increases in levels of low-density lipoprotein cholesterol (LDL-C). The oxidation of LDL-C is also enhanced, as are levels of very-low-density lipoproteins and Lp(a) lipoprotein. HDL-C levels trend downward with time, but these changes are small and inconsistent relative to the increases in LDL-C.[243]

Coagulation balance is not substantially altered as a counterbalancing change occurs. Some procoagulation factors increase (Factor VII, fibrinogen, and plasminogen activator inhibitor [PA1-1]), but so do counterbalancing factors like antithrombin III, plasminogen, protein C, and protein S.[244] Inflammation markers such as C-reactive protein and cytokines are increased; and blood flow in all vascular beds decreases after menopause. Prostacyclin production decreases, endothelin levels increase, and vasomotor responses to acetylcholine are constrictive, reflecting reduced nitric oxide synthetase activity. With estrogen, all these parameters (generally) improve and coronary arterial responses to acetylcholine are dilatory with a commensurate increase in blood flow.[245-252]

Circulating plasma nitrites and nitrates have also been shown to increase with estrogen, and angiotensin-converting enzyme levels tend to decrease. Estrogen and progesterone receptors have been found in vascular tissues, including coronary arteries (predominantly ERβ). In addition, there are membrane effects mediated by estrogen—which may or may not relate to either ERα or ERβ.[253,254]

Overall, the direct vascular effects of estrogen are viewed to be as important, or more important, than the changes in lipid and lipoproteins after menopause. While replacing estrogen has been thought to be beneficial for the mechanisms previously cited, these beneficial arterial effects may only be seen in younger (stage +1) postmenopausal women. Women with significant atherosclerosis or risk factors such as those studied in a secondary prevention trial do not respond in a beneficial manner[255-260] (Fig. 15.25). Some of this lack of effect may be accounted for by increased methylation of the promoter region of ERα, which occurs with atherosclerosis and aging.[261]

Another theory proposed to explain differences in the effects of estrogen when given early rather than later is the interfering effect of endogenous 27-hydroxy cholesterol. This endogenous metabolite of cholesterol increases with advancing levels of cholesterol and competes for binding with E_2 at the ER in the endothelium. Thus when cholesterol is elevated, high levels of 27-C may prevent estrogen action[262] (Fig. 15.26).

In normal, nonobese postmenopausal women, carbohydrate tolerance also decreases as a result of an increase in insulin resistance. This, too, may be partially reversed by estrogen.[263] Biophysical and neurohormonal responses to stress (stress reactivity) are exaggerated in postmenopausal women compared with premenopausal women, and this heightened reactivity is blunted by estrogen.[264] Whether or not these changes influence cardiovascular risk with

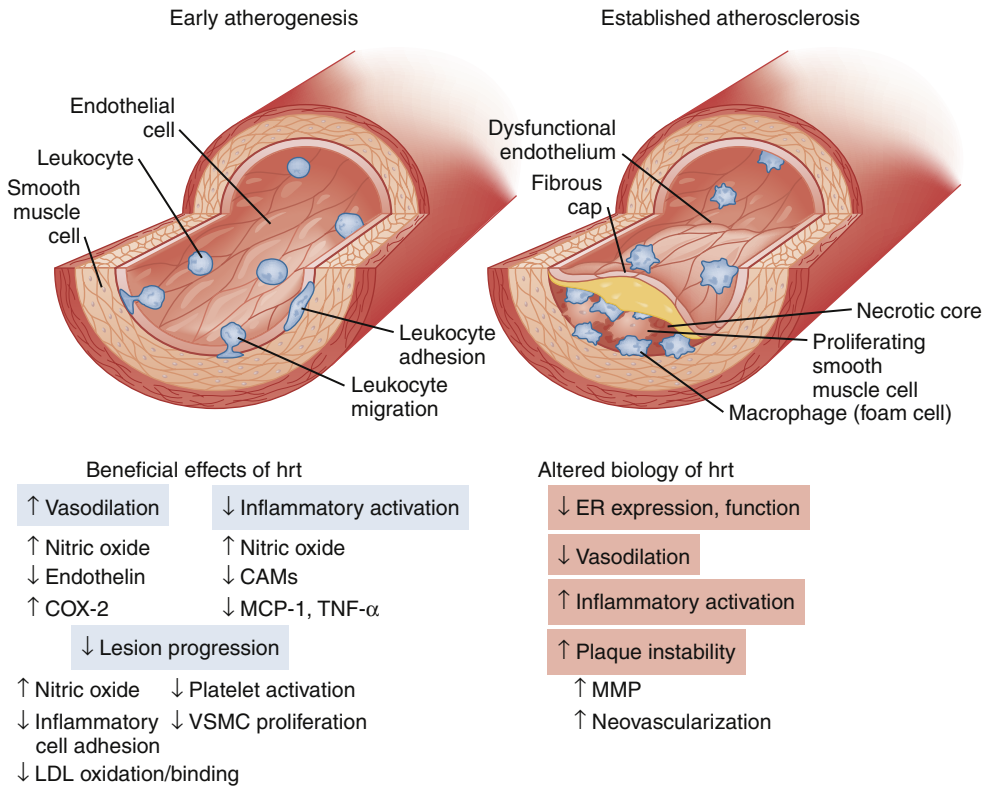


FIGURE 15.25 Early and esterified atherosclerosis with aging and the beneficial and negative effects of estrogen during these stages. ER, estrogen receptor; MMP, matrix metalloproteinase; TNF, tumor necrosis factor. *(From Mendelsohn ME, Karas RH. Molecular and cellular basis of cardiovascular gender differences. Science 308[5728]:1583–1587, 2005 [review].)*

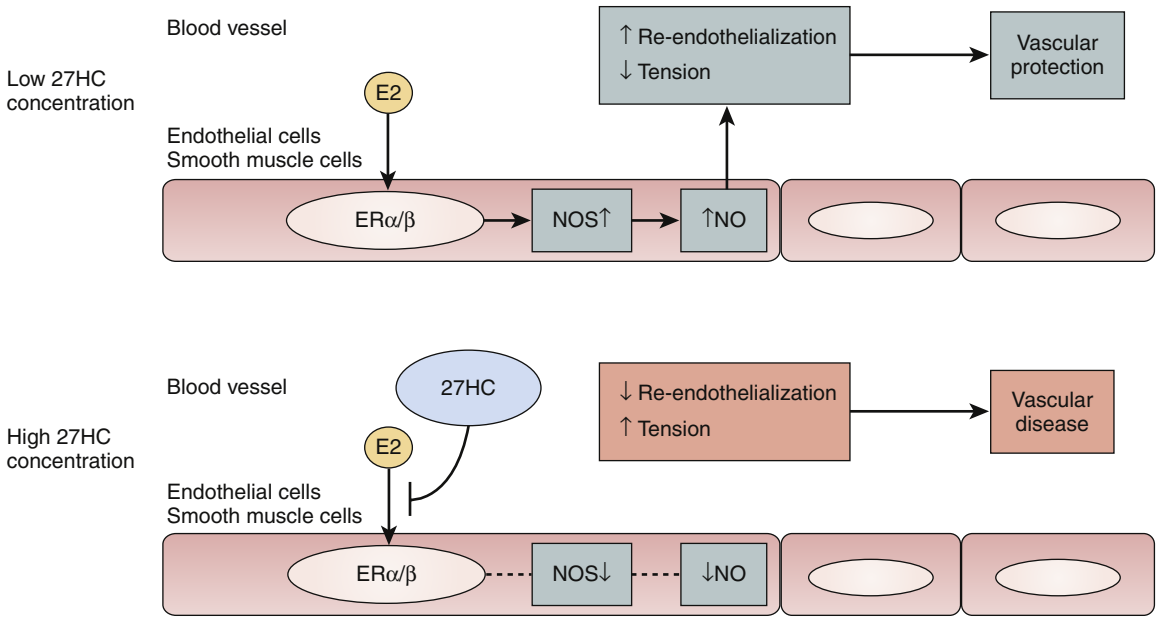


FIGURE 15.26 Hypothesis of how elevated cholesterol (27-hydroxycholesterol, 27HC) can influence the effect of estradiol (E_2). ER, estrogen receptor; NO, nitric oxide; NOS, nitric oxide synthase. *(From Umetani M, Domoto H, Gormley AK, et al. 27-Hydroxycholesterol as an endogenous SERM that inhibits the cardiovascular effects of estrogen.* Nat Med *13[10]:1185–1192, 2007.)*

estrogen deficiency is not known, but clearly estrogen treatment returns many parameters into the range of premenopausal women in early postmenopausal women.

These consistently strong basic science and clinical data for the protective effects of estrogen on the cardiovascular system, together with strong epidemiological evidence for a protective effect of estrogen (Fig. 15.27)[265] led to the belief that estrogen should be prescribed to prevent cardiovascular disease in women. However, randomized clinical trials (RCTs) have refuted this notion in women with established disease as noted previously. Results from several randomized trials in women,[255-257] have found there to be more coronary events in older women, given estrogen for the first time.[266]

This trend toward increased cardiovascular events in women who are older and/or who have documented diseased

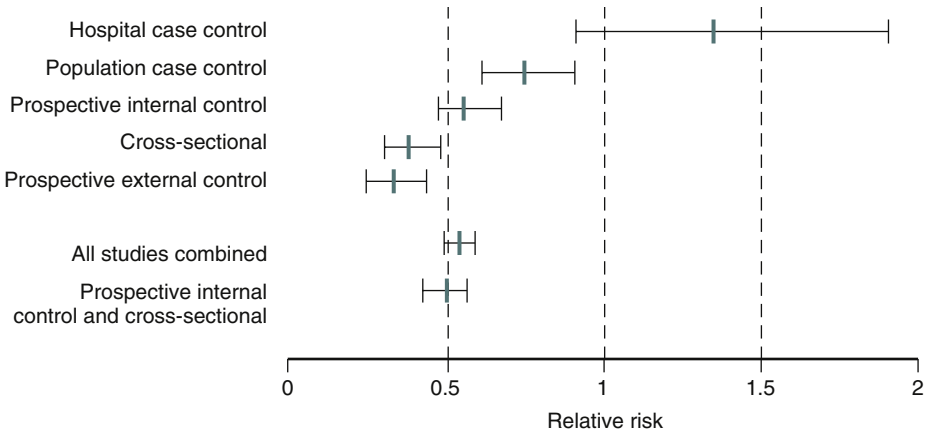


FIGURE 15.27 Estrogen replacement therapy and coronary heart disease. Relationship between relative risk and study type. *(Adapted from Stampfer MJ, Colditz GA. Estrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiologic evidence.* Prev Med *20:47–63, 1991.)*

coronary arteries ("early harm")[267,268] occurs within the first 1 to 2 years. The Women's Health Initiative (WHI) trial, which compared CEE/medroxyprogesterone acetate (MPA) with placebo, came to similar conclusions.[269] This trial was considered to be a primary prevention trial that evaluated younger women without cardiovascular risk factors; the higher frequency of cardiovascular events were reported in an older cohort of women (mean age 63). These women did not have vasomotor symptoms and had more risk factors than the healthy women studied in observational cohorts.

The protective effect of estrogen demonstrated in the Nurse's Health Study (Fig. 15.27)[265] occurred predominantly in young healthy symptomatic women. Table 15.4 compares the demographics of the participants of WHI and the Nurse's study. Trials carried out in the monkey model have shown that there is a 50% to 70% protective effect against coronary atherosclerosis when estrogen is begun at the time of oophorectomy, with or without an atherogenic diet; delaying the initiation of hormonal therapy for even 2 years (in the monkey) prevents this protective effect (Fig. 15.28).[270-274]

No clear explanation exists for what the observed "early harm" may be due to, but these effects were not observed in those women receiving statins concurrently.[275] This finding suggests that hormone therapy (in the doses used) may lead to plaque destabilization and thrombosis in some women with coronary disease. The molecular mechanisms for this may be due to estrogen up-regulating matrix metalloproteinase-9 within the mural area of the plaque; the resultant disruption of the gelatinous covering then leads to thrombosis. This process is inhibited by statins. In women who had been receiving estrogen for a prolonged time, mortality was decreased in those who sustained a myocardial infarction.[276] No increase in cardiovascular events has been found in young healthy symptomatic women during the first two years of various hormonal regimens in clinical trials.[277]

It is now well established that the late treatment of postmenopausal women with standard doses of estrogen may be harmful and affords no coronary protection. This finding pertains to various trials with end points of coronary events, or angiographically-determined disease, as noted above, and does not appear to be modified by the hormonal regimen or route of administration. This is also true whether or not the woman has sustained a known coronary event. Since atherosclerosis is highly age dependent, even women who have not had a coronary event have diseased arteries. (Fig. 15.29) Also, as shown in Figure 15.28, well over 70% of the women studied in WHI would have been expected to have atherosclerotic vessels.

Table 15.4 Baseline Characteristics: Nurses' Health Study (NHS) Compared with Women's Health Initiative (WHI)

Characteristic	NHS	WHI
Mean age or age range at enrollment (years)	30-55	63
Smokers (past and current)	55%	49.9%
BMI (mean)	25.1 kg/m^2	28.5 kg/m^2*
Aspirin users	43.9%	19.1%
HT regimen	Unopposed or sequential	Continuous-combined
Menopausal symptoms (flushing)	Predominant	Uncommon

*34.1% had BMI ≥30 kg/m^2.
BMI, body mass index; HT, hormone therapy.
Data from Colditz GA, Stampher MJ, Willet WC, et al. A prospective study of parental history of myocardial infarction and coronary heart disease in women. Am J Epidemiol 123:48-58, 1986; Grodstein F, Manson JE, Colditz GA, et al. A prospective, observational study of postmenopausal hormone therapy and primary prevention of cardiovascular disease. Ann Intern Med 133:933-941, 2001; Grodstein F, Stampfer MJ, Manson JE, et al. Postmenopausal estrogen and progestin use and the risk of cardiovascular disease. N Engl J Med 335:453-461, 1996; and Writing Group for the WHI Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women. JAMA 288:321-333, 2002.

In concert with the view that estrogen given to early (and younger) postmenopausal women may have different effects, data have been analyzed in the 50- to 59-year-old age group in WHI, and in those less than 10 years from menopause. The definitions of menopause in WHI were not precise, and more women in the 50- to 59-year-old age group were older than 55 years old. The data are in strong contrast to the results of the entire group (two-thirds over 60 years old) In the estrogen only arm of WHI (hysterectomized women) receiving CEE 0.625 mg, the 50- to 59-year-old age group had reduced coronary event scores of borderline significance and a composite coronary score of statistical significance.[278] An analysis of 20 RCTs in younger women (which included WHI) showed a statistically significant benefit in a reduction of coronary events[279] and

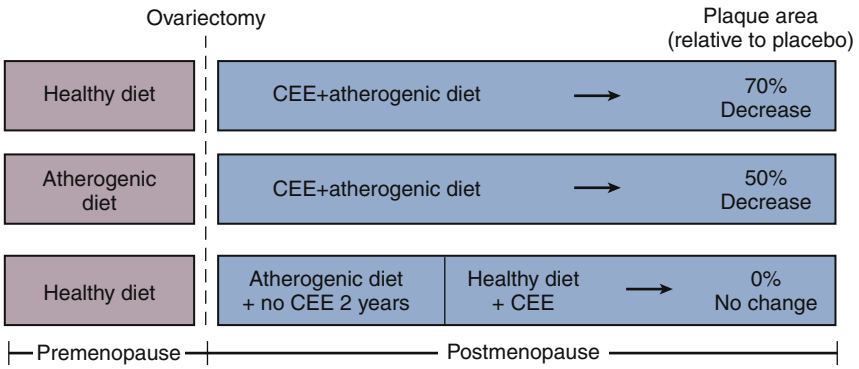
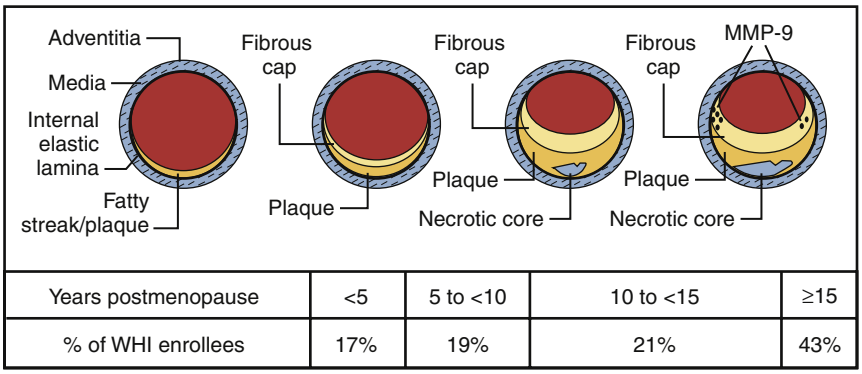


FIGURE 15.28 Importance of timing of intervention on the effect of estrogens on atherogenesis in nonhuman primates. CEE, conjugated equine estrogens. *(Adapted from Clarkson TB, Anthony MS, Jerome CP. Lack of effect of raloxifene on coronary artery atherosclerosis of postmenopausal monkeys. J Clin Endocrinol Metab 83:721-726, 1998; Adams MR, Register TC, Golden DL, et al. Medroxyprogesterone acetate antagonizes inhibitory effects of conjugated equine estrogens on coronary artery atherosclerosis. Arterioscler Thromb Vasc Biol 17:217-221, 1997; Clarkson TB, Anthony MS, Morgan TM. Inhibition of postmenopausal atherosclerosis progression: a comparison of the effects of conjugated equine estrogens and soy phytoestrogens. J Clin Endocrinol Metab 86:41-47, 2001; and Williams JK, Anthony MS, Honore EK, et al. Regression of atherosclerosis in female monkeys.* Arterioscler Thromb Vasc Biol *15:827–836, 1995.)*



Years postmenopause	<5	5 to <10	10 to <15	≥15
% of WHI enrollees	17%	19%	21%	43%

FIGURE 15.29 Relationship between number of years since menopause to progression of atherosclerosis in Women's Health Initiative (WHI) enrollees. MMP-9, matrix metalloproteinase-9. *(Adapted from Clarkson TB. The new conundrum: do estrogens have any cardiovascular benefits?* Int J Fertil *47:61–68, 2002.)*

mortality.[280] Younger women in WHI using estrogen only also had significantly reduced coronary calcium.[281] Ten-year follow up data of the estrogen alone arm published in 2011 confirmed that the 50- to 59-year-old group had a significant reduction in coronary heart disease (CHD) events and total mortality (Fig. 15.30).[281a]

While the data are not as strong for the use of combined HT (CEE/MPA) in WHI, it is difficult to conclude that MPA attenuates the benefit or may add harm in that the two trials in WHI were different, and studied different populations of women. The WHI conducted an observational trial in parallel with the RCTs. The results of the observational trials are more in keeping with the older observational data suggesting coronary protection and with no adverse effects on stroke.[282,283] These data could not be reconciled with the RCT data but reinforce the notion that early initiation of therapy and length of treatment influences these findings.[283]

The overall results of WHI for the 50- to 59-year-old age group in the two trials (CEE and CEE-MPA) were reanalyzed.[284] These data are more useful to assess in that this is the relevant population of symptomatic postmenopausal women who may be candidates for HT. For CHD, in the 50- to 59-year-old age group, the hazards ratio (HR) was 0.93 (0.65 to 1.33) for women receiving CEE & MPA; and was 0.63 (0.36 to 1.09) for CEE alone. For the entire group, total mortality was significantly decreased in this age group, 0.70 (0.51 to 0.96). These findings and point estimates are very much in line with the older observational data.

A Bayesian meta-analysis looking at randomized trials and observational data in younger women confirmed an approximate 30% reduction in mortality[284a] (Fig. 15.31), which is completely consistent with older data.

Although the "timing" hypothesis suggests that younger women receiving hormone therapy at the onset of menopause may have a reduction in cardiovascular disease, while older women may be subjected to harm, this has not been subjected to the rigors of randomized clinical trials until recently. Because cardiovascular events are rare in younger women, a very long trial would be required to demonstrate an effect. Therefore, in trials of approximately 4 to 5 years duration, only intermediate cardiovascular endpoints can be studied, such as carotid intima-media thickness and coronary calcium which may or may not relate to hard endpoints such as myocardia infarction and cardiovascular mortality. The Kronos Early Estrogen Prevention Study (KEEPS) and the Early versus Late Intervention Trial with Estradiol (ELITE) are two recently conducted trials on this point. In the 4-year KEEPS trial which compared CEE 0.45 mg to transdermal E_2, 50 μg and placebo, statistical benefit between the three arms in C-IMT was not seen although there were other beneficial effects of estrogen.[284b] There was a trend for a reduction in coronary calcium with estrogen (Fig. 15.32). It may be hypothesized that these younger healthy women at

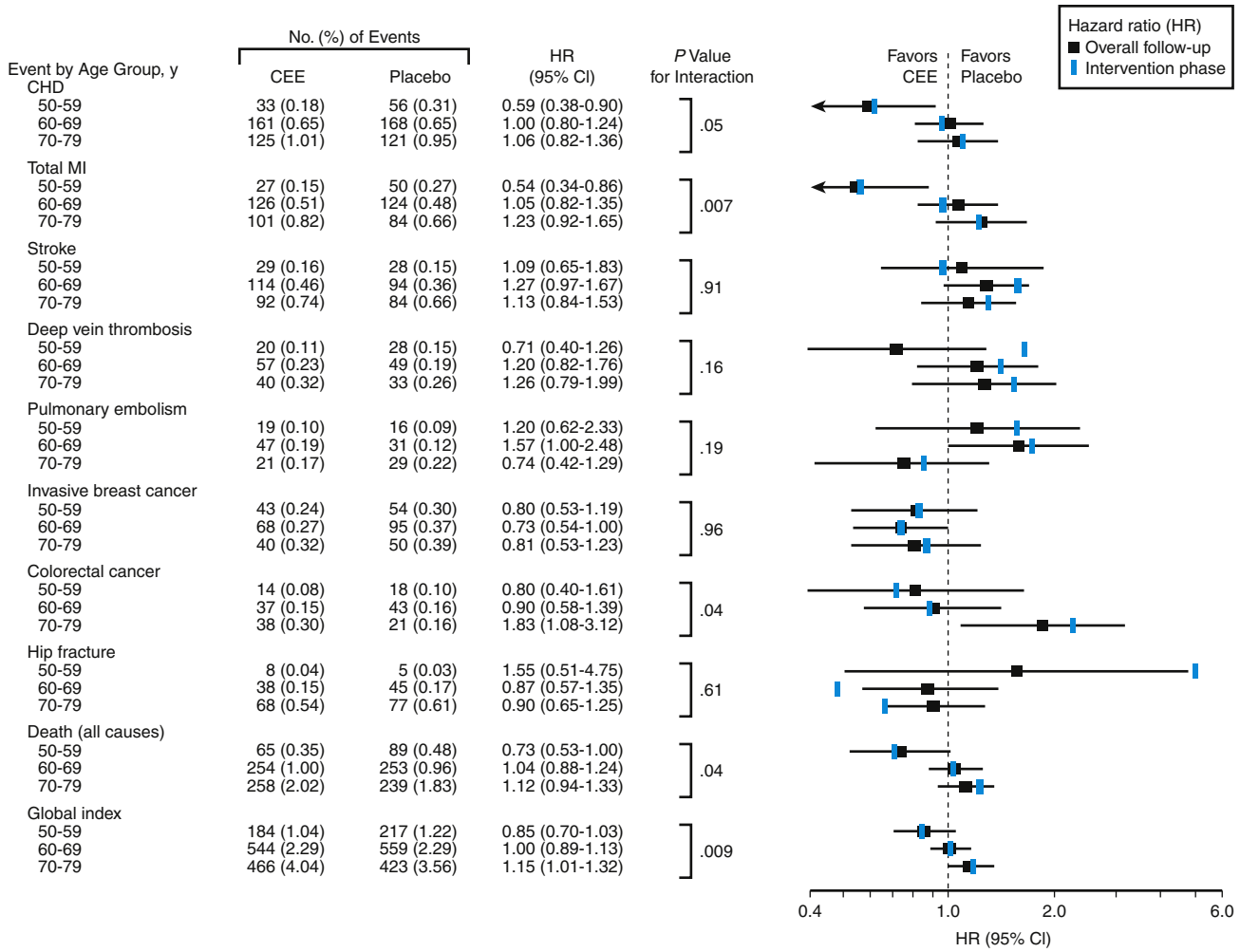


FIGURE 15.30 Ten-year follow-up of the estrogen alone arm in Women's Health Initiative (WHI) showing in the 50- to 59-year-old age group a significant reduction in CHD events and total mortality.

the onset of menopause did not have sufficient atherosclerosis progression over 4 years to differentiate placebo effect from estrogen. ELITE has not been analysed at the present time. However, a 10-year prospective trial in young Danish women treated at the onset of menopause and followed for 16 years recently showed a statistically significant reduction in cardiovascular events in users of oral estradiol, 2 mg, with and without norethindrone acetate[284c] (Fig. 15.33).

Stroke

Stroke is the third leading cause of death in women, and the interaction between hormones and stroke risk in women has been studied for some time.

In the original papers of WHI, overall stroke was significantly increased in women receiving CEE-MPA or CEE. However, the findings of increased stroke risk in younger women is more of a borderline nature and constitutes very few events. Age, obviously, and particularly hypertension, influences the risk of stroke in postmenopausal women; and this is significantly influenced by the dose and route of administration of estrogen.[285] It is ischemic stroke, and not hemorrhagic stroke which is influenced by the use of HT.

When large numbers of women are studied, both meta-analyses[285a] and observational studies[285b] have shown a small but significant increased risk of ischemic stroke with standard doses of hormone therapy. This is in the range of a 30% increase (relative risk ~1 to 3) because in younger postmenopausal women the risk of stroke is 6 to 8/10,000 women-years, the absolute risk is no more than 1 to 2 cases/10,000 women/year, a truly rare event. Our recent analysis suggests that those rare events in younger, postmenopausal women[285c] are related to thrombosis such as occurs rarely in women on oral contraceptives[285d] and is not due to atherosclerosis which occurs in older women. In concert with this, is the observation that less prothrombotic forms of hormone therapy (transdermal)[285a] and lower doses[285b] have not been found to increase the risk of ischemic stroke.

It has been acknowledged for some time that oral estrogen increases the risk of venous thrombosis and thromboembolism (VTE) in postmenopausal women. This risk tends to occur early (within the first 1 to 2 years of therapy) and is markedly increased if there is an underlying thrombophilia (about a fifteenfold increased risk).[286] However, in the absence of a family history or a prior thrombotic event, it is not standard practice to screen for factor V Leiden mutation or other abnormalities. Although the relative risk of thrombosis with oral estrogen is in the range of 2 to 3,

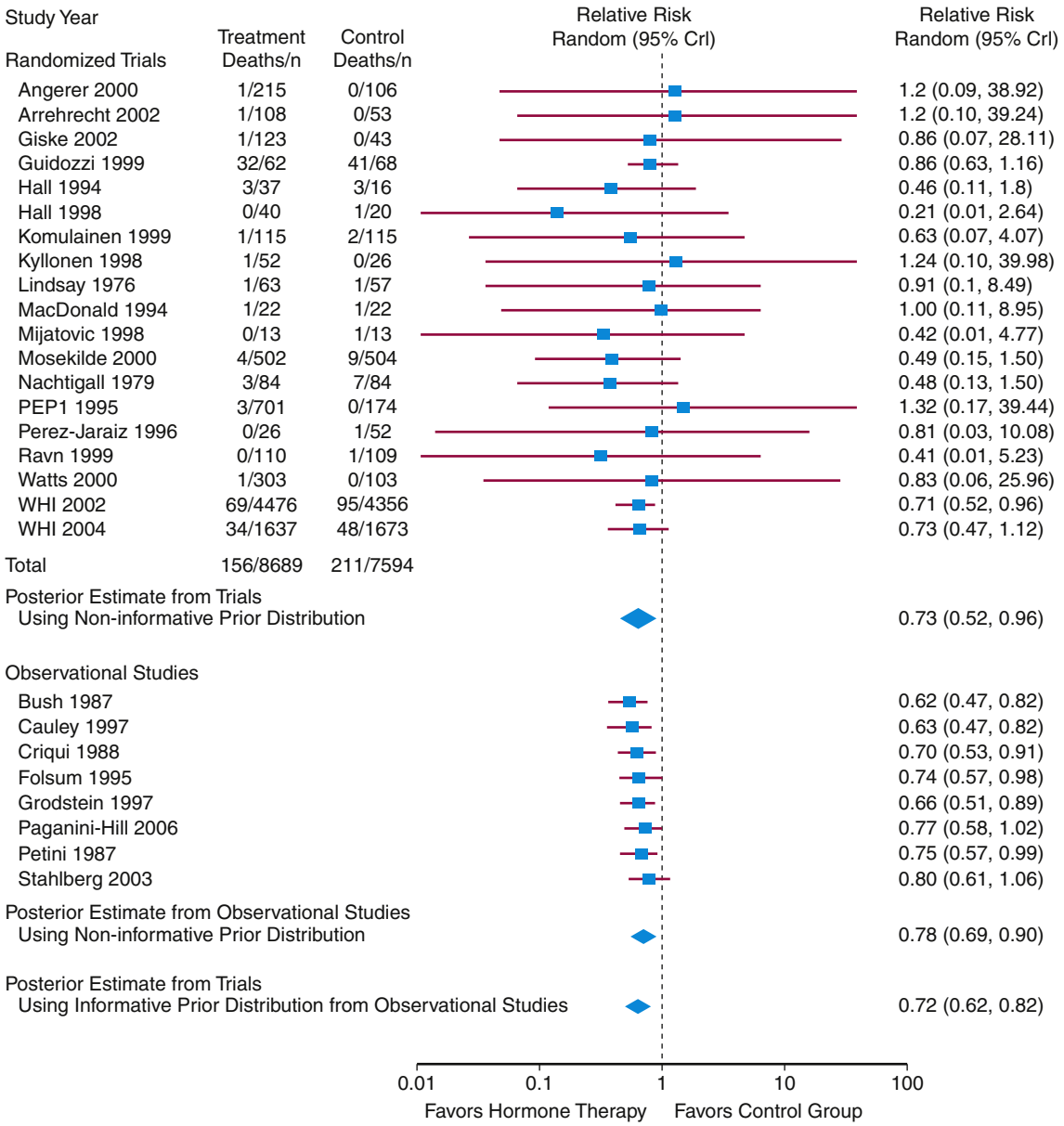


FIGURE 15.31 Bayesian meta-analysis of reduction in mortality with HT in younger women. *(Adapted from Salpeter SR. Bayesian meta-analysis of hormone therapy and mortality in younger postmenopausal women.* Am J Med *2009;122:1016–1022.)*

the prevalence of this is low, particularly in a younger population. For example, the risk of pulmonary embolism may increase from 20 to 40 cases per 100,000 women years with HT, but this is less than the general risk of VTE observed in pregnancy (60/100,000 women/year). The overall relative risk of VTE reported in WHI was in the range of 2 to 3 as noted above, but did not achieve statistical significance in the hysterectomized group using estrogen alone. Although this was a more obese group who could have been considered at higher risk, two other possibilities exist. The first is that approximately 50% of women had used estrogen in the past, and it is possible that this population of exposed women had received therapy beyond the first year or two when VTE usually occurs in susceptible women, and were not susceptible. Second, there is a possibility that the progestogen addition (MPA) with CEE increases the risk further. In a larger French observational study, the type of progestogen was considered to confer an additional risk of VTE with natural progesterone not enhancing the risk.[287] Further data from this cohort of women in the ESTHER study showed no increase in VTE risk with transdermal estrogen.[288] Several other recent studies have suggested the relative safety of transdermal over oral estrogen, even in high risk patients, unless higher doses (≥50 μg/day) are used[288a-c] (Fig. 15.34).

As noted earlier, blood pressure increases after menopause which increases CV risk. Apart from an idiosyncratic hypertensive response to oral estrogen in some women, the overall effect of estrogen on blood pressure is neutral, including women who are already hypertensive. There are studies showing some small increase, others showing no changes and others showing a decrease, even in hypertensive women.[289] Uncontrolled hypertension in postmenopausal women constitutes a major concern for stroke, and this may be aggravated in some women with the use of HT.

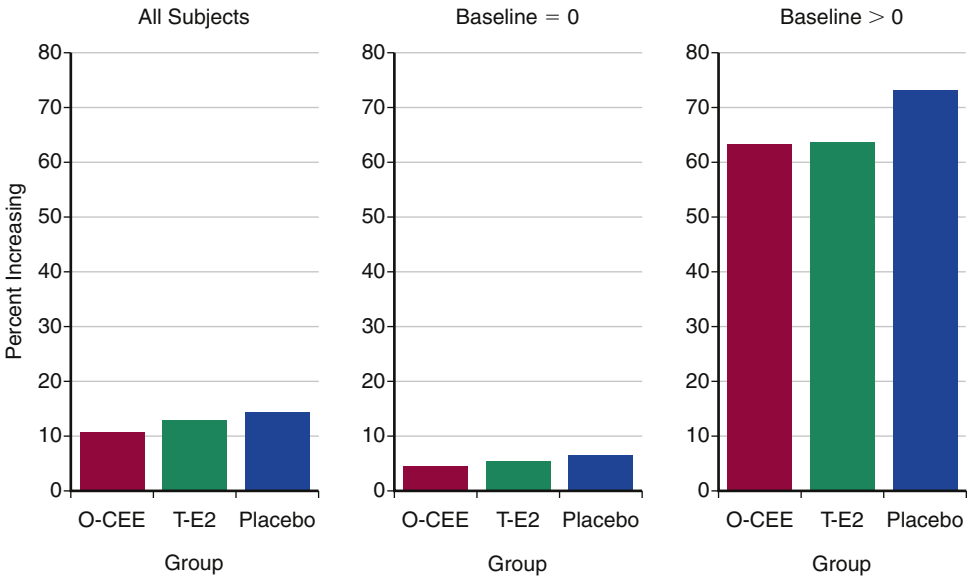


FIGURE 15.32 Data from the Kronos Early Estrogen Prevention Study (KEEPS) suggesting that there was less coronary calcium in women on HT (not statistically significant) and coronary calcium seen was very low. *(From Harman M: Primary findings of the KEEPS. 23rd annual meeting of the North American Menopause Society, Oct. 3, 2011, [submitted]).*

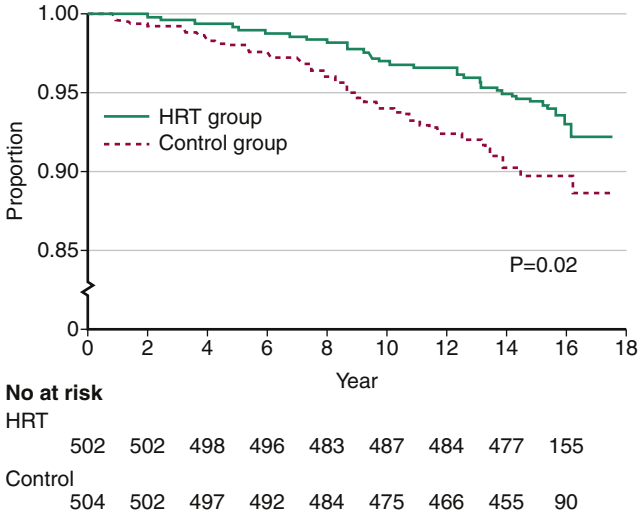


FIGURE 15.33 Sixteen-year follow-up of women randomized to hormone therapy (HT) showing a reduction in death, heart failure, and myocardial infarction (MI). *(Adapted from Schierbeck L. Effect of hormone replacement on cardiovascular events in recently postmenopausal women: randomized trial.* BMJ *345:e6409, 2012.)*

Breast Cancer

Because of the enormous importance of this topic, breast cancer and its relationship to HT use will be reviewed separately as well as in the context of the risk-benefit assessment discussed below.

Listed in Table 15.5 are the approximate rates of breast cancer by decade of life; clearly age is a major determinant of risk. More baseline risk information may be found in Chapter 11. However, postmenopausal breast cancer, fortunately, is not as lethal as lung cancer or CVD. In Figure 15.35 the age specific mortality rate is depicted, and shows that breast cancer mortality decreases after the initial age of menopause, while deaths from lung cancer are higher after menopause, and the rates of CVD mortality increase dramatically from this time onward. The effect of HT on breast cancer risk has been studied for at least 30 years, and only recently has there been a little more clarity on the issue.[290-293] Most of the data on breast cancer risk have been derived from case control and cohort observational studies. There have been several larger RCTs, such as data from WHI (at least for the use of two specific regimens)[291,292] and these will be presented separately as well.

In summary, RCTs show lower point estimates than observational data and little increased risk of estrogen alone.[292,293] With estrogen alone, the WHI showed reduced risk[292] and others, including the Million Women's Study that showed some increased risk.[294] This latter study, although large, has been widely criticized on methodological grounds. An update of the Nurses Health Study (NHS) showed no increasing risk for up to 20 years in hysterectomied women using CEE 0.625 mg.[293] As shown in Table 15.6, the overall risk going out 15 to 20 years is principally in lean women. Since obesity itself is associated with an increased risk of breast cancer, an additional risk with HT has not been demonstrated.

It has been known for some time, based on autopsy studies, that there is an "occult" or undiagnosed breast cancer rate of approximately 15.6%. These tumors have a doubling of time of 70 to 350 days leading to possible detection by mammography when they reach 0.88 to 1.66 cm in diameter. It has been estimated, based on iterative analysis and modeling that of the tumors that emerged in the E + P arm of WHI, 93.3% were occult lesions that grew and 6.7% arose *de novo*. Therefore the promotional effect of E + P shortened the doubling time from an average of 200 days to 150 days, allowing for early detection by mammography towards the end of the trial.[294a] With estrogen alone therapy in WHI the reverse was suggested with apoptosis occurring in some of the tumors, arresting the doubling time with estrogen causing a lower number of tumors to be detected compared to placebo.[294a]

In the CEE alone trial of WHI, there was a borderline significant reduction in breast cancer risk, which was statistically significant for adherent women and for ductal cancers.[292] These data are reassuring for a negligible effect of estrogen alone on breast cancer risk, but should

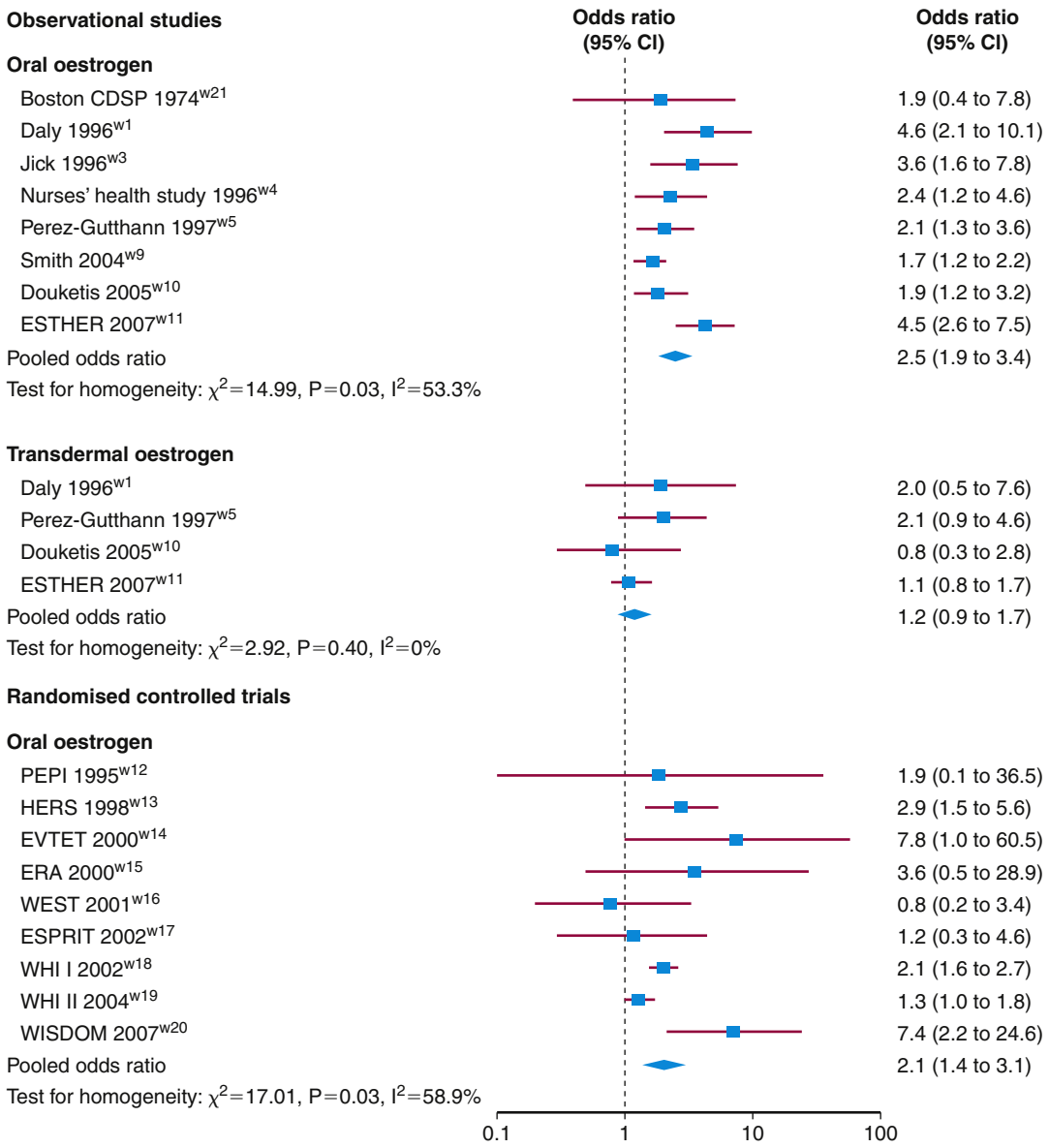


FIGURE 15.34 Meta-analysis of various studies showing no increased risk of thrombosis with transdermal therapy. *(Adapted from Canonico M, et al. Hormone replacement therapy and risk of venous thromboembolism in postmenopausal women: systemic review and meta-analysis.* BMJ *336:1227–1231, 2008.)*

Table 15.5 Women Who Will Develop Breast Cancer: Risk According to Age

Decade of Life	Incidence
Third	1 of 250
Fourth	1 of 77
Fifth	1 of 42
Sixth	1 of 36
Seventh	1 of 34
Eighth	1 of 45

Adapted from Lobo RA: Treatment of the postmenopausal woman: where we are today. *In* Lobo RA (ed). Treatment of the Postmenopausal Woman: Basic and Clinical Aspects, 2nd ed. Philadelphia, Lippincott Williams & Wilkins, 1999, pp 655-659.

not be interpreted as estrogen being protective for breast cancer.

There seems to be consistent findings of increased risk with estrogen and progestogen regimens after 5 years of use. This risk estimate ranges from 1.2 to 1.7 and is related to dose, possibly the type of progestogen[295] and duration of use. However, there are no clear data showing a difference between continuous combined and sequential regimens of progestogen therapy. In a less well known publication from WHI, when adjustments were made for breast cancer risk factors, the overall risk for the 5 to 6 years of CEE and MPA use was not significant at 1.20 (0.94 to 1.53).[291] Of great importance was the finding that in women who had never received HT (70% of the WHI population), the relative risk (RR) was 1.02 (0.77 to 1.36)[291]; that is, the trend for an increased risk was accounted for by prior use and a longer cumulative exposure to estrogen and progestogen.[295] There also has been a suggestion that estrogen and progestogen

therapy increases the relative frequency of lobular cancers which are normally more rare, but generally more well differentiated and less aggressive. This, however, has not been proven.

In a follow-up of women in the WHI estrogen-only arm, over 10 years, the significant reduction in breast cancer incidence was confirmed.[295a] Moreover, in terms of breast cancer mortality, this was significantly lower among the users of estrogen as has been suggested before. This study also confirmed a statistically significant reduction in total mortality in estrogen users (Fig. 15.36).[295a]

If standard dose estrogen and progestogen therapy over 5 years increases the risk of breast cancer by 24%, (RR 1.24), what does this translate into in absolute terms? If the background risk between the ages of 50 to 60 is 2.8% (2.8 women out of 100 will be expected to get breast cancer over this 10 year time span), then the rate would increase to 3.4% (less than 1% increase). Clearly, there is probably no increased risk in women who have never had therapy for up to 5 years and, particularly if lower than the standard dose is used. Also, observational data from France have suggested that progesterone and dydrogesterone are not associated with an increased risk, as opposed to synthetic progestogens.[295] These data, however, remain to be confirmed.

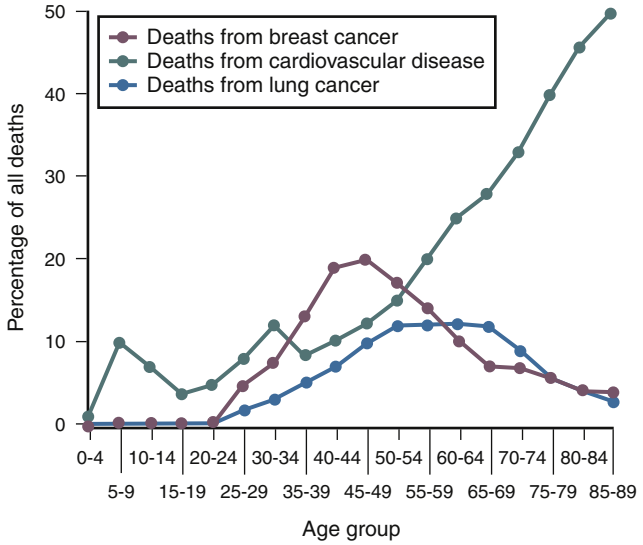


FIGURE 15.35 Risks of breast cancer and lung cancer versus cardiovascular disease in various age categories. *(Adapted from Phillips KA, Glendon G, Knight JA. Putting the risk of breast cancer in perspective.* N Engl J Med *340:141–144, 1999.)*

This RR of 1.2 to 1.6 for 5 years of estrogen and progestogen use as discussed above should also be compared to other experiences such as having a waist to hip ratio greater than 0.8: 3.3 (1.1 to 10.4) or being an airline flight attendant 1.87 (1.15 to 2.23). There are many more every day experiences such as these for breast cancer risk.[295b]

Compared to the small increase in breast cancer observed with standard dose E + P in WHI, other exposures are much higher[295c] (Fig. 15.37). The highest relative risk is seen in women with increased breast density, which also makes mammographic detection of small tumors challenging. While E + P treatment increases breast density, it is unclear if this imparts an additional risk for cancer; clearly these induced changes in breast density are reversible.

A report showing a downward trend in breast cancer rates in the U.S. suggested that this might be related to a reduction in hormone use after the WHI and other reports.[296] However, there are several other possible explanations as well, including a reduction in mammography use and other variables reviewed elsewhere. Of interest is that the reduction in breast cancer in the U.S. also occurred in older women in their seventh decade as well (not users of hormones) and has not been reported in many other countries[295c] where HT use has dropped as well. A 3-year follow-up of WHI showed that the breast cancer risk also persisted (nonsignificantly) over this follow-up period.[297]

Table 15.6 Risk of Invasive Breast Cancer by Duration of ET Use Among All Postmenopausal Women Who Had Undergone Hysterectomy and Those With ER+/PR+ Cancers Only*

	All Postmenopausal Women Who Had Undergone Hysterectomy				ER+/PR+ Cancers Only			
	All		Screened Cohort†		All		Screened Cohort†	
ET Use and Duration (yr)	Cases	Risk	Cases	Risk	Cases	Risk	Cases	Risk
Never current	226	1.00	104	—	87	—	48	—
<5	99	0.96 (0.75-1.22)	59	1.06 (0.76-1.47)	38	1.00 (0.67-1.49)	26	1.04 (0.64-1.70)
5-9.9	145	0.90 (0.73-1.12)	95	0.91 (0.68-1.21)	70	1.19 (0.86-1.66)	50	1.08 (0.72-1.62)
10-14.9	190	1.06 (0.87-1.30)	141	1.11 (0.85-1.44)	85	1.27 (0.93-1.73)	77	1.29 (0.89-1.86)
15-19.9	129	1.18 (0.95-1.48)	95	1.19 (0.89-1.58)	61	1.48 (1.05-2.07)	58	1.50 (1.02-2.21)
≥20	145	1.42 (1.13-1.77)	127	1.58 (1.20-2.07)	69	1.73 (1.24-2.43)	74	1.83 (1.25-2.68)
P for trend for current use	—	<0.001	—	<0.001	—	<0.001	—	<0.001

*All cases are reported as number of cases; risks are reported as multivariate relative risk (95% CI), controlled for age (continuous), age at menopause (continuous), age at menarche (continuous), BMI (quintiles), history of benign breast disease (yes or no), family history of breast cancer in first-degree relative (yes or no), average daily alcohol comsumption (0, 0.5-5, 5-10, 10-20, or ≥20 g/day), parity/age at first birth (nulliparous; 1-2 children and age at first birth ≤22 years; ≥3 children and age at first birth 23-25 years; ≥3 children and age at first birth >25 years).

†Screened cohort defined as those women starting in 1988 who reported either a screening mammogram or clinical breast examination in the previous 2 years. All cases before 1988 are excluded.

BMI, body mass index; CI, confidence interval; ER+/PR+, positive for both esgtrogen and progesterone receptors; ET, estrogen therapy, unopposed.
From Chen WY, Manson JE, Hankinson SE, et al. Unopposed estrogen therapy and the risk of invasive breast cancer. *Arch Intern Med* 166:1027–1032, 2006.

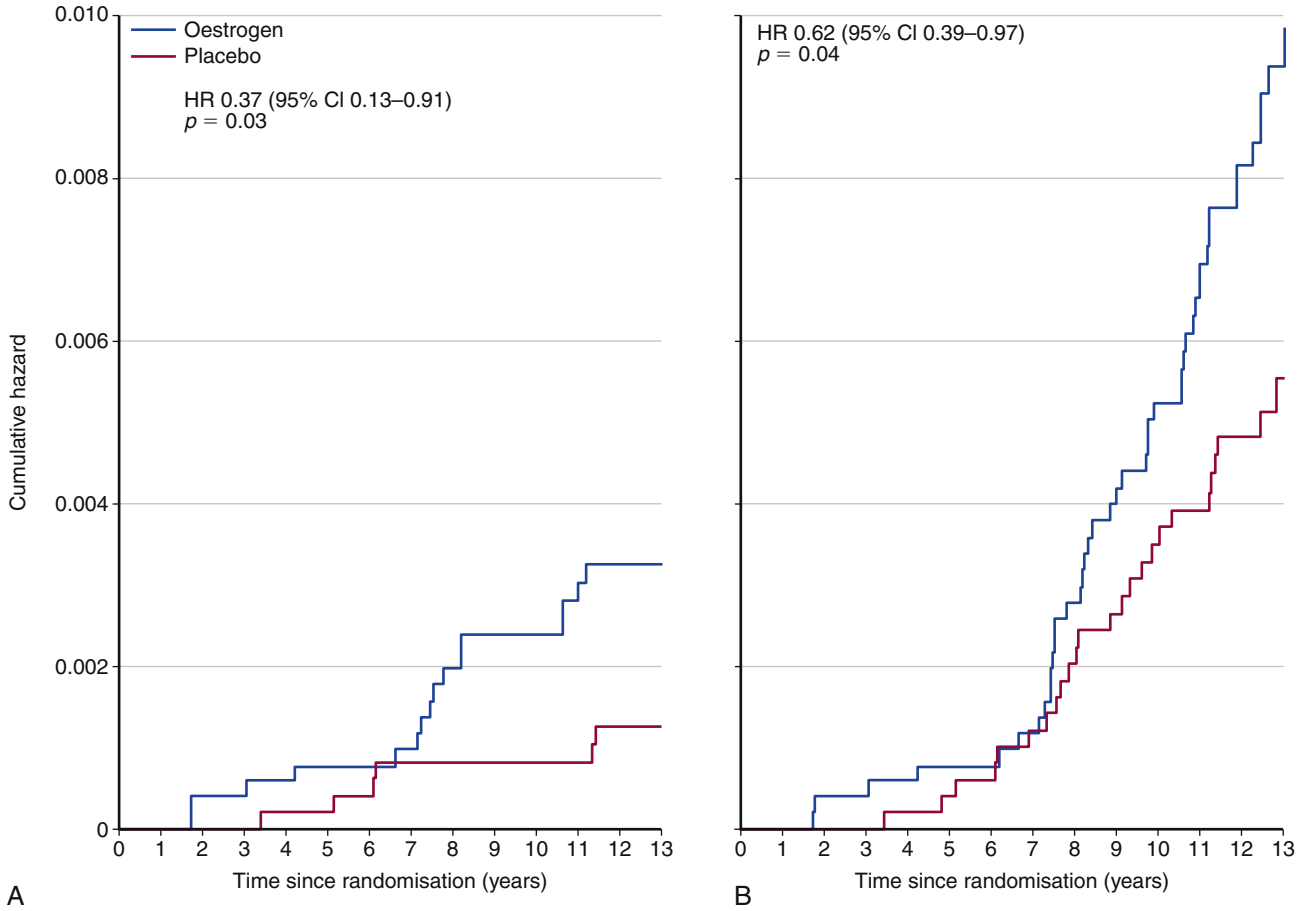
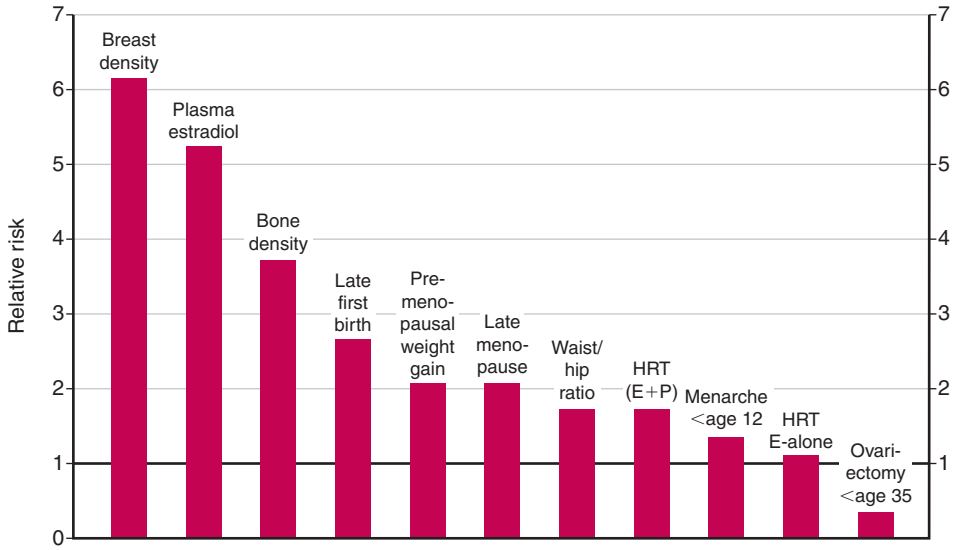


FIGURE 15.36 In women with breast cancer, women taking E2 had significantly reduced Mortality: Breast Cancer **(A)** and Total **(B)**. *(Adapted from Anderson G, et al. Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow-up of the Women's Health Initiative randomized placebo-controlled trial.* Lancet Oncol *13:476–486, 2012.)*



Hormonal factors and the relative risk of breast cancer.[52]
HRT - Hormone Replacement Therapy; E - Estrogen; P - Progestogen

FIGURE 15.37 Risks of breast cancer with various exposures and endogenous traits, particularly increased breast density. *(Adapted from Gompel A, Santen RJ. Hormone therapy and breast cancer risk 10 years after the WHI.* Climacteric *15:241–249, 2012.)*

The Decision to Use Estrogen

Whether or not hormonal therapy should be considered is a very individual decision. The woman must take into account symptoms, risk factors, and individual preferences and needs. The predominant indication for estrogen is for symptoms (vasomotor and vulvo-vaginal).

Alternatives should also be considered. If hormonal therapy is chosen, there should be flexibility in prescribing because there is no ideal regimen for every woman, and each woman has individual risks and needs.

Because hormone therapy is clearly the most efficacious treatment for symptoms, it should be considered a first line therapy. Since the results of WHI, which were misinterpreted and generalized to all women, caused most women and many providers to move away from hormone therapy, it was deemed important for several prominent medical societies to come together and make a definitive statement 10 years after WHI was first published. This statement of agreement amongst "experts" caring for women clearly states that estrogen is appropriate for younger, healthy women at the onset of menopause[297a]; in most cases the benefits far outweigh the risks. Since the original release of the findings of WHI, over 10 years later the following comment from a review seems most appropriate: "We have come full circle in some ways, with mounting evidence supporting the benefit of hormone replacement therapy started near menopause and with hard lessons learned about pathophysiology, publicity, and interpreting data."[297b]

Risk-Benefit Assessment

Several recent guidelines provide details regarding the use of HT for control of symptoms (International Menopause Society,[298] North American Menopause Society,[299] Medscape,[300] and American Association of Clinical Endocrinologists.[301] These guidelines stress the relative safety in symptomatic women, and that lower doses should be used with individualization regarding the length of treatment. Whether osteoporosis prevention alone is a sufficient indication for HT is controversial, but this should be considered as well.

At present there is no indication for using HT for the prevention of CVD or Alzheimer risk. Nevertheless, for both of these diseases there is the suggestion that early initiation of therapy (as was carried out in observational trials) may show protective effects.

Individualization of therapy is what is key in that there is no way to tally the risks and benefits of therapy in a global way. The "global index" used by WHI is an unvalidated instrument which gives equal weight to a number of parameters that are not expected to be equal in terms of risks for an individual woman. Further, data obtained to generate the global index for the trials (+ or – a certain number of events) included findings which were not statistically significant, making numbers of absolute risk inapplicable, although they were applied as such to create the composite score.

Changes in Mortality with Estrogen Use

In several cohort studies,[302-304] an overall 40% reduction in all-cause mortality has been observed with long-term estrogen use. Two studies have shown that the benefit in mortality is related to the duration of use; one study has suggested that the effect is decreased beyond 10 years of use because of an increase in breast cancer mortality (only reported in this cohort and not in the others).[304] Most studies have shown either no change in breast cancer mortality with estrogen use, or a decrease in mortality as was noted earlier from the updated data from WHI.[295a] In the re-analysis of data from WHI, in the 50- to 59-year-old age group, total mortality in the two trials was reduced significantly by 30% (HR 0.70 [0.51-0.96]) corroborating the findings of the earlier observational data. Figure 15.31 clearly points out the reduction in mortality in younger women combining randomized trials and observational data.[284a]

In these observational cohorts looking at all-cause mortality, the overall reduction was found to be attributable to a reduction in cardiovascular mortality, although there was a small effect in cancer mortality as well. It is important to note that the women in these observational (epidemiological) trials received estrogen therapy (ET) or estrogen/progestogen therapy (EPT) at the onset of menopause, and the women observed were healthier. In older women (10 or more years after menopause) who may have silent or established cardiovascular disease, there may not be a protective effect on mortality.

Several studies have also found hormone therapy to be a cost effective therapy for younger women; particularly looking at quality adjusted life years.[304]

Endometrial Disease Risk and Other Issues with HT

Among the risks that have been associated with estrogen are endometrial disease; breast cancer; side effects such as vaginal bleeding; somatic complaints; and idiosyncratic reactions, including hypertension. Cardiovascular events and thrombosis risk have been discussed earlier. All of the risks are, in part, dose-related, lending to the credence of using lower doses. Endometrial disease occurs with unopposed estrogen therapy in women who have a uterus. Although a woman's risk for endometrial cancer with unopposed estrogen use is twofold to eightfold higher than that for the general population, precursor lesions (primarily endometrial hyperplasia) signal the presence of an abnormality in most patients. Thus, the risk is far less for endometrial cancer than it is for varying degrees of hyperplasia.

One study showed that the risk of endometrial hyperplasia was 20% after 1 year of use of 0.625 mg of oral CEE.[305] In another study, the 3-year postmenopausal Estrogen/Progestin Interventions Trial,[306] the risk was approximately 40% at the end of 3 years. No cancers were reported in either of these two studies, and the addition of a progestin essentially eliminated the hyperplasia. Using a dose of 0.3 mg of CEE results in a risk in the range of 5% to 10% after 2 to 3 years. With the same dose of esterified estrogens (which is less potent), no hyperplasia was found after 2 years.[221]

The risk for endometrial cancer is the same for a woman taking estrogen and progestin EPT as for the general population. The addition of a progestin merely eliminates the excess risk attributed to estrogen. However, there are a few studies published suggesting a reduced risk with continuous

combined HT. Other endometrial cancers occurring in postmenopausal women are not thought to be hormonally related (see Chapter 29). Although the risk for endometrial cancer is increased substantially in estrogen users, the risk of death from this type of endometrial cancer does not increase proportionately.[307,308] Endometrial cancers associated with estrogen use are thought to be less aggressive than spontaneously occurring cancers, in part because tumors in women taking estrogen are more likely to be discovered and treated at an earlier stage—thus improving survival rates.

Several studies have also suggested an increased risk of ovarian cancer with long duration of use of ET and HT. However, the data are inconsistent and the purported risk is in the range of less than a twofold relative risk.[309,310] An analysis found no association, and there was no increase reported in the WHI studies.[311]

One of the concerns of women receiving estrogen is the return of menstrual bleeding. Somatic complaints such as breast tenderness and bloating may also occur with ET, but these can be alleviated by alterations in the dose and type of preparation. Such concerns should be addressed with a view of being flexible in prescribing, because there is no ideal product for any particular woman.

It has been estimated that the absolute increased risk for venous thromboembolic events is 15 per 100,000 women per year, which is categorized as an uncommon finding. Nevertheless, all patients must be informed of these findings. Women who have a family history of thrombosis or have had thrombotic events linked to oral contraceptives or any prior estrogen use should be counselled very carefully and monitored closely. A low-dose, nonoral form of estrogen would be a consideration for these patients; again, the need for choosing estrogen as a treatment should be clearly documented.

Approach to Therapy

In developed countries, much of what women know about healthcare is gleaned from the mass media.[312] In general, the more sensational an item is, the more noteworthy. For example, breast cancer risk is a real and serious concern for all women, but the fear of breast cancer seems to drive *all* decision-making, particularly regarding hormonal options.

American women believe that the leading cause of death in women is breast cancer and attribute only a small percentage of deaths to cardiovascular disease. In reality, the opposite is true. Statistics indicate that one in three women older than age 65 has some evidence of cardiovascular disease. Despite public perception, the overall incidence of breast cancer has remained constant in recent years. Nevertheless, what is not commonly appreciated by those healthcare professionals counselling patients is the age-associated relationship in the incidence of breast cancer.

The rationale for choosing estrogen at the onset of menopause for symptom control is expected to have a minimal effect on breast cancer incidence, particularly if the dose of estrogen is lowered and with a reduction in progestogen exposure in women with a uterus. Whether or not there is also some cardiovascular benefit in this setting cannot be determined with certainty at this time, but should not be used as an indication.

Table 15.7 Mean Serum Estradiol (E_2) and Estrone (E_1)

Estrogen Dose (mg)	Level (pg/mL) E_2	E_1
CEE (0.3)*	18	76
CEE (0.625)	39	153
CEE (1.25)	60	220
Micronized E_2 (1)	35	190
Micronized E_2 (2)	63	300
E_1 sulfate (0.625)	34	125
E_1 sulfate (1.25)	42	220

*Conjugated equine estrogen (CEE) contains biologically active estrogens other than E_2 and E_1.

Hormone Regimens

There are many hormonal preparations available for treatment, and the most comprehensive listing can be downloaded from the North American Menopause Society: www.menopause.org. Also medications for osteoporosis may be found, including the SERMS, bisphospohonatesdenosumab, tibolone, and human parathyroid hormone tariparatide.[1-34] For the clinician and patient, the decision to start estrogen therapy need not involve a long-term commitment. For short-term treatment of symptoms, estrogen should be used at the lowest dose that can control hot flushes or can be administered via the vaginal route for symptoms of dryness or dyspareunia.

Oral ET results in higher levels of E_1 than E_2; this is true for oral estradiol as well as estrone products. CEE is a mixture of at least 10 conjugated estrogens derived from equine pregnant urine. Estrone sulfate is the major component, but the biological activities of equilin, 17β-dihydroequilin, and several other B-ring unsaturated estrogens, including Δ dihydroestrone, have been documented. Table 15.7 compares the standard doses of the most frequently prescribed oral estrogens and the levels of E_1 and E_2 achieved.[313,314] Much of the clinical information contained below may be found in systematic reviews.[315]

Synthetic estrogens, given orally, are more potent than natural estradiol. Ethinyl estradiol is used in oral contraceptives, with a dose of 5 μg being equivalent to the standard ERT doses used (0.625 mg CEE or 1 mg micronized estradiol). Standard ERT doses are five or six times less than the amount of estrogen used in oral contraceptives.[316] For hepatic markers, CEE 0.625 mg is generally more potent than E_2 and is closer to 1.5 mg E_2.

Oral estrogens have a potent hepatic "first pass" effect that results in the loss of approximately 30% of their activity with a single passage after oral administration. However, this results in stimulation of hepatic proteins and enzymes. Some of these changes are not particularly beneficial (an increase in procoagulation factors), whereas other changes are beneficial (an increase in high density lipoprotein cholesterol [HDL-C] and a decrease in fibrinogen and plasminogen activator inhibitor-1).

E_2 can be administered in patches, gels, and subcutaneously. These routes of administration are not subject to major hepatic effects as with oral therapy. Standard doses in the United States of alcohol-based or matrix patches are 0.05 mg or 0.1 mg. Lower-dose patches of 0.025 mg are also

available for administration once or twice weekly. Matrix patches are preferable because there is less skin reaction and estrogen delivery is more reliable. Whereas levels of E_2 vary widely among women, levels with transdermal therapy are more constant in individual women than with oral ET. With the 0.05-mg patch, OK E_2 levels are in the 40 to 50-pg/mL range; with the 0.1-mg patch, levels are typically 70 to 100 pg/mL. Levels in excess of 200 pg/mL are not unusual for some women.[317] An ultralow dose patch, 0.014 mg has been approved for osteoporosis protection in older women.

In women with vulvovaginal or urinary complaints, vaginal therapy is most appropriate. Creams of estradiol or CEE are available, as well as tablets and an estrogen ring. With creams, systemic absorption occurs but with levels that are one fourth of that achieved after similar doses administered orally. Absorption decreases as the mucosa becomes more estrogenized. For CEE, only 0.5 g (0.3 mg) or less is necessary; for micronized E_2, doses as low as 0.25 mg are sufficient. Other products (tablets and rings) are available that have been designed to limit systemic absorption.[318,319] A silastic ring of E_2 is available that delivers E_2 to the vagina for 3 months with only minimal systemic absorption.[320]

Estrogen may be administered continuously (daily) or for 21 to 26 days each month. If the woman has a uterus, a progestogen should be added to the regimen.[321] For women who are totally intolerant of progestogens (regardless of the dose and route of administration) and take unopposed estrogen, annual endometrial sampling is necessary. Alternatively, different progestogens may be tried; micronized progesterone can be used vaginally, or an intrauterine system (IUS) can be used. The current IUS, releasing 20 μg levonorgestrel, is too large a dose for routine HRT and the 10 μg IUS, although well studied, will not be marketed as such.[322]

Colon Cancer and Other Changes

Observational studies have reported a reduced risk of colorectal cancer in menopausal women who are current and past users of EPT. This was confirmed in the WHI trial using EPT (HR 0.65). This protective effect is believed to increase with duration of use. With aging, there is a decline in macular function.[323] In observational studies, estrogen therapy has been reported to reduce macular degeneration and maintain visual acuity in menopausal women. There are also several studies showing that estrogen is associated with a reduction in tooth loss[324,325]

Androgen Therapy

In a very subtle way, some women are relatively androgen deficient.[326] Clinicians have proposed adding androgen to ET or EPT for complaints or problems relating to libido and energy, which are not relieved by adequate estrogen[327] though well-controlled trials using parenteral testosterone have shown benefit in younger oophorectomized women, there have been few data showing benefit using more physiological therapy, until recently. Recent data using a testosterone patch (with near physiological levels) have shown improvement in several scales of well-being and sexual function.[328-332] An oral preparation (esterified estrogens 0.625 mg with 1.25 mg of methyl testosterone) was shown to improve sexual motivation and enjoyment in women with hypoactive sexual desire who were unresponsive to estrogen alone.[333] The latter findings correlated with an increase in circulating unbound testosterone levels. As newer forms and doses of androgen become available, perhaps more women may benefit from this approach. At present, androgen therapy should be individualized and considered for those women who have symptoms that are not adequately relieved with traditional ET or EPT.[327] At lower doses, androgenizing side effects are very infrequent but should be discussed before prescribing testosterone. At present, small doses of methyltestosterone (1.25 and 2.5 mg) added to esterified estrogens are available in tablets, as are testosterone patches that are available for men (and therefore require dose reductions) and testosterone subcutaneous pellets. The testosterone patch (300 μg) has not been approved for use in women in the U.S. but is available in Europe. Administration of dehydroepiandrosterone at 25 to 50 mg/day may also be an option.[334]

Another SERM-like compound that is used worldwide but is not yet approved in the United States is tibolone. This progestin-like compound exhibits estrogenic, antiestrogenic, and androgenic effects by virtue of its structure and metabolites. At 2.5 mg, tibolone suppresses hot flushes, prevents osteoporosis, and has a positive effect on mood and sexual function.[335] There is also very limited (or no) uterine stimulation. However, there is a suppression of HDL-C, but at the same time a decrease in triglycerides. In the monkey, there was no deleterious effect of tibolone on coronary arteries.[336] In older women it may increase the risk of stroke.

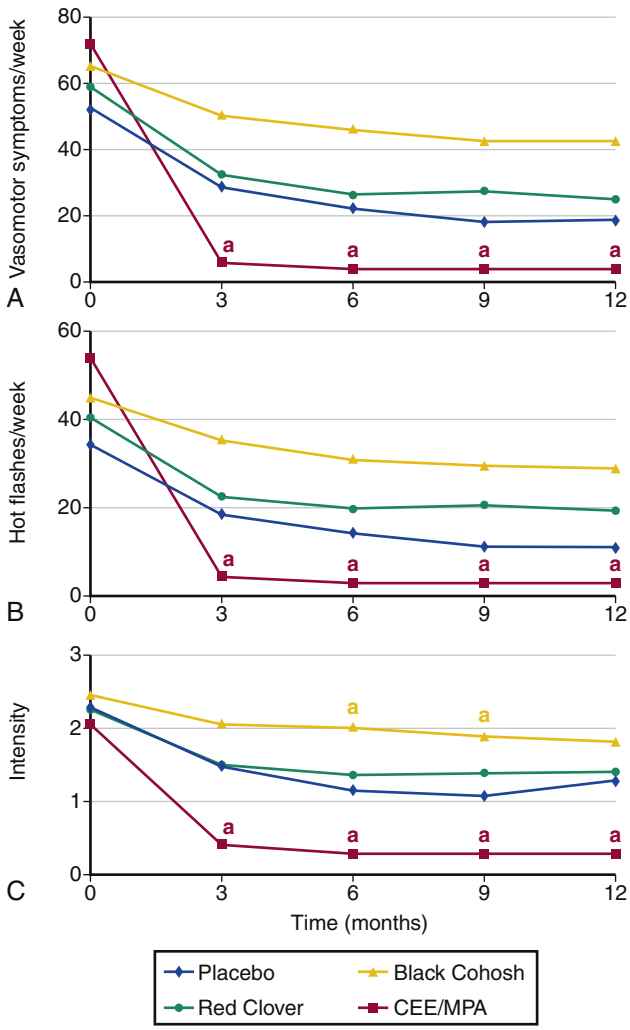
Alternative Therapies for Menopause

Phytoestrogens

Phytoestrogens are a class of plant-derived estrogen-like compounds conjugated to glycoside moieties. Phytoestrogens are not biologically active in their native forms unless taken orally. After oral ingestion, colonic bacteria cleave the glycosides, producing active compounds that are subject to the enterohepatic circulation. These compounds can produce estrogen-agonistic effects in some tissues, whereas in other tissues they can produce antagonistic effects.

Few randomized trials have examined the efficacy of phytoestrogens. For large daily doses (60 mg isoflavone) there appears to be some limited efficacy in relieving hot flushes.[337] With doses of 30 to 40 mg, cholesterol levels are reduced, but it is no longer recommended as a strategy.[338] It should be noted that there is an important reduction in hot flushes with *any* placebo treatment. Phytoestrogens do not appear to have much of an effect on bone loss or on vaginal atrophy. A recent trial of longer duration has shown no benefit of red clover and soy (Fig. 15.38).[338a]

Estimates are that between 30% and 60% of women use so-called alternative interventions for the symptoms of menopause, including "natural" estrogens, plant estrogens, herbal medicines, and acupuncture. Botanicals, herbals, and many steroid products are sold over the counter, and some do in fact exert significant hormonal activity. The use of botanicals to alleviate the symptoms of menopause is extremely popular. This popularity is fostered by the notion that plant sterols might provide all the benefits of estrogen



CEE - Conjugated Equine Estrogens
MPA - Medroxyprogesterone Acetate
a - Statistically significant difference

FIGURE 15.38 Black Cohosh and Red Clover are not different from Placebo. *(Adapted from Geller SE. Safety and efficacy of black cohosh and red clover for the management of vasomotor symptoms: a randomized controlled trial.* Menopause *16:1156–1166, 2009.)*

replacement therapy without the risks. However, most plant products recommended for menopause have performed poorly in clinical trials. The Dietary Supplement Health and Education Act of 1994 classifies most botanical medicines as food supplements and removes them from regulatory oversight and scrutiny by the U.S. Food and Drug Administration (FDA). Adulteration, contamination, and poor quality control in their harvesting, manufacture, and formulation yield products of questionable efficacy and safety.

The FDA has determined that more than 25% of Chinese patent medicines are adulterated with hidden pharmaceutical drugs. These kinds of deficiencies make it difficult for consumers and practitioners to employ botanicals with confidence and security. Furthermore, clinical trial data obtained using one brand of herbal product cannot necessarily be extrapolated to other brands using the same plant. DHEA is marketed as a dietary supplement for a variety of purported benefits. There are no data in women to support its role in well-being or immune function. As an androgen, DHEA is converted to androstenedione and testosterone. Doses of 25 to 50 mg raised testosterone and has been mentioned as an option for androgen therapy.[334]

In recent years, more women have turned to "bioidentical" hormones. In essence, estradiol and progesterone, which are available as FDA-approved products, are "bioidentical"—that is, identical to the steroids produced by the body. However, an industry has arisen where compounding pharmacies add various combinations of steroids to be administered in cream form. These products are not FDA-approved and quality control cannot be guaranteed. There is no evidence that these preparations are safer than standard products. Use of saliva to quantify levels is extremely inaccurate. The major societies [e.g., North American Menopause Society (NAMS), Endocrine Society, American Society for Reproductive Medicine (ASRM), American Congress of Obstetricians and Gynecologists (ACOG)] do not endorse this use. A joint statement has recently been issued by ASRM and ACOG.[339]

Use of a Progestogen

There are many ways to administer progestogens. The most commonly used oral progestins are MPA in doses of 5 to 10 mg, norethindrone (NET) in doses of 0.3 to 1 mg, and micronized progesterone in doses of 100 to 300 mg. Equivalent doses to prevent hyperplasia when administered for at least 10 days in a woman receiving ET (equivalent to 0.625 mg GEE) are as follows: MPA, 5 mg; NET, 0.35 mg; and micronized progesterone, 200 mg.[340] Larger doses of estrogen may require larger doses and more prolonged regimens of progestins. In sequential administration of progestins, the number of days (length of exposure) is more important than the dose. Thus, if a woman is receiving oral ERT continuously, a regimen of at least 10 to 12 days of exposure is preferable to a seven-day regimen.

When progestogens are administered sequentially (10 to 14 days each month), withdrawal bleeding occurs in about 80% of women. Continuous administration of both estrogen and progestogen (continuous combined therapy) was developed to achieve amenorrhea. In the first 3 to 6 months, breakthrough bleeding and spotting is common. In some women on this regimen, amenorrhea is never completely achieved. The most common combinations in the United States are single tablets containing 0.625 mg CEE and 2.5 mg (or 5 mg) MPA, and the lower dose combination with 0.45 mg and 0.3 mg. (CEE) 5 μg of EE with 1 mg NET; 1 mg micronized E_2 with 0.5 mg NET, and 1 mg of E_2, with 0.5 mg of drosperinone. Patches with E_2 NET of levonorgesterone are also available. Currently, the only marketed sequential regimen is one that contains 0.625 mg CEE and 5 mg MPA, which is added for 14 days each cycle.

Equal efficacy to standard doses has been demonstrated for several low dose combinations in terms of reduction of hot flushes, maintenance of bone mass, metabolical profiles, and a reduction in the incidence of bleeding in the first year of treatment has been observed.

Progesterone administered vaginally (in low doses) avoids systemic effects and results in high concentrations of progesterone in the uterus. Intrauterine delivery of progestogens is ideal for targeting the uterus but is not approved in the United States.

Progestogens, particularly when taken orally, may lead to problems of continuance or compliance because of adverse effects, including mood alterations and bleeding. These have to be dealt with effectively, and they usually require more flexibility in prescribing habits. Most short-term clinical trials have demonstrated an attenuating effect of progestogens on cardiovascular end-points that are improved with estrogen; these effects include lipoprotein changes (an attenuation of the rise in HDL-C) and arterial and metabolical effects. A reduction in blood flow and some brain effects may also be found.

How progestogens influence cardiovascular risk is not clear at present. The observational studies, which showed a benefit for ET, did not find any diminution of this effect with EPT. Although there were differences in the effects of CEE with MPA and CEE alone in WHI, there were separate trials which are difficult to compare. The addition of progestogens probably is responsible for the observed increase in the risk of breast cancer in susceptible women. Progestogens should not be prescribed in women who have undergone hysterectomy.

Tissue Selective Estrogen Complex

The concept of a tissue selective estrogen complex (TSEC) is to pair estrogen (CEE) with a SERM (bazedoxifene) which has certain estrogen-antagonistic effects in certain organ systems (breast, uterus). Not all SERMS are successful in this pairing. The effects of bazedoxifene are less marked in the brain (affecting hot flush susceptibility) and is also agonistic in bone. Thus, a combination of CEE and bazedoxifene could be used to prevent hot flushes without the need for a progestogen while also having beneficial effects for bone and neutral effects on the breast. While not approved for use in the U.S. as yet, CEE 0.625 and 0.45 paired with bazedoxifene 40 mg have gone through successful phase 3 clinical trials demonstrating efficacy and safety.[340a,b]

Aging

It is extremely difficult to separate the effects of menopause from that of natural aging. Some of these problems have been reviewed in detail elsewhere, and the much needed research in this area has been outlined.[341] The key areas where it is important to dissect out aging effects from the effects of estrogen deficiency are in bone loss and osteoporosis, cardiovascular disease, cognitive decline, decline in sexual function, and depression. While premenopausal estrogen therapy may play a role in protecting against these entities, aging also has significant effects.

From a reproductive endocrine perspective, the hormonal systems that are most notably affected by aging are declines in the somatotropic axis, declines in adrenal steroids, and changes in cortisol secretion. Table 15.8 outlines the endocrine metabolical changes with aging.

The Somatotrophic Axis

In men and women, GH decreases with aging,[341] beginning around age 40[342,343]; this decrease may be responsible for some of the changes in body composition. A redifferentiated proportion of body fat increases by 100%. Concomitantly there is a 20% to 50% decrease in muscle mass and a 20% decrease in body fat. In women, declines in growth hormone-releasing hormone secretion and a loss of the priming effect of estrogen explain the decline in GH.[344-347]

The anabolical effects of replacing GH can result in stimulated muscle development and strength, a loss of fat, and an increase in bone mass.[348] Accordingly, GH releasing peptides have been studied for this purpose.[349,350]

Table 15.8 Endocrine-Metabolical Changes with Aging

Substance	Change
Luteinizing hormone	↑
Follicle-stimulating hormone	↑
Growth hormone	↓
Adrenocorticotropic hormone	→*
Prolactin	↓
Thyroid-stimulating hormone	→*
Estradiol	↓
Estrone	↓
Progesterone	↓
Triiodothyronine	→
Thyroxine	→*
Cortisol	↑
Dehydroepiandrosterone	↓
Dehydroepiandrosterone sulfate	↓
Testosterone	↓
Androstenedione	↓
Inhibin	↓
Melatonin	↓
Insulin-like growth factor-1	↓
Dopamine	↓
Norepinephrine	↑

*May be slightly increased or decreased in response to specific secretagogue (e.g., corticotropin-releasing hormone, thyrotropin-releasing hormone, somatostatin, growth hormone-releasing hormone, adrenocorticotropin) with age.

Metabolical Changes

With aging, weight gain and fat accumulation occurs, as stated above. These changes begin a few years before menopause and parallel increases in FSH[351] (Fig. 15.39). It has recently been confirmed in longitudinal studies that weight changes generally precede the hormonal changes, such as the decrease in SHBG.[351a] These changes are associated with increases in leptin. In early menopause, associated with normal BMI, adiponectin increases.[352] However, in late menopause, as with obesity, adiponectin is reduced increasing CV risk.[353] With aging, there is a reduction in pancreatic β cell secretion increasing the risk of DM. Adding to this risk, insulin resistance increases with age but is accelerated with weight gain and obesity.

The major concern with increasing adiposity and reduction in muscle mass (sarcopenic obesity) is the evolution of chronic diseases. Increasing immobility compounds the transition to metabolical syndrome and overt diabetes and cardiovascular disease. It has been shown in 18 randomized trials that the best way to combat sarcopenic obesity is by the combination of aerobic and strengthening exercise 2 to 3 days per week, combined with caloric restriction leading to a daily deficit of 750 Kcalories.[353a] Diet and exercise should be a mainstay for combating the aging process.[353a]

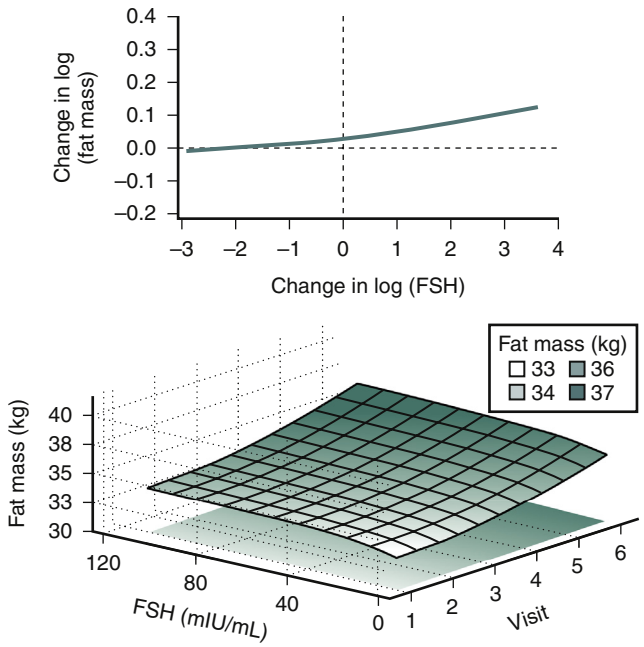


FIGURE 15.39 Changes in fat mass during the perimenopause and relationship to follicle-stimulating hormone (FSH). FMP, final menstrual period.

The increase in fat mass and decrease in lean muscle mass in postmenopausal women may be enhanced by estrogen deficiency. Indeed, hormone therapy does not increase fat mass and tends to decrease abdominal obesity.[353b,c] It appears that oral estrogen tends to slightly increase fat mass (not visceral fat mass) while transdermal estrogen does not affect fat mass.[353d] The effect of oral estrogen may be mediated by a decrease in IGF-1.[353e]

Adrenal Steroids

The age-related decline in adrenal androgens has been noted previously, as well as the possibility of administering DHEA for androgen support in postmenopausal women. While higher DHEAS levels in men are associated with a better cardiovascular survival, this correlation has not been found in women. Similarly, the data on enhancing insulin sensitivity and immune function, as well as quality of life assessments in postmenopausal women, are inconsistent,[354] requiring more study.

Because DHEA and DHEAS may be produced locally in the brain[355,356] and bind to the γ-aminobutyric (GABA) receptors,[357] there may be an important role for DHEA in remedying the mood and cognition declines that come with aging. Positive effects of DHEA on mood and cognition in the elderly have been reported.[358,359] Unfortunately, most clinical trials and DHEA in postmenopausal women have not shown benefit.

Cortisol levels are higher in older postmenopausal women.[360] This relates to a higher nocturnal rhythmicity which has been related to loss of resiliency and could explain sleep disturbances in the elderly.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 16

Male Reproductive Aging

Peter J. Snyder

As men age, reproductive function declines in several ways. Although the change is gradual, unlike the relatively abrupt decline that occurs in women at the time of menopause, it is progressive and probably has some adverse consequences. The decline is sometimes called *andropause*, *male menopause*, *late onset hypogonadism*, and other terms. This chapter reviews the changes in male reproductive function with aging and what is known about the consequences of attempting to prevent the changes by treatment with testosterone.

Changes in Male Reproductive Function with Age

Spermatogenesis and Semen Parameters

The little information available suggests that spermatogenesis does not decline severely with increasing age. Testicular volume, which reflects largely seminiferous tubular volume, averaged 20.6 mL, as determined by ultrasound, in 114 elderly men compared to 29.7 mL in 42 young men.[1] Sperm production was studied in an autopsy study of 89 men aged 21- to 50-years-old and 43 men aged 51- to 80-years-old who died suddenly. The older men had a daily sperm production rate per testis that was approximately 30% less than that in the younger men.[2] Ejaculated sperm were studied in 20 fathers aged 24- to 37-years-old and 22 grandfathers aged 60- to 88-years-old. Sperm density was somewhat higher in the older men, and percentage of sperm motility was somewhat lower in the older men, so that total number of motile sperm was similar in the two groups.[3] In 189 ambulatory men, the serum concentration of inhibin B, a reflection of Sertoli cell function, in men over 70-years-old was about 75% of that in men younger than 35-years-old ($P = 0.002$).[4] In the same study, the serum follicle-stimulating hormone (FSH) concentration was more than three times as high in the men over 70 years old than in the men under 35 years old ($P < 0.001$). From these data it appears that sperm production declines with age, but marginally.

Sex Steroid Hormone and Gonadotropin Concentrations

Serum Testosterone Concentration

The serum testosterone concentration has been found to decrease with age in both cross-sectional and longitudinal studies. The decrease appears to occur gradually beginning in the third decade of life. The magnitude of the decrease appears to be greater in longitudinal than cross-sectional studies, and greater in the free testosterone concentration than in the total. This decrease reflects a decrease in testicular secretion of testosterone, since in men testosterone is principally of testicular origin.

Several cross-sectional studies show a decrease in serum testosterone concentration with age. One cross-sectional study was performed on 302 healthy men in Belgium, in whom the serum testosterone concentrations decreased with increasing age.[5] The mean (± SD) total testosterone concentration in that study in the 70 men, 20- to 39-years-old, was 683 ± 289 ng/dL and that in 51 men, 70- to 79-years-old, was 428 ± 128 ng/dL. The calculated free testosterone concentration fell by a relatively greater amount; in 70- to 79-year-old men it was approximately half of that in 20- to 39-year-old men. In a study of 4263 men, 70- to over 85-years-old, total serum testosterone remained stable with age, but sex hormone binding globulin (SHBG) increased and free testosterone decreased.[6] In the European Male Aging Study of 3200 community dwelling, men ages 40- to 79-years-old, total serum testosterone was not associated with increasing age, but free testosterone was lower and luteinizing hormone (LH) higher (Fig. 16.1), suggesting that primary hypogonadism was the cause of the fall with age.[7] Comorbid conditions and obesity (Fig. 16.1) were also correlated with lower free testosterone concentrations but not higher LH, suggesting that these conditions cause secondary hypogonadism.

Longitudinal studies all show a decrease in testosterone with increasing age. In the Baltimore Longitudinal Study of Aging, the serum total testosterone concentration and a calculated free testosterone index fell from the third to the ninth decades in 890 men who were followed longitudinally.[8] By the eighth decade, approximately 30% of men were hypogonadal using the total testosterone concentration and 70% were hypogonadal by the free testosterone index. In the Massachusetts Male Aging Study, a population-based, random sample cohort of men aged 40 to 70 at baseline, 1156 men were followed for 7 to 10 years.[9] The serum concentration of total testosterone fell at a rate of 0.8% per year cross-sectionally, but 1.6% per year longitudinally.

The decrease in serum testosterone concentration with increasing age appears to be primarily the result of a decrease in the morning peak that younger men experience (Fig. 16.2).[10] When the serum testosterone concentration was measured once an hour for 24 hours in 17 men, aged 23- to 28-years-old, and in 12 men, aged 58- to 82-years-old, the

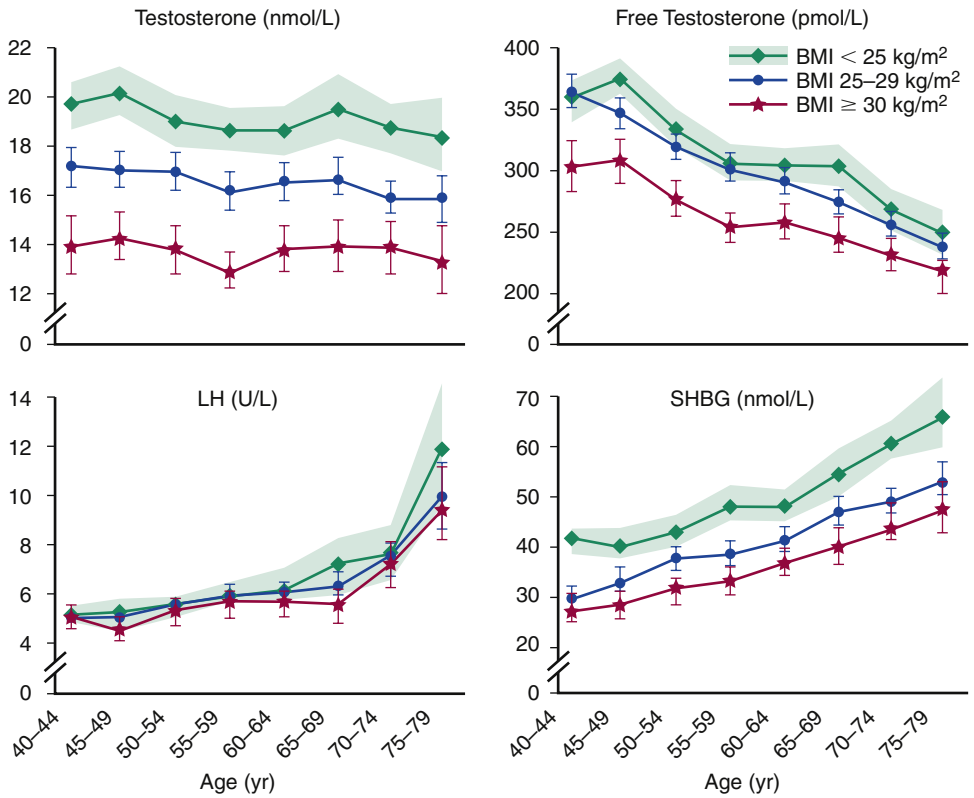


FIGURE 16.1 Serum testosterone, free testosterone, LH and SHBG concentrations as functions of aging in 3220 men in the European Male Aging Study. Free testosterone was significantly lower and LH and SHBG significantly higher in the older men. BMI ≥ 30 kg/m^2 was associated with significantly lower free testosterone and SHBG, but not higher LH. *(Adapted from Wu FCW, Tajar A, Pye SR, et al. Hypothalamic-pituitary-testicular axis disruptions in older men are differentially linked to age and modifiable risk factors: The European Male Aging Study.* J Clin Endocrinol Metab *93: 2737, 2008.)*

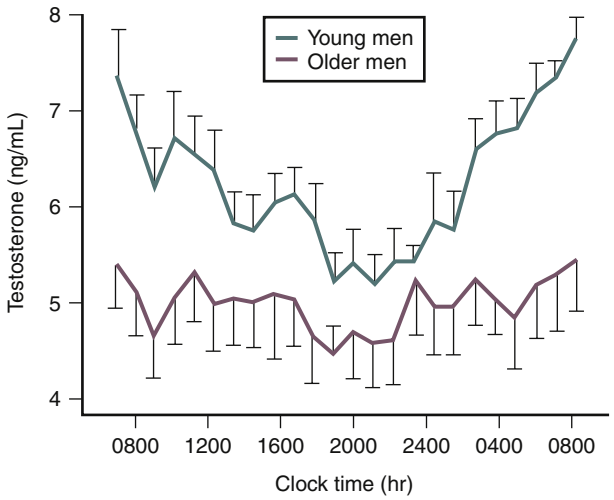


FIGURE 16.2 The pronounced diurnal variation in the serum testosterone concentration in young men but much less pronounced variation in elderly men. *(Adapted from Bremner WJ, Vitiello V, Prinz PN. Loss of circadian rhythmicity in blood testosterone levels with aging in normal men.* J Clin Endocrinol Metab *56:1278, 1983.)*

young men exhibited a clear diurnal variation: the peak at 8 AM and the nadir at 8 PM. The older men exhibited much less of a diurnal variation, so that the young men had a significantly higher serum testosterone concentration than the older men from 2 AM to 1 PM, but not from 2 PM to 1 AM. The decrease in serum testosterone concentration with increasing age also appears to be affected by other health factors. In the Massachusetts Male Aging Study, a decrease in body mass index of 4 to 5 kg/m^2 or a loss of a spouse resulted in a similar decrease in testosterone as 10 years of aging.[11]

Serum Estradiol Concentration

The serum total estradiol concentration does not appear to change with increasing age, but the serum bioavailable estradiol concentration, that not bound to SHBG, appears to decrease slightly. In a study of 810 men aged 24- to 90-years-old in Rancho Bernardo, California, the serum concentration of total estradiol decreased by only 0.3 pg/mL per year of age, but the concentration of bioavailable estradiol decreased by 0.12 pg/mL per year of age.[12] In Rochester, Minnesota, 130 men, aged 66- to 90-years-old, did not have a significantly different concentration of total estradiol than did 88 men, 22- to 39-years-old, but the older men had a significantly lower serum bioavailable estradiol concentration than did the younger men, 40 pmol/L compared to 59 pmol/L.[13] In a study of 206 men, 18- to 95-years-old in Parma, Italy, both the total and free serum concentrations of free estradiol fell with age.[14]

Serum Gonadotropin Concentration

Both cross-sectional and longitudinal studies show an increase in the serum concentrations of luteinizing hormone (LH) and FSH with increasing age. In 15 years of observation in the New Mexico Aging Process Study, the mean serum LH concentration increased from 9.4 mIU/mL to 13.7 mIU/mL and the FSH from 14.1 mIU/mL to 27.4 mIU/mL.[15] In the Massachusetts Male Aging Study, LH increased by 0.9% per year and FSH by 3.1% per year.[9] This increase in serum gonadotropins suggests that a degree of primary hypogonadism is responsible for the decline in testosterone, which is supported by the smaller testosterone response to human chorionic gonadotropin (hCG)[16] or recombinant human LH[17] stimulation of elderly men than young men. The increase in basal serum LH, however,

is not as great as one would expect for the magnitude of testosterone decline, suggesting secondary hypogonadism as well. In several aspects of LH secretion, the magnitude appears to be less in elderly men than in younger men. The LH response to a bolus dose of gonadotropin-releasing hormone (GnRH) is slightly less in older men than in younger men, and the peak response is somewhat delayed.[18] Spontaneous LH pulses are also different in elderly men compared to young men. LH secretory burst amplitude is less in elderly men than in young men.[19] The fall in testosterone with increasing age, therefore, appears to be the result of a combination of primary and secondary hypogonadism. As shown in the European Male Aging Study, age itself appears to result more in primary hypogonadism and comorbidity and obesity more in secondary hypogonadism.[7]

Consequences of Decreased Serum Testosterone Concentrations

The parallels between the consequences of frank hypogonadism due to known hypothalamic-pituitary or testicular disease and the consequences of normal aging in men suggest that the fall in testosterone with increasing age might contribute, at least in part, to the consequences of aging in men. These consequences include decreases in bone density and quality, muscle mass and strength, physical function, energy, and sexual function, and an increase in heart disease. Treatment of these deficiencies in frankly hypogonadal men often reverses those consequences.

Bone

The bone mineral density of men who are evaluated because of hypogonadism is lower than that of eugonadal men.[20,21] When men are made hypogonadal by GnRH agonist treatment for prostate cancer, their bone mineral density decreases. In a study of 152 men with prostate cancer—30 who had been kept hypogonadal for less than 6 months, 50 who had been kept hypogonadal for ≥6 months, and 72 who had prostate cancer but were eugonadal, as well as men who did not have prostate cancer—had areal bone mineral density (BMD) determinations on two occasions one year apart.[22] During this year, the men who had been kept hypogonadal for less than 6 months experienced a 4.0% decrease in BMD of the spine and 2.5% of the total hip, but the men who had been hypogonadal for ≥6 months and the eugonadal men did not experience a decrease. Men who are made hypogonadal to treat prostate cancer also are at greater risk for bone fracture, as shown in data from the Surveillance, Epidemiology, and End Results program and the Medicare database, in which the prevalence of fractures was determined in 50,613 men age >66 years with prostate cancer who had been made hypogonadal by a GnRH agonist or bilateral orchiectomy.[23] Men who had been made hypogonadal had a significantly higher prevalence of fracture (19.4%) than men not made hypogonadal (12.6%, $P < 0.001$). There was also significant relationship between the number of doses of GnRH agonists and the risk of fracture.

As men age, bone mineral density also decreases. In a study of normal men who had no history of hip fracture, bone mineral density of the spine and hip deceased linearly from age 20 to more than 80 years (Fig. 16.3); the decline

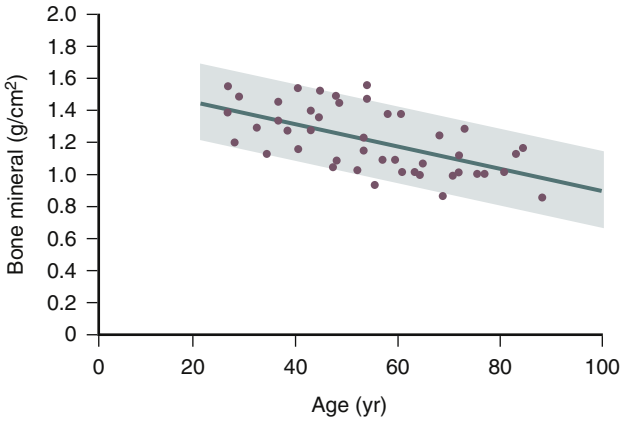


FIGURE 16.3 Decrease in bone mineral density of the intertrochanteric region of the proximal femur in 52 men with increasing age. *(From Riggs BL, Wahner HW, Seeman E, et al. Changes in bone mineral density of the proximal femur and spine with aging.* J Clin Invest *70:716, 1982.)*

was less than that in women,[24] similar to the findings of other cross-sectional studies.[25,26] However, a cross-sectional study in which bone density was measured by quantitated computed tomography (QCT) showed a greater rate of bone loss,[27] perhaps reflecting the greater sensitivity of QCT in detecting changes in trabecular bone than bone densitometry. A longitudinal study showed as rapid a loss of bone density in men as in women.[28] Within the 2908 men of an average age of 75.4 years in the Swedish Osteoporotic Fractures in Men (MrOS) study, serum free testosterone was a modest predictor of bone density and prevalent osteoporotic fractures.[29] In 728 men at two sites in the European Male Aging Study, increased age was associated decreased cortical BMD by peripheral QCT.[30] The association of the decrease in cortical bone with the serum concentrations of estradiol and testosterone was less clear.

Replacement of testosterone in unequivocally hypogonadal men greatly increases bone mineral density. Administration of 100 mg of testosterone enanthate for 18 months to 29 men with previously untreated hypogonadism resulted in a 5% increase in bone mineral density in the lumbar spine as determined by dual energy x-ray absorptiometry (DEXA).[21] Trabecular bone mineral density, as measured by quantitative computed tomography, increased by 14%. Administration of testosterone transdermally for 3 years to 18 hypogonadal men who had previously untreated hypogonadism resulted in increases in bone mineral density as determined by DEXA of 7.7% at the lumbar spine and 4.0% at the trochanter.[31]

Body Composition

When men become hypogonadal as the result of hypothalamic-pituitary or testicular disease, they experience a decrease in lean body mass and an increase in fat mass, and testosterone treatment of hypogonadal men usually reverses those changes. The same changes occur as men age. Whole-body lean mass in one study was about 15% less at age 70 than at age 20, and whole body fat mass was about 100% more.[32] In another study, appendicular skeletal muscle mass was 16% less in men over 75 years old than in men 18- to 34-years-old.[33] Percentage of body

fat was 26% in 36 hypogonadal men compared to 19% in 44 eugonadal men.[21] When six normal young men were made hypogonadal for 10 weeks by the administration of the GnRH agonist leuprolide, their fat-free mass decreased from 56.5 to 54.4 kg and their fat mass increased from 15.8 to 16.9 kg.[34]

Administration of testosterone reverses these changes in body composition. Administration of 100 mg of testosterone enanthate to previously untreated hypogonadal men resulted in a 14% decrease in total body fat and a 12% decrease in subcutaneous fat, as assessed by quantitated computed tomography.[21] Lean muscle mass, assessed by the same technique, increased 6.8%. When testosterone was administered transdermally to 18 previously untreated hypogonadal men, lean body mass increased 5.8%.[31]

Muscle Strength

Muscle strength has long been known to decrease with increasing age, but demonstration that hypogonadism leads to decreased muscle strength and that testosterone treatment improves strength have been much more difficult to demonstrate. In a study of 72 normal men in three age groups ages 20 to 86, strength of knee extension, both isometric and isokinetic, decreased with increasing age.[35] In another study in 114 men ages 11- to 70-years-old, quadriceps strength increased up to the third decade, was stable until age 50, then declined with increasing age.[36] The only clear demonstration that hypogonadal men have lower muscle strength than eugonadal men was provided by the study, described earlier in this chapter, in which six healthy young men were made severely hypogonadal for 10 weeks by administration of leuprolide. At the end of the 10 weeks, their strength of knee extension was 6% less than prior to treatment when measured isokinetically at 60 degrees angular velocity, but was not significantly different during treatment than before when measured isokinetically at 180 degrees angular velocity or isometrically at either angular velocity.[34]

Administration of testosterone transdermally to 18 previously untreated hypogonadal men did not increase their strength of knee extension or flexion or handgrip strength.[31] Administration of 100 mg testosterone enanthate once a week to men with wasting secondary to human immunedeficiency virus (HIV) infection resulted in a 16% increase in leg press strength compared to no significant change in those treated with placebo, whereas men who were treated with testosterone and resistance exercise did not exhibit a greater increase than those treated with placebo and exercise.[37] In short, it is clear that muscle strength decreases with increasing age, but it is not clear how much, if any, the decline in testosterone contributes to the decrease in muscle strength.

Physical Function

The relationship between testosterone and physical function in middle-aged to elderly men was evaluated in the Framingham Offspring Study, in which 1445 men, of average age 61 years, were evaluated twice, an average of 6.6 years apart. By logistical regression analysis, free testosterone, but not total, was associated with incident Short Performance Physical Battery score and usual walking speed but not with grip strength.[38] Free testosterone at the first visit was significantly associated with increased risk of self-reported mobility disability at the second visit.

Cardiovascular and Metabolical Risk

Because the prevalence of heart disease is greater in men than in women, the possible role of testosterone in the development of arteriosclerotic cardiovascular disease has long been discussed. An alternative explanation for the difference is higher estradiol concentrations in women before menopause. This possibility is suggested by the gradual decrease in the gap between the genders, the greater the length of time after menopause. A recent study, however, suggests that it may be low testosterone that is harmful. When 794 men of median age 73.6 years had a serum testosterone measurement and were followed for an average of 11.8 years, men whose serum testosterone concentrations were in the lowest quartile (<241 ng/dL) were 40% more likely to die of all causes and 38% more likely to die of cardiovascular causes[39] than men with higher values. This association was independent of age, adiposity, and lifestyle.

If low testosterone does cause increased mortality rate, one possible mechanism is via metabolical risk factors, because in epidemiological studies low testosterone precedes the development of central obesity, the metabolical syndrome, and diabetes.[40-42] However, in the study cited earlier in this chapter, the association between low testosterone and higher mortality rate persisted even after adjusting for metabolical syndrome and diabetes mellitus. Further, associations between testosterone and metabolical syndrome and diabetes are equivocal. In the Baltimore Longitudinal Study of Aging, the prevalence of the metabolical syndrome was associated with a lower total testosterone but a higher index of free testosterone.[43] Conversely, in an analysis of data from the Third National Health and Nutrition Survey (NHANES III), men in the lowest tertile of an index of free testosterone were four times as likely to have a diagnosis of diabetes mellitus than men in the highest tertile, but that relationship was not observed with total testosterone.[44] Perhaps the best evidence that testosterone influences glucose metabolism is a randomized, placebo-controlled crossover trial in which testosterone treatment of men for 3 months who had type II diabetes and low-normal to low serum testosterone concentrations improved their fasting glucose, hemoglobin A_1c, and homeostatic model assessment (HOMA) index, compared to placebo treatment.[45]

Other possible mechanisms by which testosterone could affect cardiovascular disease are via serum lipids, clotting factors, and markers of inflammation, but treatment of hypogonadal men with testosterone does not consistently change these parameters.[31,46-50]

Energy

Men's sense of energy decreases with increasing age, and when men who are hypogonadal due to known disease are treated with testosterone, their self-reported energy increases markedly. Administration of testosterone transdermally to 18 men with severe, untreated hypogonadism resulted in an increase in self-reported energy, reaching an apparent peak in about 3 months.[31]

Cognition

The association of low testosterone with declining cognitive function was evaluated in the Baltimore Longitudinal Study of Aging, in which 407 men ages 50- to 91-years-old at baseline assessment were followed for an average of 10 years. Men who were classified as hypogonadal, defined for this study as a free testosterone index (total testosterone/SHBG) below the 2.5 percentile, had significantly lower scores on measures of memory and visuospatial performance and a faster decline in verbal memory.[51]

Sexual Function

Low testosterone appears to be associated with decreased sexual function in middle aged and elderly men, as shown in data from the European Male Aging Study.[52] In a random population sample of 3369 men ages 40- to 79-years-old, the combination of three sexual symptoms (low sexual desire, poor morning erection, and erectile dysfunction) was significantly related to total and free testosterone concentrations; the relationship was stronger the lower testosterone. Hypogonadism also causes a decrease in libido. When men who were unequivocally hypogonadal were treated with a single dose of testosterone enanthate, sexual function increased much more within a few weeks after a dose of 100 mg than a dose of placebo, and even more after a dose of 400 mg than 100 mg.[53] When 18 severely hypogonadal men were treated with testosterone transdermally, sexual function increased markedly when first reassessed at 3 months but did not increase further when the observation was continued for a total of 3 years.[31]

Attempts to Reverse the Consequences of Aging by Testosterone Treatment

A few attempts have been made to administer testosterone to relatively small numbers of elderly men to raise their serum testosterone concentration to that of young men to determine if the changes of aging can be reversed. Improvements in some parameters occurred.

Testosterone Preparations Available for Treatment of Male Hypogonadism

Several preparations of testosterone are available for replacing testosterone in hypogonadal men (Table 16.1). Testosterone gels are the most commonly used preparations in countries where they are available, because they increase the serum testosterone concentration to within the normal range in most men, keep the concentration relatively steady within a 24-hour period and from day-to-day, and are easy to use.[54-57] A testosterone patch also keeps the serum testosterone relatively normal,[58] but about one-third of men cannot use it because of severe skin rash, and sometimes it falls off.

The enanthate[59] and cypionate esters of testosterone, when given by deep intramuscular injection every 1 to 2 weeks, are effective in treating hypogonadism, but produce wide fluctuations in the serum testosterone concentrations from supranormal the day after an injection, to low-normal to subnormal by the time of the next injection. Some patients notice these fluctuations in their energy, mood, and sexual interest. The undecanoate ester of testosterone, which is available in some countries, increases the serum testosterone concentration to normal when administered every 3 months.[60] This ester is also available in some countries for oral use, but that route requires ingestion with a fatty meal and results in serum concentrations that are variable and usually subnormal.[61] Several 17-α-alkylated androgens have been available for oral use for decades, but they may cause hepatotoxicity,[62,63] so they are not recommended. Testosterone pellets, which are available in some countries, must be implanted via trochar using a surgical incision and may extrude. The limited data suggest that the resulting serum testosterone concentrations peak at 4 weeks and decrease gradually to baseline at 24 weeks.[64]

Table 16.1 Testosterone Preparations Available for Treatment of Male Hypogonadism

Types of Preparations	Specific Preparations	Dosing	Comments
Transdermal	Testosterone gels	Daily	Normal, steady serum T levels usually achieved
	Testosterone patch	Daily	Normal serum T; high incidence of skin reactions
Esters for IM use	Testosterone enanthate	1-2 weeks	Peak serum T level supranormal; nadir subnormal
	Testosterone cypionate	1-2 weeks	Same as above
	Testosterone undecanoate	3 months	Steady serum T levels for 2-3 months
Oral	17-Alkylated androgens	Daily	Hepatic toxicity
	Testosterone undecanoate	3 times/day	Variable absorption, subnormal serum testosterone
Pellets implanted by incision	Testosterone pellets	3 months	May extrude; serum T data

Parameters of Improvement

Bone

In one study, 108 healthy elderly men over 65 years old with a serum testosterone concentration below 475 ng/dL were randomized to receive testosterone or placebo transdermally for 3 years. Bone mineral density of the lumbar spine increased somewhat more in the testosterone-treated group than in the placebo-treated group, but overall the difference was not statistically significant. When the data were analyzed by a linear regression model, there was an inverse correlation between the pretreatment serum testosterone concentration and the effect of testosterone on bone mineral density above that of placebo, called the testosterone treatment effect (Fig. 16.4). For example, for a pretreatment serum testosterone of 400 ng/dL, the testosterone treatment effect on the percentage of increase in bone mineral density above baseline was only 0.9%, but when the pretreatment serum testosterone concentration was 300 ng/dL, the testosterone treatment effect was 3.4%, and when the pretreatment serum testosterone was 200 ng/dL, the testosterone treatment

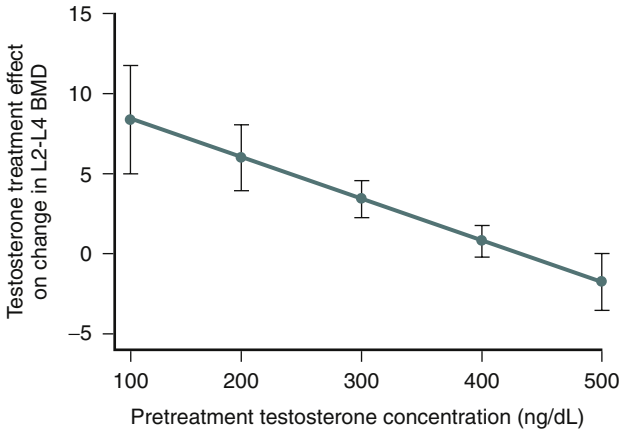


FIGURE 16.4 Effect of testosterone treatment for 3 years on spine bone mineral density (BMD) of elderly men. Shown is a linear regression model of the testosterone treatment effect (the effect of testosterone over placebo) as a function of the pretreatment serum testosterone concentration, showing that only men who have a lower than normal serum testosterone concentration would be expected to experience an increase in spine bone mineral density if treated with testosterone. *(Adapted from Snyder PJ, Peachey H, Hannoush P, et al. Effect of testosterone treatment on bone mineral density in men over 65 years of age.* J Clin Endocrinol Metab *84:1966, 1999.)*

effect was 5.9%.[65] No significant difference was observed in hip bone mineral density. In another study, 67 men over 65 years old who had subnormal bioavailable testosterone concentrations were randomized to receive testosterone transdermally for 1 year. In the 44 men who completed the trial, femoral neck bone mineral density increased 0.3% in the testosterone-treated group and decreased 1.6% in the placebo-treated group, a difference that was statistically significant, but there were no significant differences in bone mineral density at other sites in the hip or in the spine.[66] In a third study, in which elderly men were randomized to receive testosterone enanthate alone (24 men), testosterone enanthate plus finasteride (22 men), or placebo (24 men) for 3 years, the men treated with testosterone alone or with finasteride exhibited 10.2% and 9.3% increases in spine bone density, whereas the men treated with a placebo increased only 1.3%.[67] Increases in the total hip were less: 2.7% and 2.2% versus 0.2%, but still statistically significant.

Body Composition

Several studies show that testosterone treatment of elderly men increases lean body mass and decreases fat mass. In the 108 elderly men described earlier,[68] lean mass increased by 1.9 kg and fat mass decreased by 3.0 kg in the testosterone-treated men during the 3 years of treatment (Fig. 16.5). In the study of 67 men described earlier,[66] lean mass increased by 1.0 kg and fat mass decreased by 1.7% in the 1 year of treatment. In a study of elderly men treated with placebo or testosterone enanthate for 6 months,[69] 21 testosterone-treated men experienced a 1.4 kg increase in lean mass and a 1.2 kg decrease in fat mass, whereas 17 placebo-treated men experienced no change. In the study of 70 men treated with testosterone enanthate with or without finasteride or with placebo for 3 years, lean body mass increased by about 4 kg and fat mass decreased by about 5 kg in the two testosterone-treated groups.[70]

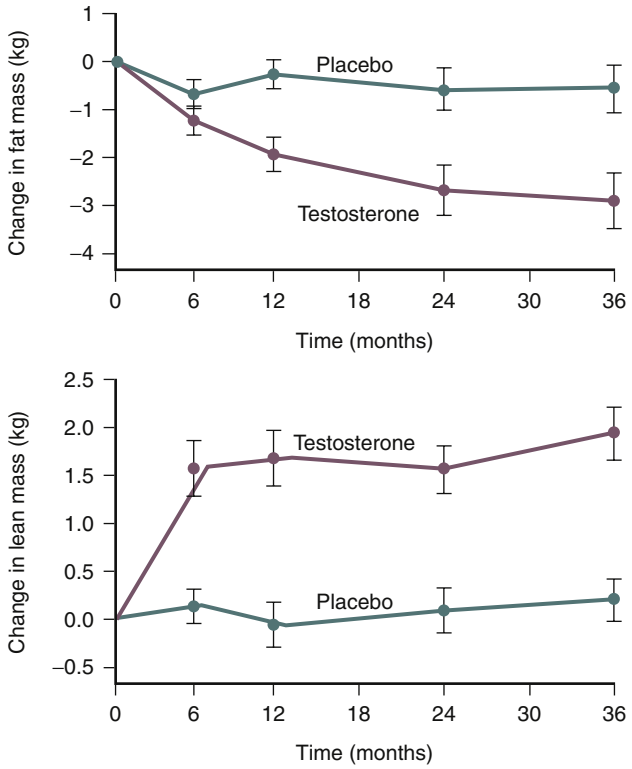


FIGURE 16.5 Effect on fat mass and lean mass when 108 men were treated with testosterone or placebo for 3 years. *(Adapted from Snyder PJ, Peachey H, Hannoush P, et al. Effect of testosterone treatment on body composition and muscle strength in men over 65 years of age.* J Clin Endocrinol Metab *84:2647, 1999.)*

Muscle Strength

Although an increase in muscle mass should result in an increase in muscle strength, controlled studies in which a physiological dose of testosterone has been administered to elderly men have generally not demonstrated an increase in muscle strength compared to placebo-treated men. In the study of 108 men described earlier in this chapter[68] who were randomized to receive either testosterone or placebo for 3 years, the testosterone-treated men did not differ from the placebo-treated men in change from baseline in strength of knee extension or flexion at 60 degrees or 180 degrees angular velocity at 3 years or earlier, nor in handgrip strength. In the study of 67 men described earlier[66] who were randomized to receive testosterone or placebo for 1 year, strength of knee extension by leg press was no different at the end of the year in the testosterone-treated group than in the placebo-treated group. In 40 men randomized to receive human chorionic gonadotropin (hCG) or placebo for 3 months,[71] shoulder and knee strength as measured by dynamometry did not differ in the two groups at the end of treatment. In the 70 men treated with testosterone enanthate with or without finasteride or treated with placebo, the testosterone-treated men exhibited a greater improvement than placebo-treated in right handgrip strength but not in left handgrip strength or in ankle or knee strength.[70] In 274 men ≥65-years-old chosen because of low-normal or low serum testosterone concentrations and a mild degree of frailty, and randomized to receive a testosterone gel or placebo for 6 months,

testosterone treatment was associated with greater isometric knee extension strength, but not with greater isokinetic knee strength, isokinetic or isometric knee flexion strength, or grip strength.[72]

Physical Function

Parameters of physical function also have not changed significantly following administration of testosterone to elderly men. In 108 men randomized to receive testosterone or placebo, walking time and stair climbing did not differ between the two groups at the end of 3 years of treatment.[68] The testosterone-treated men, however, had a more favorable perception of their physical function than the placebo-treated men had of theirs. In 67 men randomized to receive testosterone or placebo, physical function, as estimated activity by Physical Activity for the Elderly questionnaire, did not differ between the two groups.[66] In 40 men who were randomized to receive hCG or placebo; maximum reach, static and dynamic balance, gait, and chair rise did not differ between the two groups at the end of 3 months of treatment.[71] In the men treated with testosterone enanthate with or without finasteride or treated with placebo, the testosterone-treated men exhibited greater improvement than placebo-treated men after 3 years.[70] In men selected for a moderate degree of frailty, testosterone treatment was not associated with better performance in any of six tests of physical function.[72]

Energy

Testosterone treatment of elderly hypogonadal men has generally not affected their energy. In 108 elderly men randomized to receive either testosterone or placebo, self-reported energy and sense of well-being was no different in the two groups at the end of 3 years of treatment.[68] In 67 men randomized to receive testosterone or placebo, sense of well-being was no different between the two groups at the end of 1 year of treatment.[73] However, in the men selected for a moderate degree of frailty, those treated with testosterone indicated a better quality of life on the Aging Male Symptom scale than those treated with placebo.[72]

Cognition

The effects of testosterone treatment on cognition have been studied in elderly men who have low-normal serum testosterone concentrations, but the results have been variable. Some studies have shown in improvement in various aspects of cognitive function,[62] but others have not.[61]

Libido

Testosterone treatment of elderly men has not been shown to increase libido consistently. In 108 men randomized to receive testosterone or placebo for 3 years, the two groups did not differ in libido, as determined by questionnaire, either at the end of treatment or at any earlier time.[68] In 40 men randomized to receive either hCG or placebo for 1 year, libido also did not differ at the end of treatment.[71] In the men chosen because of moderate frailty, the testosterone-treated men exhibited a greater improvement in the sexual subscale of the Aging Male Symptom scale than those treated with placebo.[72]

Potential Deleterious Consequences of Testosterone Treatment of Elderly Men

Several diseases to which elderly men are prone are testosterone-dependent. These diseases include prostate cancer and benign prostatic hyperplasia, and possibly erythrocytosis and sleep apnea. Prostate cancer, for example, was the first cancer shown to be hormonally dependent,[74] and treatment of metastatic prostate cancer even now is based on blocking the secretion and action of testosterone.[75,76] An additional reason for concern that testosterone treatment of elderly men might increase the risk of prostate cancer is that approximately 50% of men who are 50- to 60- years-old harbor clinically silent prostate cancer.[77] If these men were treated with testosterone, would these clinically silent tumors become clinically significant? Benign prostatic hyperplasia has been known to be testosterone-dependent for over a century, and a current treatment for this condition is also based on blocking the action of testosterone in the prostate.[78,79] Testosterone stimulates erythropoiesis, which is why men have higher hemoglobin concentrations than do women, but it probably does not cause erythrocytosis unless the dose is excessive or there is a concomitant illness that treatment of hypogonadism unmasks. The evidence that testosterone exacerbates sleep apnea is based on studies of small numbers of patients.[80]

Prostate Events

Testosterone treatment of elderly men has generally not been associated with a significant increase in prostate disease, but the studies performed so far have been too small to draw any conclusions from these results. In a study of 108 men randomized to receive testosterone or placebo, the serum prostate specific antigen (PSA) concentration increased from 1.4 ng/mL to 2.1 ng/mL, which was statistically significant but still well within the normal range.[65] The testosterone-treated men experienced somewhat more prostate events, such as persistently elevated PSA, urinary retention, and urosepsis, than did the placebo-treated men. A meta-analysis of five testosterone studies reported that prostate cancer did not occur more often in men treated with testosterone than in men treated with placebo, but this analysis involved too few men upon which to do draw firm conclusions.[81]

Erythrocytosis

In 108 men randomized to receive either testosterone or placebo transdermally, hemoglobin and hematocrit increased significantly in the men treated with testosterone—although within the normal range—but not in men treated with placebo.[54] In men treated with 200 mg of testosterone enanthate every 2 weeks, 30% experienced an increase in hematocrit above the upper limit of normal (52%).[56]

Cardiovascular Risk

Administration of testosterone to elderly hypogonadal men has little effect on serum lipid or apolipoprotein concentrations, but other cardiovascular risk factors have not been well studied. In 108 elderly men randomized to receive either testosterone or placebo, there was no difference between the two groups in serum concentrations of total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, apolipoprotein

A_2, apolipoprotein B, triglycerides, or lipoprotein a at the end of 3 years of treatment.[82] In 67 men randomized to receive testosterone or placebo, total cholesterol and LDL cholesterol did not differ between the two groups after a year, but HDL cholesterol decreased in the men who received testosterone.[83] Endothelial brachial artery reactivity did not change in either group.

Cardiovascular adverse events have been reported in only a few studies. A study of elderly moderately frail men, of whom 106 were randomized to receive testosterone and 103 to receive placebo for 6 months, was stopped by its Data Safety Monitoring Board because 23 men who were receiving testosterone developed cardiovascular adverse events compared to 5 receiving placebo.[56] In a meta-analysis of seven prior testosterone trials, however, testosterone was not associated with an increased risk of myocardial infarction.[57]

Sleep Apnea

Although there are a few reports that men with frank hypogonadism who were treated with testosterone experienced an increase in apneic episodes during sleep, sleep apnea has not been shown to increase in elderly men treated with testosterone.[65,71]

Conclusions

Although the serum testosterone concentration falls as men age, the potential beneficial effects and potential risks of reversing that fall are still being discovered. Small, placebo-controlled trials of raising the serum testosterone of elderly men to that of normal young men suggest that this treatment increases lean muscle mass and decreases fat mass. They also suggest that testosterone treatment increases bone mineral density of the spine. Whether or not this treatment increases the risks of prostate cancer, benign prostatic hyperplasia, erythrocytosis, or sleep apnea, however, remains to be determined.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 17

Disorders of Sex Development

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Eric Vilain

Introduction to Human Sex Development

From the moment of our conception, each of us has a sex. Our individual sex has a major role in determining the physical attributes of our bodies, the structure of our brains, our behavioral tendencies, and our self-concept. Understanding the development of sex differences has informed and been informed by the advances in the molecular dissection of genes responsible for disorders of sex development. New genes have been identified, allowing for rapid diagnosis, understanding of the pathophysiology, and prediction of future fertility.

In parallel to the progress in the biology of sexual development, the management of individuals with disorders of sexual development has started a gentle revolution. From a practice based on the opinions of a few leaders in the field, the clinical approach to disorders of sex development (DSD) is slowly entering the era of evidenced-based medicine, under the pressure of patient advocacy groups, who have been highly instrumental in establishing a dialogue between practitioners and their patients. As a result, physicians are beginning to understand the difficulties involved in defining normalcy, as well as the ethical dilemma of acting medically on children before they reach an age of consent.

For the first time in 2005, a consensus on the management of "intersex" disorders emerged, bringing together experts from a variety of fields (endocrinology, genetics, surgery, psychology, advocacy groups). The consensus statement provides guidelines in all aspects of management of "intersex," including genital surgery, the requirement of a multidisciplinary team and of mental health professionals, and the diagnostic approach. This chapter reflects the advances made in the field as well as the recommendations of the consensus statement.[1] One area that changed considerably was the nomenclature of intersexuality. The term *Disorders of Sex Development (DSD)* was proposed, as defined by "congenital conditions in which development of chromosomal, gonadal or anatomical sex is atypical." This broad definition replaces the word "intersex," which has social connotations and reflects a concept of sexual identity. It includes patients that do not necessarily have ambiguous external genitalia (such as complete androgen insensitivity, or Turner syndrome) and yet belong to the broad family of DSD.

The new nomenclature was guided by the following principles. First, although a modern categorization should integrate the important progress in molecular genetic aspects of sex determination and differentiation, it should not overemphasize one particular aspect of the biology of sex (for instance, gonadal sex) and should accommodate the spectrum of phenotypic variations. Second, terms should be as precise as possible and should reflect the genetic etiology when available. Finally, the new nomenclature should be understandable by patients and families and should be psychologically sensitive. In particular, gender labeling in the diagnosis should be avoided, and use of the words "hermaphrodite," "pseudohermaphrodite," and "intersex" should be abandoned, as they either are confusing or have a negative social connotation that may be perceived as harmful by some patients and parents.

The new nomenclature is summarized in Tables 17.1 and 17.2. This chapter will refer to the new nomenclature rather than the old nomenclature.

Normal Human Sex Development

Sex determination refers to the developmental decision that directs the orientation of the bipotential, undifferentiated embryo into a sexually dimorphic individual. In mammals, this decision occurs during the development of the gonads. In 1947, physiologist Alfred Jost, performed a series of elegant experiments showing that all mammalian embryos that are castrated early in development and reimplanted into the uterus develop into females, regardless of their genetic sex (Fig. 17.1).[2,3] This study established the

Table 17.1 Proposed Revised Nomenclature

Previous	Proposed
Intersex	DSD
Male pseudohermaphrodite, undervirilization of and XY male, and undermasculinization of an XY male	46,XY DSD
Female pseudohermaphrodite, overvirilization of and XX female, and masculinization of an XX female	46,XX DSD
True hermaphrodite	Ovotesticular DSD
XX male or XX sex reversal	46,XX Testicular DSD
XY sex reversal	46,XY complete Gonadal Dysgenesis

DSD, disorder of sex development.
From Lee PA, et al. Consensus statement on management of intersex disorders. International Consensus Conference on Intersex. *Pediatrics* **118**(2):e488–500, 2006.

Table 17.2 An Example of a DSD Classification

Sex Chromosome DSD	46,XY DSD	46,XX DSD
45,X (Turner syndrome and variants)	Disorders of gonadal (testicular) development: (1) complete gonadal dysgenesis (Swyer syndrome); (2) partial gonadal dysgenesis; (3) gonadal regression; and (4) ovotesticular DSD	Disorders of gonadal (ovarian) development: (1) ovotesticular DSD (e.g., SRY translocation, RSPO1 mutation); (2) testicular DSD (e.g., SRY+; dup-SOX9); (3) Gonadal Dysgenesis
47,XXY (Klinefelter syndrome and variants)	Disorders in androgen synthesis of actions; (1) androgen biosynthesis defect (e.g., 17-hydroxy dehydrogenase deficiency, 5αRD2 deficiency, StAR mutations); (2) defect in androgen action (CAIS, PAIS); (3) luteinizing hormone receptor defects (Leydig cell hypoplasia); and (4) disorders of anti-müllerian hormone and anti-müllerian hormone receptor	Androgen excess: (1) Fetal (e.g., 21-hydroxylase deficiency, 11-hydroxylase deficiency); (2) fetal-placental (aromatase deficiency, POR [P450 oxidoreductase] deficiency); and (3) maternal (luteoma)
45,X/46,XY (Mixed Gonadal Dysgenesis, ovotesticular DSD)		
46,XX/46,XY (Chimeric, ovotesticular DSD)		

Adapted from Lee PA, et al. Consensus statement on management of intersex disorders. International Consensus Conference on Intersex. *Pediatrics* **118**(2):e488–500, 2006.

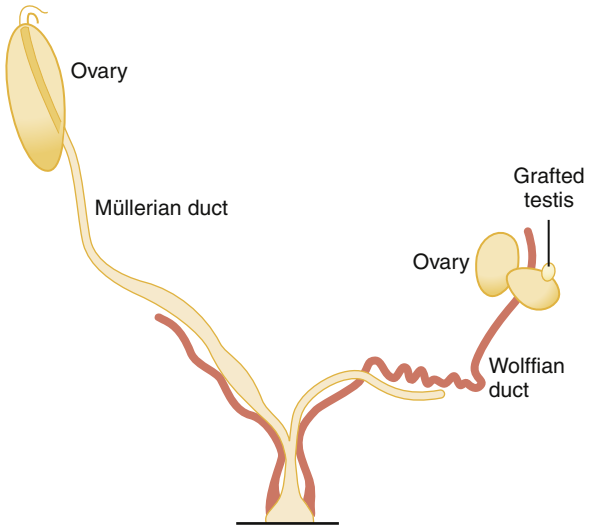


FIGURE 17.1 Alfred Jost's classic experiment defined the current paradigm of sex development by grafting testicular tissue to the right ovary of a rabbit fetus. The testis was able to stimulate the wolffian duct and inhibit müllerian development, but the effect was only ipsilateral to the grafted testes. *(From Austin C, Short RV.* Embryonic and fetal development [reproduction in mammals, book 2]. *New York: Cambridge University Press, 1972, p 58.)*

current paradigm of sex determination and differentiation in placental mammals: genetic sex determines gonad development, and gonadal sex in turn governs anatomical sex.

At conception, genetic sex is determined based on whether the X or Y chromosome was paternally inherited.[4,5] *Sex determination* is defined by the developmental choice of the bipotential and undifferentiated gonad to become either testis or ovary. Following this sex determination decision, the process of *sex differentiation* begins and the testes start producing the male hormones testosterone (T) and anti-müllerian hormone (AMH), which are responsible for male internal and external genitalia, while the lack of these hormones result in female internal and external genitalia (Fig. 17.2).

The molecular mechanisms of mammalian sex determination are still poorly understood. The vast majority (about 75%) of sex-reversed patients cannot be explained at the molecular level yet, suggesting the existence of a number of unknown sex determining genes.[6] The next section outlines the known molecular mechanisms affecting sex determination and sex differentiation.

Sex Determination: Testes or Ovary?

This paradigm for sex development established by Jost led to the search for a sex-determining gene that was a Testis Determining Factor (TDF). When the karyotypes of patients with Klinefelter syndrome who are male (47,XXY)[7] and Turner syndrome who are female (45,X)[8] were determined, it became clear that the Y chromosome was sex-determining and that TDF had to be located on the Y chromosome.

In the early 1990s, a series of elegant experiments found SRY to be the elusive mammalian testis determining gene. Positional cloning located a 35-kb fragment of the Y chromosome translocated to the X chromosome of 46,XX testicular and ovotesticular DSD patients.[9,10] Further sequence analysis defined a conserved sequence within this region. Sry gene expression analysis revealed a male-specific increase in transcript consistent with earliest known divergence of male and female gonadal development. Furthermore, XX mice transgenic for the 14kb fragment containing *Sry* developed testes and the full male phenotype. This was followed up with experiments showing that deletions and mutations in *SRY/Sry* result in XY sex reversal in both humans and mice.[11-13]

In humans, 8 weeks after conception the bipotential gonad progresses towards testis organogenesis in the presence of SRY and can be recognized on ultrasound by week 10. Factors expressed earlier than SRY prime the bipotential gonadal ridge to differentiate in the male pathway. Mutations in the MAP-kinase signaling pathway result in 46,XY sex reversal and is important in promoting SRY expression within the developing gonad.[14,15] Chromobox-2 *(CBX2)* has also been shown to be required for SRY expression in mouse sex determination and mutations result in 46,XY sex reversal.[16,17]

SRY expression in the Sertoli cell precursors initiates testis determination by activating downstream effectors

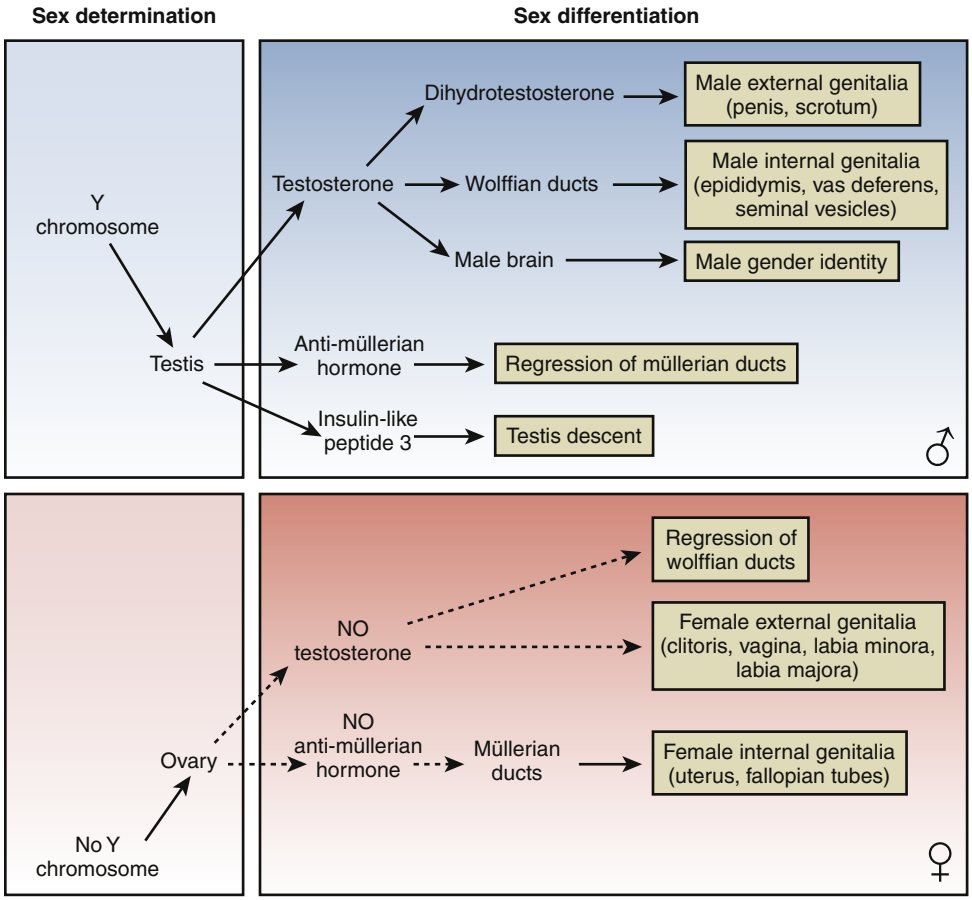


FIGURE 17.2 Sex determination is the process of genetic sex (presence of absence of the Y chromosome) to gonadal sex. Sex determination is the process of sex development after gonadal sex has been determined. In XY males, sex differentiation is an active process requiring testes derived hormones, testosterone, anti-müllerian hormone, and Insulin-3 (Insl3). In XX females, sex differentiation is a default pathway which occurs in the absence of testes-derived hormones.

such as *SOX9* (*SRY*-related HMG-box 9). Sertoli cell proliferation and organization into tubular cords is directed by *SOX9* (*SRY*-related HMG-box 9) and *SF1/NR5A1 (Steriodogenic Factor-1)*. At 9 weeks of gestation, after the organization of discrete tubules, Sertoli cells begin the secretion of AMH.[18,19] Other genes involved in sex determination are *DAX-1*, in which a single copy is required for testes development, however two copies in XY fetuses result in XY gonadal dysgenesis by repressing testis organogenesis.[20] Another gene involved in sex determination is Desert hedgehog *(DHH)*, which up-regulates SF1 and in turn regulates Leydig cell proliferation.[21,22]

The cellular fate of murine ovarian and testicular tissues is not sealed at the point of sex determination. In murine models of sex determination, cellular fates of the testis or ovary are actively maintained throughout adult life by *DMRT1* (Doublesex and mab-3 related transcription factor 1) and *FOXL2* (Forkhead box L2), respectively.[17,23] This lack of finality in the gonadal cell fate determination indicates that cell fate is carefully cultivated throughout adulthood, and if the factors important in maintaining cell fate are mutated, it may theoretically lead to decreased secondary sex characteristics and infertility.

Relative to testis organogenesis, there is much less information on the genes involved in ovary organogenesis. Studies suggesting that ovary development does not require a large number of genes are incomplete in that they look at timepoints that are important in testis determination but may not be critical in ovarian determination.[24,25] Despite this, researchers have uncovered several autosomal genes required for proper ovarian development, including *WNT-4* (wingless-related MMTV integration site 4), *RSPO-1* (R-spondin-1), and *FST* (Follistatin).[26]

Sex Differentiation

The type of internal and external genitalia is decided by the presence of a viable testis. Therefore, the testis actively governs *sexual differentiation*. Conversely, the growth of female genitalia is the default process in sexual differentiation. The bipotential ductal system consists of the müllerian and wolffian ducts which give rise to female and male internal genitalia, respectively (Fig. 17.3).

Hormones secreted from testes are essential to the development of male internal and external genitalia. Normally developed testes have both Sertoli cells and testicular cords. Sertoli cells secrete AMH causing müllerian duct regression. At the same time, Leydig cells, the steroidogenic cells of the testes, secrete testosterone and INSL3 (insulin-like 3) to promote development of wolffian structures (epididymis, vas deferens, and seminal vesicles) and mediate transabdominal descent of the testes to the internal inguinal ring, respectively.[27] To mediate the development of the male external genitalia, testosterone is converted into 5α-dihydrotestosterone (DHT), a more potent androgen, by the enzyme 5α-reductase (Fig. 17.4). Except for phallic growth and inguinoscrotal descent in the third trimester, male sexual differentiation is essentially complete by week 14 of gestation. Defects in labioscrotal fusion and urogenital sinus growth cannot be modified by high doses of androgens after week 14.

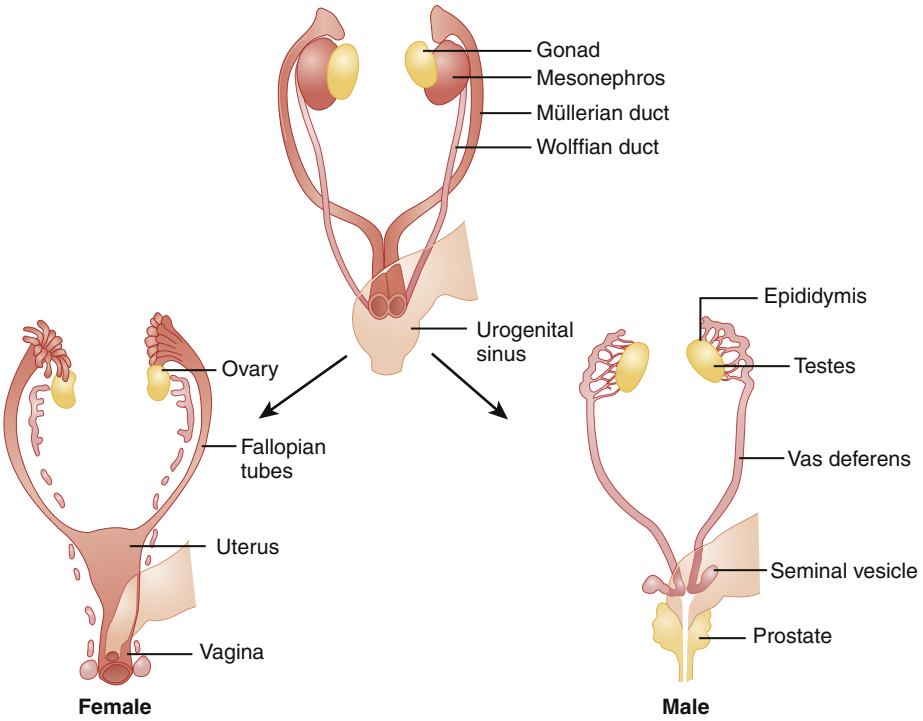


FIGURE 17.3 Internal genital development of male and female ductal system. *(Courtesy of Dr. J. Wilson, University of Texas, Southwestern Medical School, Dallas, TX.)*

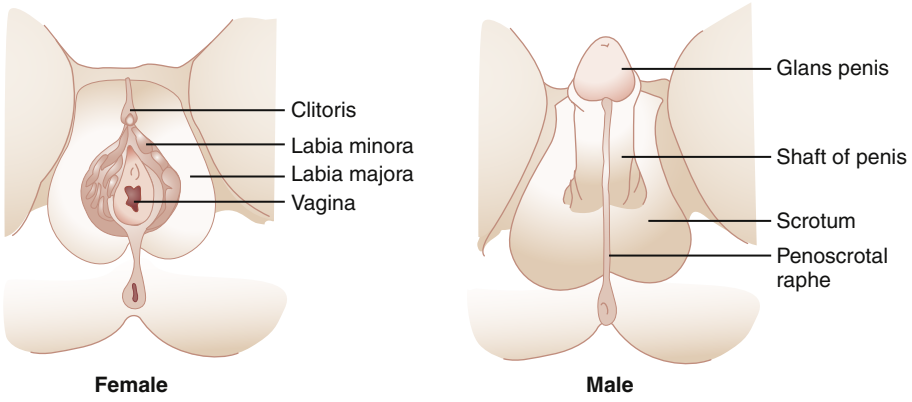


FIGURE 17.4 External genital development, demonstrating homologies and common anlagen in male and female. *(Courtesy of Dr. J. Wilson, University of Texas, Southwestern Medical School, Dallas, TX.)*

In females, the absence of a testicular tissue and its' associated hormones, such as AMH or testosterone, result in müllerian structure development (fallopian tubes, uterus, upper vagina) and wolffian duct regression, around 12 weeks of gestation. Finally, absence of DHT results in no virilization of external genitalia.

Disorders that can cause ambiguous genitalia but are not associated with dysgenic gonads (e.g., complete and partial androgen insensitivity syndrome [CAIS, PAIS] and congenital adrenal hyperplasia [CAH]) are considered disorders of sexual differentiation, not disorders of sex determination. Overview of the diagnostic differences in disorders of sex development, including both disorders of sex determination and sex differentiation, are presented in Table 17.3.

Sex Chromosome Disorders of Sexual Development (DSD)

Sex Chromosome DSDs are defined by aneuploidy of the sex chromosomes, X and Y. In disorders of aneuploidy (i.e., Trisomy 21, 18, etc), maternal errors in meiotic non-disjunction during meiosis-I account for the majority of cases. With few exceptions, such as in Klinefelter Syndrome, maternal and paternal errors are equally likely (47,XXY).[28]

Turner Syndrome

In 1938, a group of females with short stature, primary amenorrhea, and a lack of secondary sex characteristics was described by Turner in 1938. Turner syndrome is classified as a sex chromosome DSD, with the majority having a 45,X karyotype, and the remaining having a 46,XX karyotype with a deletion in part of one X chromosome or having various mosaics involving 45,X and 46,XX, and 46,XY cells or more complex combinations. In 100% of patients with 45,X and 80% of mosaic patients, the universal feature in patients is short stature (Fig. 17.5), with average height less than 58 inches. The genetic locus for short stature in both Turner syndrome and idiopathic short stature lies in the pseudoautosomal region (PAR1) of the X chromosome. This region is

Table 17.3 Syndromes Associated with DSD

	Syndromes Associated with Ambiguous Genitalia	Phenotype	Gene	Locus	OMIM #
Syndromes associated with gonadal dysgenesis	Denys-Drash	Early diffuse mesangial sclerosis of kidneys, Wilms' tumor, XY gonadal dysgenesis	WT-1	11p13	194080
	Frasier	Early adolescent development of focal segmental glomerulosclerosis, gonadoblastoma, XY gonadal dysgenesis	WT-1	11p13	136680
	Campomelic dysplasia	Congenital bowing of long bones, hypoplastic scapulae and thoracic vertebrae pedicles, ambiguous genitalia	SOX9	17q24.3-q25.1	114290
	GD with neuropathy	XY gonadal dysgenesis with associated minifascicular neuropathy (one of 4 cases)	DHH	12q12-13.1	607080
	X-linked alpha-thalassemia/mental retardation	Hemoglobin H disease, mental retardation, dysmorphic facies, genital abnormalities	XH2	Xq13.3	301040
	Palmoplantar hyperkeratosis with squamous cell carcinoma and XY sex reversal	Variable degrees of XY gonadal dysgenesis, ranging from full sex reversal to ovotesticular DSD	RSPO1	1p34.3	610644
	Blepharophimosis-ptosis-epicanthus inversus syndrome Type I	Blepharophimosis, ptosis, and epicanthus inversus syndrome, either with premature ovarian failure	FOXL2	3q23	110100
Syndromes associated with small penis and/or cryptorchidism	VACTERL/VATER	Vertebral defects, anal atresia, tracheoesophageal fistula with esophageal atresia, and radial dysplasia. Limb anomalies	Unknown	Unknown	192350
	Goldenhar syndrome (Hemifacial Microsomia)	Unilateral deformity of the external ear and small ipsilateral half of the face with epibulbar dermoid and vertebral anomalies	Unknown	Unknown	164210
	SmithLemli Opitz syndrome (SLOS)	Multiple congenital malformation and mental retardation syndrome	DHCR7	11q12-q13	270400
	Pallister Hall syndrome (PHS)	Hypothalamic hamartoblastoma, postaxial polydactyly, and imperforate anus	GLI3	7p13	146510
	Robinow syndrome	Mesomelic limb shortening associated with facial and genital abnormalities	ROR2	9q22	180700
	Prader Willi syndrome	Obesity, muscular hypotonia, mental retardation, short stature, hypogonadotropic hypogonadism	SNRPN	15q12, 15q11-q13	17620
	Kallmann syndrome	Hypogonadotropic hypogonadism and anosmia	FGFR-1	8p11.2-p11.1	147950
	Holoprosencephaly	Craniofacial dysmorphology	Many	21q22.3, 2q37.1-q37.3	236100
	Malpeuch facial clefting syndrome	Short stature, hypertelorism, eye anomalies, facial clefting, hearing loss, urogenital abnormalities, mental retardation	Unknown	Unknown	248340
	Najjar syndrome	Genital anomaly, mental retardation, and cardiomyopathy	Unknown	Unknown	212120
	Varadi-Papp syndrome	Big toes, hexadactyly, cleft lip/palate or lingual nodule, and somatic and psychomotor retardation. Some showed absent olfactory bulbs and tracts, cryptorchidism	Unknown	Unknown	277170
	Juberg-Marsidi syndrome	Cleft lip/palate with abnormal thumbs and microcephaly	Unknown	Unknown	216100
	Johanson-Blizzard syndrome	Aplasia or hypoplasia of the nasal alae, congenital deafness, hypothyroidism, postnatal growth retardation, malabsorption, mental retardation, midline ectodermal scalp defects, and absent permanent teeth	Unknown	15q15-q21.1	243800
	Borjeson-Forssman-Lehmann syndrome	Severe mental defect, epilepsy, hypogonadism, hypometabolism, marked obesity	Unknown	Xq26.3	301900
	Torticollis, keloids, cryptorchidism, renal dysplasia	Torticollis, keloids, cryptorchidism, renal dysplasia	Unknown	Xq28	314300
	Hypertelorism with esophageal abnormality and hypospadias	Laryngotracheoesophageal cleft; clefts of lip, palate, and uvula; swallowing difficulty and hoarse cry; genitourinary defects, mental retardation; congenital heart defects	Unknown	22q11.2	145410

Continued

Table 17.3 Syndromes Associated with DSD—cont'd

	Syndromes Associated with Ambiguous Genitalia	Phenotype	Gene	Locus	OMIM #
Syndromes associated with small penis and/or cryptorchidism—cont'd	Faciogenitopopliteal syndrome	Cleft palate and webbing intercrural pterygium	Unknown	1q32-q41	119500
	Dubowitz syndrome	Short stature, microcephaly, mild mental retardation with behavior problems, eczema, and distinctive facies	Unknown	Unknown	223370
	Noonan syndrome	Hypertelorism, a downward eye slant, and low-set posteriorly rotated ears, short stature, webbed neck, cardiac anomalies	PTPN11	12q24.1	163950
	Aarskog syndrome (Faciogenital Dysplasia)	Embryonic ocular hypertelorism, anteverted nostrils, broad upper lip, and peculiar penoscrotal relation	FGD1	Xp11.21	305400
	Cornelia de Lange syndrome	Low anterior hairline, anteverted nares, maxillary prognathism, long philtrum, 'carp' mouth in association with prenatal and postnatal growth retardation, mental retardation	NIPBL	5p13.1	122470
	Rubinstein-Taybi syndrome	Mental retardation, broad thumbs and toes, and facial abnormalities	CREBBP	16p13.3, 22q13	180849
	Seckel syndrome	Growth retardation, microcephaly with mental retardation, and a characteristic 'bird-headed' facial appearance	SCKL1	3q22-q24	210600
	Miller Dieker syndrome	Microcephaly and a thickened cortex with 4/6 layers	LIS1	17p13.3	247200
	Lenz-Majewski hyperostosis syndrome	High palate; short, yellow, carious teeth; progeroid appearance; short, increased venous pattern of the forehead and thorax	Unknown	Unknown	151050
	Lowe syndrome	Ophthalmia, cataract, mental retardation, vitamin D-resistant rickets, amino aciduria	OCRL1	Xq26.1	309000
Syndromes associated with müllerian malformations	MURCs	Müllerian duct aplasia, renal aplasia, and cervicothoracic somite dysplasia	Unknown	Unknown	601076
	Mayer-Rokitansky-Kuster-Hauser syndrome (MRKH)	Müllerian duct aplasia	WNT4 in subset with hyperandrogenism	Unknown; 1p35	277000
	McKusick Kaufman syndrome (MKKS)	Hydrometrocolpos, congenital heart malformations, post-axial polydactyly	Unknown	20p12	236700

deleted in individuals with short stature caused by the lack of one copy of the short stature homeobox gene, *SHOX*.

The classic Turner 45,X karyotype is thought to be one of the most common human chromosomal abnormalities and is estimated to occur in 0.8% of all zygotes. However, less than 3% of these zygotes survive to term, and this karyotype is commonly found in spontaneous abortions.[29] The incidence of 45,X karyotypes is approximately 1 in 2500 live newborn phenotypic females.[30,31]

The gonads of Turner syndrome females do not develop normally, having reduced growth and follicle formation *in utero*.[31] A normal number of eggs develop in girls with Turner syndrome, but, for unknown reasons, they disappear prematurely.[32] The gonads appear as streaks of white tissue next to the fallopian tubes—called "streak gonads" which on histology have primitive connective tissue stroma without primary follicles. Patients with Turner syndrome have fewer follicles and therefore less estrogen secretion from granulosa cells than normal females, resulting in delayed puberty.

Presentation and Diagnosis

In the prenatal period, fetuses with 45,X DSD and its variants frequently have intrauterine growth restriction. Suspicion for Turner syndrome should be raised if the presence of large septate cystic hygromas, nuchal thickening, short femur, total body lymphangiectasia, or cardiac defects is detected by ultrasound. At birth, Turner syndrome babies often have low birth weights; 30% of babies will present with lymphedema of the upper and lower extremities (Fig. 17.6), an extension of the lymphangiectasias *in utero*, which disappears in the first few months of life. Additionally, the presence of a webbed neck (pterygium colli) (Fig. 17.5) and dysmorphic features including: high-arched palate, low set prominent ears, low posterior hairline, epicanthal folds, micrognatia, hypoplastic nail beds, or hypoplastic fourth and fifth metacarpals at birth should raise the suspicion for 45,X DSD.[33] Turner syndrome patients may have increased carrying angle of the arms (cubitus valgus), shield-like chest with wide set nipples, hearing loss, and a higher frequency of cardiovascular disease such as coarctation of the aorta.

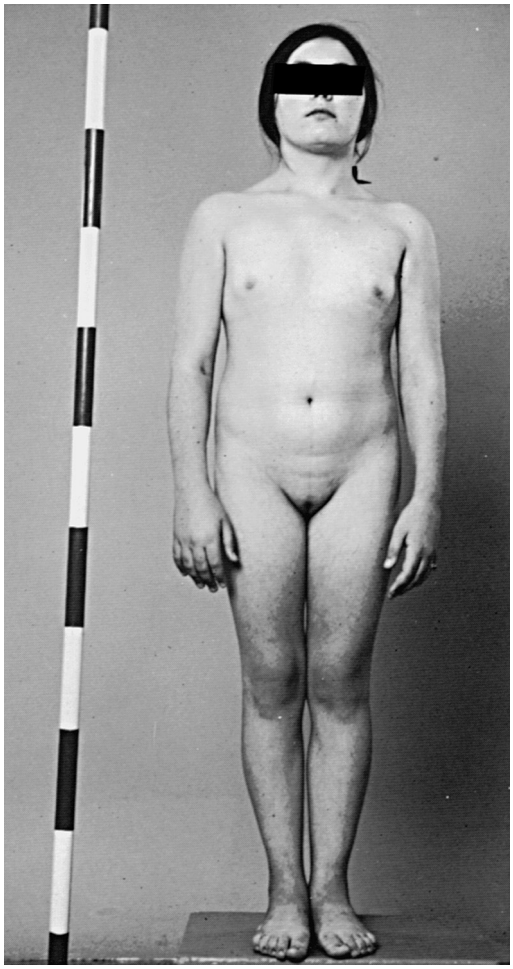


FIGURE 17.5 Patient with Turner syndrome. Note the short stature, webbed neck, and lack of secondary sex development.

Furthermore, the incidence of renal anomalies is between 30% to 50%, with the most common being a horseshoe kidney followed by abnormal vasculature.

During adolescence, the most common presentation is short stature, amenorrhea, and lack of secondary sex characteristics. Patients may also have any of the other characteristics mentioned above. 45,X DSD patients have normal intelligence. Depending on the degree of gonadal dysgenesis, up to 30% of Turner patients undergo some degree of spontaneous puberty.[34]

Diagnosis is made by karyotype, which will show in a 45,X or mosaic variant of cells. If the karyotype appears normal, but the presentation is suggestive of Turner syndrome, a fluorescent *in situ* hybridization (FISH) or comparative genomic hybridization (CGH) study should be done, as there may be a cryptic deletion in the pseudoautosomal region in one of the X chromosomes. Typically patients with 45,X or 46,XX with a chromosomal abnormality in one X chromosome have more prominent phenotypic features than those who are a mosaic.

Management

For most patients with 45,X DSD, the greatest concern is for short stature and secondary sexual development. The treatment of both of these should be carefully titrated. For cases diagnosed in childhood, the use of low dose anabolical steroid coupled with growth hormone has been shown to increase final adult height with increased beneficial effects compared to those who started therapy late.[35-37] At the onset of the age of puberty, high dose estrogen and progesterone should be given to promote the development of secondary sex characteristics. Estrogens and progesterone should be taken throughout life to prevent complications, such as osteoporosis.[38]



FIGURE 17.6 Lymphedema in the lower extremities of an infant with Turner syndrome.

In young patients diagnosed with Turner syndrome, spontaneous puberty is a good indicator of the presence of functional ovarian tissue. Overall, it is estimated that 2% to 5% of patients with Turner syndrome have the potential for spontaneous pregnancy.[39]

Klinefelter Syndrome

In utero, the XXY fetal testis has the normal complement of primordial germ cells in the XXY testes. However, these germ cells degenerate during childhood, most likely due to a defect in Sertoli cells and germ cell communication during testes maturation.[40] In theory, the etiology of the nondisjunction in Klinefelter can be from maternal meiosis I or II or paternal meiosis II, and thus each situation should contribute to 33% of cases. However, nearly 50% of Klinefelter patients show a paternal origin[41] with some studies showing increased frequency of XY sperm with advanced paternal age.[42]

Presentation and Diagnosis

Klinefelter syndrome is largely undiagnosed in the general population.[43] In early life, Klinefelter may be diagnosed in boys with behavioral disorders, abnormally small testes, and long legs (Fig. 17.7). The presence of only long lower extremities distinguishes Klinefelter syndrome from the other forms of eunuchoidism that results in equally long upper and lower extremities. In patients with Klinefelter syndrome, IQ typically falls in the normal range, however it tends to be below that of other siblings.[42,44] Most patients present in adolescence with small firm testes and hypogonadism with varying degrees of androgen deficiency.[42] In later life, many males present at infertility centers with azoospermia.

Diagnosis of Klinefelter syndrome is performed by lymphocyte karyotype. Some mosaic cases will only be detected by karyotype of skin fibroblasts and occasionally of testicular biopsy specimens.

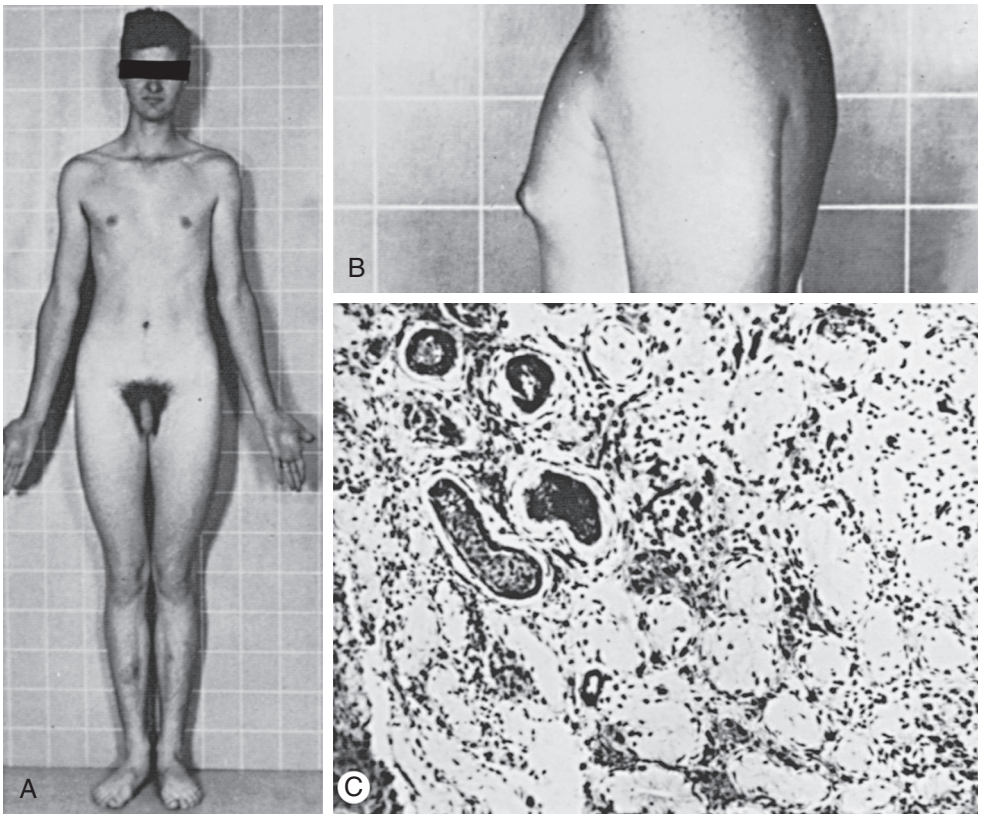


FIGURE 17.7 Klinefelter syndrome. **A**, Phenotypic male with XXY. Note the long lower extremities as compared with upper extremities **B**, Well virilized patient with gynecomastia. **C**, Testicular biopsy of patient in **B**. Note marked hyalinization of seminiferous tubules and Leydig cell hyperplasia. *(From Gumbach, MM, Conte FA. Disorders of Sex Differentiation. In Wilson JS, Foster DW, Kronenberg HM, Larsen PR [eds],* Williams textbook of endocrinology. *Philadelphia: WB Saunders, 1998, p 1368.)*

Management

Early detection of Klinefelter allows for early intervention for cognitive and behavioral disorders. Current studies are underway exploring the role of androgen replacement therapy in childhood, to help with cognitive and behavioral disabilities. Replacement of androgens also allows for development of masculine secondary sex characteristics, improved self-esteem, and increased libido, strength, and bone mineral density.[45] With regards to fertility, testicular mosaicism is an important factor to determining spermatogenesis and the potential for fertility. The vast majority of 47,XXY patients are azoospermic, but since the advent of Intra Cytoplasmic Sperm Injection (ICSI) technology have the potential to be fertile. It should be noted that there is a higher rate of sex chromosomal hyperploidy and autosomal aneupoloidy[46] in the sperm of Klinefelter patients. However, the risk of passing sex chromosome aneuploidy to the offspring remains unclear. Regardless, preimplanation genetic diagnosis (PGD) or fetal karyotype should be implemented to prevent chromosomal abnormalities in the progeny. Using testicular sperm extraction (TSE) technology, sperm can be successfully extracted from the testes[47] and injected into a donor oocyte.

46,XY Disorders of Sex Development

46,XY Disorders of Gonadal Determination

46,XY Gonadal Dysgenesis

Etiology and Pathophysiology of Pure-Complete Gonadal Dysgenesis

XY gonadal dysgenesis (GD) is a result of abnormal testis development *in utero*. There are three types of GD, pure (or complete), partial, or mixed, all of which can be differentiated by the extent of normal testicular tissue within the gonad and karyotype of the individual. In pure-complete GD, individuals have intra-abdominal, bilateral, fibrous streaks which do not secrete AMH or testosterone. Phenotypically, pure XY GD individuals are unambiguously phenotypic females (previously known as Swyer syndrome) but usually internally possess hypoplastic müllerian structures.

Given the primary importance of *SRY* in human testes determination, it is surprising that mutations in SRY only account for approximately 15% of all pure XY *GD*.[15,48-50] This suggests the existence of many other genes involved in primary human sex determination. SRY is a transcription factor, but its mechanism of action remains poorly understood. For instance, it is still unclear whether it acts as an activator or repressor[51] and its only identified and confirmed target is SOX9, another transcription factor from the Sox (Sry-box) family of genes. To date, there are over 50 verified mutations within the *SRY* gene. Mutations which result in streak gonads primarily occur within the high mobility group (HMG) box, and cause reduced DNA binding,[48,50] alterations in DNA bending,[52] or prevents nuclear import of the *SRY* protein.[53] There are over 50 known mutations of the *SRY* open reading frame. Larger cytogenetic deletions of Yp which include *SRY* have also been implicated in XY GD.[48,49,54]

Steroidogenic factor 1 (SF1, also known as NR5A1) is an orphan nuclear receptor necessary for adrenal and testicular development. Interactions between SF1 and the transcription factors GATA binding protein 4 (GATA4) and friend of GATA 2 (FOG2) appear to be necessary for *SRY* expression in developing testes.[55,56] Recent studies have underlined the importance of SF1 in testicular development, as nearly 15% of isolated XY GD can be attributed to *SF1* haploinsufficiency.[57]

However, three cases have reported *SF1* mutations with XY GD and adrenal hypoplasia congenita, which reflect the role of SF1 in adrenal development. Together, mutations or deletions of *SRY* and *SF1* account for 30% of XY gonadal dysgeneses, indicating that other genes critical to the testes determination cascade still remain to be discovered.

Mutations in genes involved in testis sex determination *(XH2, SOX9, WT-1, DHH)* and duplications of putative "anti-testis" genes *(WNT-4, DAX1)* are responsible for a minority of all XY GD,[58-60] but are usually associated with other syndromic features, which are outlined in Table 17.3.

Etiology and Pathophysiology of Partial or Mixed Gonadal Dysgenesis

Like many developmental disorders, there is a wide range in phenotypic variability in patient presentation. When testes dysgenesis does not involve the entirety of both gonads and the phenotype is ambiguous rather than female, the condition is called "partial" gonadal dysgenesis. The internal genitalia display varying degrees of wolffian and müllerian development, which correspond with the percent of the gonads, which are dysgenic, or "streak." The medical literature is often confusing in regards to the difference between "partial" and "mixed" GD, which are often used interchangeably. Partial XY GD refers to intermediate stages of dysgenic testes, between streak gonads and normal testes, and usually has a 46,XY, nonmosaic karyotype. However, mixed 46,XY GD typically refers to a situation in which one gonad is a streak gonad while the opposite gonad is partially dysgenic or a normal testis. Mosaicism for 45,X/46,XY is the most frequent cause of mixed GD, although a minority have a 46,Xi (Yq) karyotype.[61] The evidence of the variable phenotypic spectrum of individuals with 45,X/46,XY mosaicism was reported in a series of 10 45,X/46,XY patients in which 4 individuals were undervirilized males with bilateral testis, 3 were diagnosed with mixed GD and genital ambiguity, and 3 were diagnosed with Turner syndrome.[62] While not proven, there is preliminary evidence that percent of normal testicular tissue and phenotypic maleness is correlated with an increase in the proportion of gonadal Y chromosome.[63,64]

Familial cases of pure and partial 46,XY, GD[13,14,50,65] have been reported. In some of these cases, *SRY* mutations present with phenotypic variability in which the father and male relatives are phenotypically normal and fertile, while their XY offspring have gonadal dysgenesis.[50,65,66] From these cases, it is clear that autosomal genetic modifiers influence the sex determination cascade and ultimate phenotypic appearance of the individual. There are also cases of fathers who are mosaic for mutant and normal *SRY* transmitting the mutation to their XY female daughter.[67,68] Presumably, the normal father's mosaicism reflects a postzygotic mutation event. These unique cases provide more hints about the molecular basis of sex determination. This is particularly relevant since the genetic cause of 70% of 46,XY GD remains unknown.

Presentation and Diagnosis of Gonadal Dysgenesis

Pure XY GD presents as a phenotypic female with normal or tall stature, bilateral streak gonads, delayed puberty, amenorrhea, small or normal müllerian structures, and without signs of Turner syndrome. If patients are not diagnosed at birth (e.g., when *in utero,* karyotype does not match the phenotypic sex at birth) most patients will be diagnosed during adolescence due to pubertal delay and primary amenorrhea. Patients can rarely present with an abdominal or pelvic mass, which often is a gonadoblastoma. Occasionally, XY GD can be part of a constellation of other symptoms, which are outlined in Table 17.3.

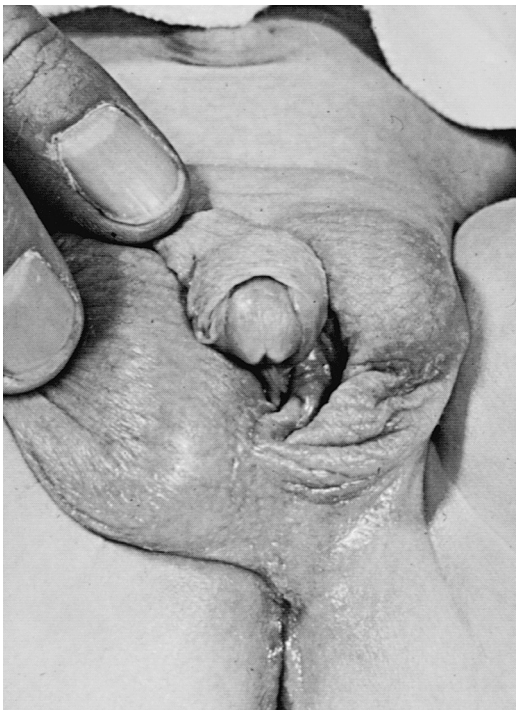


FIGURE 17.8 External genitalia of infant with XO/XY mosaicism. Note enlarged phallus, labioscrotal fusion, and gonadal bulges (histologically, testes) in labioscrotum.

Individuals with partial or mixed 46,XY GD typically present at birth with varying degrees of masculinization of external and internal genitalia. Depending on the percent of testicular tissue, dysgenetic testes can be found anywhere along the line of testes descent, from the abdomen, and in cases of normal testes, in the scrotum. However, the streak gonad in mixed 46,XY GD is always abdominally located. Patients with partial 46,XY GD usually have female external genitalia with some degree of virilization, such as clitoromegaly or a bifid scrotum (Fig. 17.8). Uterus and fallopian tubes are usually well-formed, but occasionally may be hypoplastic. In mixed GD, the development of wolffian and müllerian structures as well as the virilization of external genitalia correlates with the degree of development of the ipsilateral testis resulting in asymmetric virilization of the external or internal genitalia and unilateral cryptorchidism. Pelvic ultrasound often can detect presence of absence of male or female internal genitalia and in the case of mixed XY GD, asymmetry in development of the müllerian and wolffian structures. There are rare reported cases of patients presenting with premature adrenarche in an otherwise unambiguous female, due to a testosterone-producing gonadal tumor.[69]

Isolated 46,XY pure GD is considered in an adolescent with primary amenorrhea and sexual immaturity with a full female external phenotype, while partial or mixed GD should be considered more likely in the differential diagnosis of a 46,XY patient with ambiguous genitalia. The biochemical changes in complete, partial, and mixed GD are outlined in Table 17.4.

Table 17.4 Diagnostic Criteria for Disorders of Sex Development

	DSD	Biochemical Changes	Differentiating Features	Major Genetic Diagnostic Criteria
46,XY Disorders of Gonadal Development	46,XY pure/partial/mixed gonadal dysgenesis	Pure/partial/mixed ↑FSH, LH Nml to ↓ AMH No ↑ with hCG NmL adrenal hormones and precursors Pure ⇓; T, DHT, E2 ⇓ AMH Partial/mixed ↓ T, DHT, E2 ↓ T, DHT, E2	Presence of partial testicular function (T, AMH) points towards partial or mixed GD. However, histological examination of testes after prophylactic gonadectomy can differentiate between pure, partial, and mixed GD. There is little genotype-phenotype correlation, however the presence of mosaicism 45,X/46,XY is often associated with mixed GD.	Clinical whole exome sequencing or Sanger sequencing can be performed to assess for mutations in known DSD genes. Genetic mutations in SF1, SRY, MAP3K1 provide a definitive genetic diagnosis. If partial/mixed GD suspected, patient should be tested for mosaicism. CGH should be performed to look for copy number[71] variants as a source for XY, GD.
	Testicular regression	↑ FSH, LH ⇓ T, DHT, E2 ⇓ AMH No ↑ with hCG	Complete absence of gonad or fibrosis of gonad (as opposed to streak of dysgenic testes in GD patients). Completely virilized male phenotype.	Fibrous nodules on laporoscopic exam, (not streak of dysgenic testes)
Disorders of Sex Differentiation: Disorders in Androgen Synthesis and Action	StAR deficiency; P450scc deficiency	↑ Renin ↓ Aldo ↑ K ↓ Na ↓ All adrenal hormones ↓17-OHP	Presence of lipid vacuoles in adrenals on histology. P450scc deficiency does present with enlarged adrenals. No HTN and hyperkalemia differentiates from CYP 17 deficiency	Presence of lipid filled vacuoles on histology; clinical whole exome sequencing or Sanger sequencing can be performed to assess for mutations in known DSD genes. Identification of mutations in the StaR or P450scc genes gives a definitive diagnosis.
	3βHSD type II	↑ Renin ↓ Aldo, F ↑ K ↓ Na ↑ Ratio Δ^5-17-pregnenolone: cortisol ↓ Δ4A, T	Baseline and ACTH-stimulated ratios of Δ^5-17-pregnenolone:cortisol consistently distinguished between patients affected and non-affected patients.	Clinical whole exome sequencing or Sanger sequencing can be performed to assess for mutations in known DSD genes. Mutations, deletions, and insertions in the 3βHSD type II gene provide a definitive genetic diagnosis.
	17α-hydroxylase/17,20-lyase	↓ Renin ↓ Aldo, F ↓17-OH Progesterone ↑ LH, FSH ↑ Progesterone, DOC, B ↑ Na ↓ K ↓ DHEA-S, Δ4A, T; no response to hCG stim	Hypertension and hypokalemic alkalosis in the presence of low 17-OH Progesterone. Isolated 17,20-lyase deficiency would have decreased levels of sex steroids with normal mineralocorticoids and normal glucocorticoids.	Clinical whole exome sequencing or Sanger sequencing can be performed to assess for mutations in known DSD genes. Identification of mutations of CYP17 or CYPB5 gene provide a definitive diagnosis.
	P450 oxidoreductase	↑ 17OH-progesterone ↑ Progesterone ↓ F, DHEA-S ↓ Δ4A, T	Hypertension, in the presence of elevated 17-OH progesterone (differentiates from CYP17 deficiency); sometimes presence of Antley-Bixler skeletal malformations.	Clinical whole exome sequencing or Sanger sequencing can be performed to assess for mutations in known DSD genes. Mutations in CYP450 oxidoreductase provide a definitive diagnosis.
	17βHSD Type 3	Nml to ↑ Δ4A ↑ ratio Δ4A/T (>15) ↓ T, DHT	Differentiated from 5α-reductase Type 2 by levels and ratios of serum hormones.	Clinical whole exome sequencing or Sanger sequencing can be performed to assess for mutations in known DSD genes. Sequencing of the 17βHSD gene for deletions, insertions, and point mutations.
	Leydig Cell Hypoplasia	↑ LH Nml FSH ↑ AMH ↓ T, DHT, E_2 ↓hCG response Nml Δ4A/T ratio	To differentiate LCH from GD, AMH is used as a marker of testicular function.	Clinical whole exome sequencing or Sanger sequencing can be performed to assess for mutations in known DSD genes. Deletions, insertions, and point mutations in the *LHR* gene provide a definitive diagnosis.

Table 17.4 Diagnostic Criteria for Disorders of Sex Development—cont'd

	DSD	Biochemical Changes	Differentiating Features	Major Genetic Diagnostic Criteria
	5α-Reductase Type 2 Mutations in the backdoor pathway to androgen activity.	Nml FSH, LH Nml T, E_2 ↓ DHT ↑ ratio T/DHT (>30) Nml ratio T/DHT (>30) Nml AMH Nml cortisol ↓ DHEA-S after hCG ↑ 17OH-pregnenolone	Development of male secondary sex characteristics in puberty with fine and sparse facial hair. These can be differentiated clinically from 17βHSD and AIS by the lack of gynecomastia during puberty. Similar in phenotype to 17βHSD and 5-alpha reductase deficiency but can be differentiated by serum hormone levels.	Clinical whole exome sequencing or Sanger sequencing can be performed to assess for mutations in known DSD genes. Point mutations, deletions, insertions, and parental isodisomy in *SRD5A2* gene provides a definitive genetic diagnosis. Clinical whole exome sequencing or Sanger sequencing can be performed to assess for mutations in known DSD genes. Mutations in *AKR1C2* and *AKR1C4* gene that result in a reduction of their enzymatic activity provides a definitive genetic diagnosis.
	Androgen receptor	Nml FSH, LH (PAIS) ↓ FSH, LH (CAIS) Nml to ↑ AMH Nml Δ4A, T, DHT; nml ratios ↑↑ hCG response	Female phenotype with breast development at puberty, with sparse pubic and axilla hair	Clinical whole exome sequencing or Sanger sequencing can be performed to assess for mutations in known DSD genes. Sequencing of AR gene looking for single amino acid substitutions, which account for 90% of reported cases.
	AMH/AMHR	Nml hormonal profile	Presence of both müllerian and wolffian derivatives, usually discovered incidentally.	
46,XX Disorders of Sex Determination	XX Testicular/ovo-testicular DSD	↑ FSH, LH ↓T, DHT Nml AMH No ↑ with hCG	Only way to differentiate between 46,XX testicular and ovotesticular DSD is by complete gonadal histological examination looking for the presence of ovarian tissue.	Testicular DSD: FISH for presence of SRY or SOX9 or if clinical phenotype appropriate, sequence RSPO1 gene. If no molecular diagnostic is successful, CGH should be performed. Ovotesticular DSD: Search for XX/XY mosaicism should be done (30%-33% ovotesticular DSD) and sequencing for SRY should be done (7%-10% of ovotesticular DSD).
	XX Gonadal dysgenesis	↑ FSH, LH No ↑ with hCG No AMH	Full female phenotype with amenorrhea and lack of secondary sex development	For cases of isolated 46,XX GD, sequencing for FSH mutations can be performed If clinical presentation matches, sequence FOXL2 mutation. CGH can also be done to identify delirious duplications or mutations.
Disorders of Androgen Excess	21α-hydroxylase	↑ Renin ↓Aldo, DOC, F ↑ K ↓ Na ↑ 17OH-progesterone ↑ DHEA-S, Δ4A, T	17-OH Progesterone elevated in the absence of hypertension, which is typical in 11βHSD1 deficiency.	Sequencing of CYP21 gene gives definitive diagnosis. Knowledge of the mutation can inform parents about genetic counseling and fetal diagnosis to prevent adverse effects of hyperandrogenism *in utero.*
	11βHSD1	↓ Aldo, F, Renin ↑ DOC ↑ K ↓ Na ↑ 17OH-progesterone ↑ DHEA-S, Δ4A, T	Differentiate from CYP21 deficiency by presences of hypertension and hypokalemic alkalosis.	Clinical whole exome sequencing or Sanger sequencing can be performed to assess for mutations in known DSD genes. Mutations in 11βHSD1 gives a definitive diagnosis.
	P450 aromatase	↑ 16OH-Δ4A (maternal) ↑ FSH, LH, Δ4A, T ↓Estrone, E_2	The presence of maternal virilization during pregnancy and XX virilization which stops after delivery.	The presence of maternal virlization during pregnancy and XX virilization which stops after delivery. Clinical whole exome sequencing or Sanger sequencing can be performed to assess for mutations in known DSD genes. Mutations in p450 aromatase gives definitive diagnosis.

The major criteria for the diagnosis of pure, partial, or mixed gonadal dysgenesis are the appearance and histology of both gonads. Therefore, the ultimate diagnosis of any of the forms of gonadal dysgenesis, particularly the distinction between mixed and partial GD, requires biopsy of both gonads. Since the risk of gonadoblastoma in these patients is so high, precise diagnosis of the type of gonadal dysgenesis is usually determined after prophylactic or therapeutic gonadectomy. In pure 46,XY GD both gonads are streak gonads. Partial GD is defined by bilateral dysgenetic gonads, while mixed GD typically has one streak gonad. Karyotype of peripheral leukocytes shows 46,XY in pure and partial GD, and mosaic 45,X/46,XY is frequent in mixed GD. However, if there is a streak gonad on one side and a normal testis contralaterally, but the peripheral karyotype is 46,XY, cryptic mosaicism can often be revealed within the gonad.

Once a presumptive diagnosis is made in 46,XY patients, FISH for *SRY* or for Yp can be performed. Only a minority of cases of XY GD can be explained by complete or partial *SRY* deletion or mutation, or *SF1* mutation. The majority of patients are 46,XY and positive for *SRY* and *SF1*. Sequencing of the *SRY* or *SF1* open reading frames for mutations is only positive in up to 30% of 46,XY pure GD.[49,50,57,67] Certain isolated and syndromic forms of XY gonadal dysgenesis can be diagnosed molecularly with cytogenetics, comparative genomic hybridization, or sequencing of known genes (Table 17.4). 46,XY DSD in conjunction with congenital heart disease may increase the suspicion for a *GATA4* mutation.[68] Whole-genome CGH array studies have identified many rare copy number variants associated with patients with 46,XY gonadal dysgenesis and 46,XX testicular and ovotesticular DSD,[70,71] including deletions and duplications in and around *SOX3*,[72] *GATA4*,[70] *WWOX*,[73] and *DMRT1*.[71] Deletions or duplications in the up-stream promoter region of *SOX9* are associated with isolated and familial cases of 46,XY gonadal dysgenesis and 46,XX testicular DSD.[74,75]

If a mutation or translocation involving *SRY* is found, the father should be tested for a possible familial mutation since *SRY* mutations can result in a full spectrum of phenotypes, from 46,XY fertile males to ambiguous individuals to females with partial and pure GD. Due to potential autosomal modifiers, it is very difficult to predict the risk of recurrence of a GD phenotype in XY offspring.

Management of XY Gonadal Dysgenesis

The major concern in the treatment of patients with XY GD is the risk of gonadoblastoma, a mixed germ-cell, sex-cord tumor. The risk of gonadoblastoma formation in XY GD patients increases with age and has been estimated to be as high as 30% by 30 years of age.[54,76,77] Due to the high risk of gonadoblastoma formation, patients should undergo prophylactic or therapeutic gonadectomy preferably in the first decade of life. Patients are followed with regular ultrasounds every 6 months starting at age 2 until gonadectomy can be performed. With the advent of laparoscopic techniques, the invasiveness of these surgeries is decreasing and the results are improving.[77]

In patients with XY pure GD, genitals and gender identity are unambiguously female. There are no reported cases of gender dysphoria. In patients with partial or mixed GD, the degree of genital masculinization can be used for initial gender assignment. However, issues of gender identity do arise in partial and mixed GD and are outlined further in the management section.[78]

In order to complete secondary sex development, sex steroid replacement should be initiated at puberty. Sex steroid replacement is essential for not only development of secondary sex characteristics but also growth spurt, and normal accrual of bone mineral density. Height, weight, and bone density should be monitored regularly. Furthermore, a dual-energy x-ray absorptiometry (DEXA) scan for bone density should be performed prior to induction of puberty with exogenous hormone replacement, and yearly thereafter for the first 2 years. As long as the first three DEXA scans are reported as normal, they can be done every 2 to 3 years.

Since a normal uterus is often present, hysterectomies are not common in order to preserve child-bearing potential with *in vitro* fertilization methods. Despite this, most patients do not carry successful pregnancies for unknown reasons.[7] Mixed XY GD individuals with more masculinization of the external genitalia are often raised as males and consequently require lifetime testosterone therapy, through intramuscular injections or transdermal patches or gel.

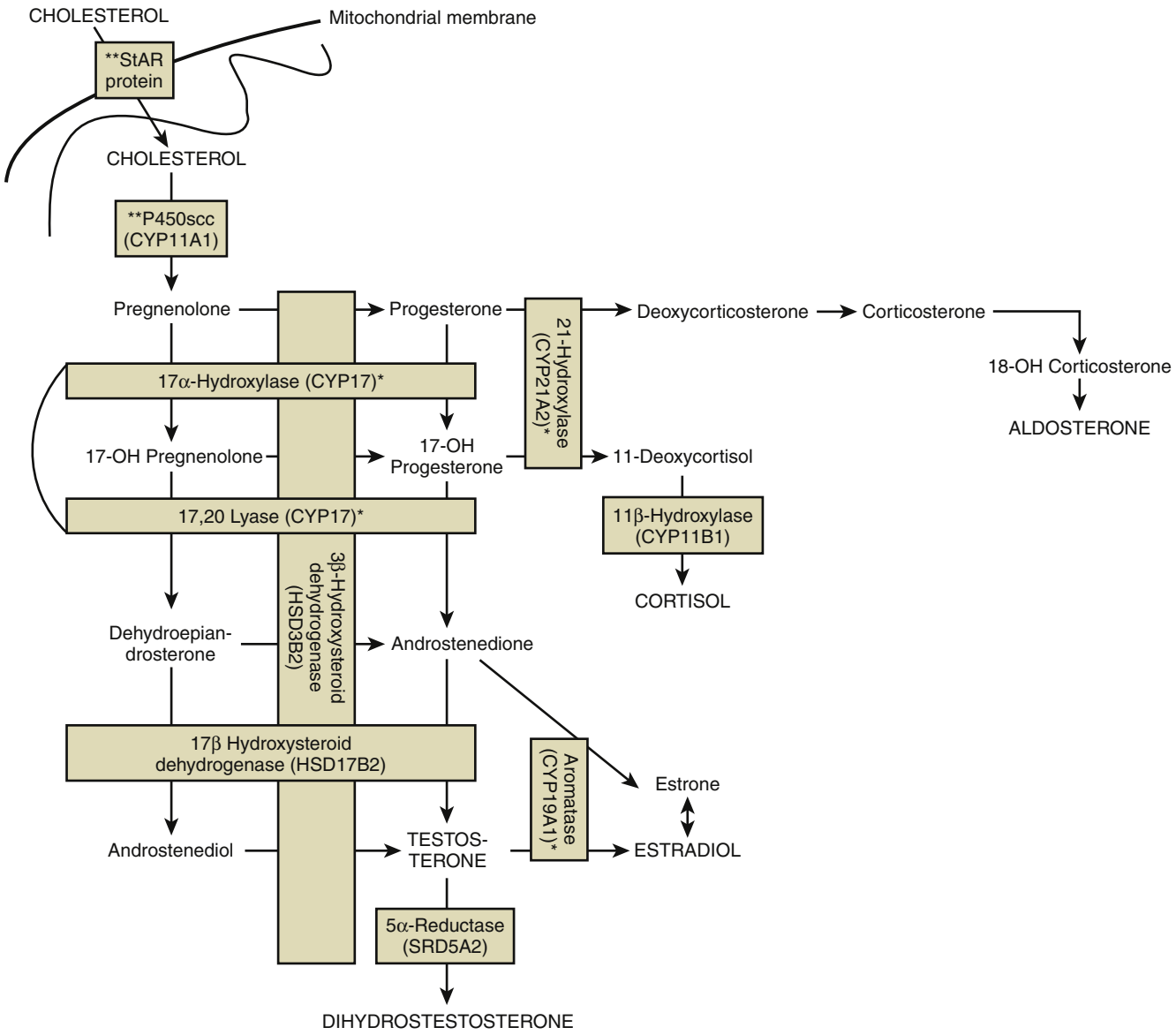
Testicular Regression Syndrome

In some cases, initial testicular development occurs normally, but there is an insult to the developing testes during sex differentiation, resulting in variable degrees of masculinization of the internal and external genitalia. Testicular regression syndrome (TRS), also known as bilateral anorchia, is distinct in that the insult typically occurs after initial testes determination.[79-81] Internal and external genitalia are variably developed, which presumably depends on when the loss of fetal testes occurred *in utero*.[80-83] Rarely, patients can present in adulthood as females with primary amenorrhea.[82,83] Typically, however, TRS is characterized by primitive epididymis and spermatic cord in the presence of a fibrous nodule rather than streak, or dysgenetic gonads.[79,80,84] The presence of spermatic cord structures, the absence of müllerian derivatives, and normal male external phenotype suggest viable testes existed early in development, and imply a late fetal or early neonatal regression. Diagnostic criteria are outlined in Table 17.4. In the literature, this variant is often called "vanishing testes."[79]

The incidence of TRS has been estimated at 5% of males presenting with cryptorchidism,[79] and as high as 12% of cryptorchid patients older than 1 year.[79,85] Correct diagnosis of TRS versus XY, GD is essential because of significant malignant potential of abdominal or dysgenic testes. Mutational analysis of *SF1* as a cause of TRS did not find any association between mutations in *SF1* and TRS.[86]

Disorders in Sex Differentiation: 46,XY Disorders of Androgen Biosynthesis and Action

46,XY DSD, with phenotypes ranging from full sex reversal to undervirilized XY males, can be a result of defects in steroidogenic enzymes (Fig. 17.9) or their receptor action. The biosynthesis of testosterone is essential for development of secondary sex characteristics in XY individuals, including differentiation of wolffian structures, inguinoscrotal



*P450 oxidoreductase deficiency results in partial activity of this enzyme.
**Both of these proteins result in congenital lipoid adrenal hyperplasia.

FIGURE 17.9 Adrenal steroidogenesis pathways. Many disorders of sex differentiation are due to defects in steroidogenesis, with the majority of them occurring in the adrenal gland.

testes descent, and masculinization of external genitalia after conversion to DHT. Additionally, mutations in Sertoli cell products, such as anti-müllerian hormone or its respective receptor can result in incomplete regression of female internal genitalia. Mutations in the synthesis and action of testosterone, or up-stream regulators of testosterone production, can all cause incomplete virilization or müllerian regression in the XY male. However, in these individuals, the testes are of normal size.

Congenital Lipoid Adrenal Hyperplasia

Mutations in genes encoding proteins involved in the initial enzymatic regions of steroidogenesis, such as steroidogenic acute regulatory protein (STAR) and cytochrome P450 side chain cleavage (P450scc, also known as *CYP11A1)*,[87,88] result in global silencing of adrenal and gonadal steroidogenesis. The accumulation of cholesterol in the adrenals and gonads, ultimately results in primary adrenal and gonadal failure.[89] StAR protein transports cholesterol across the inner and outer mitochondrial membranes of steroidogenic cells. P450scc catalyzes the initial reaction in all steroidogenic tissues, the conversion of cholesterol to pregnenolone (Fig. 17.9).[90,91] On adrenal histology, patients with mutations in StAR protein or P450scc have lipid vacuoles. However, patients with *STAR* mutations have adrenal hyperplasia, while the six patients with *P450SCC* mutations did not exhibit adrenal enlargement.

Presentation and Diagnosis

Newborns with congenital lipoid adrenal hyperplasia usually present with a salt wasting adrenal crisis at birth which

is usually fatal if not immediately diagnosed and treated, however there are reports of delayed presentation. All XY patients present with a female phenotype or, in cases of partial defect, some degree of genital ambiguity. XX patients only exhibit the features of adrenal insufficiency at birth.

46,XY patients have testes, no müllerian structures due to the presence of AMH, partial or absent wolffian derivates due to a lack of testosterone biosynthesis, and a blind vaginal pouch. The spectrum of external genital phenotype ranges from female external genitalia to ambiguous genitals. Testes can be in the abdomen, inguinal canal, or labia. Additionally, intrauterine glucocorticoid deficiency results in elevated adrenal corticotropic hormone ACTH levels, which manifests clinically as generalized hyperpigmentation at birth.

Partial deficiency of StAR protein may result in 46,XX patients who can experience spontaneous puberty, menarche, and anovulatory menses because their ovaries are able to produce estrogen through StAR-independent pathways.[89,92,93] However, at puberty, XX females develop multiple cysts in their ovaries, possibly from anovulation. Since the ovaries do not produce steroids until puberty, they are spared from the cholesterol-induced damage, which occurs in the adrenals from birth.[93]

Definitive diagnosis of congenital lipoid adrenal hyperplasia is done by sequencing of *StAR* or *CYP11A1*. Characteristic biochemical abnormalities are outlined in Table 17.4.

Management

Patients should be given physiological replacement doses of glucocorticoid and mineralocorticoids. At the onset of puberty patients should be given sex hormones in concordance with phenotypic sex.

3β-Hydroxysteroid Dehydrogenase Deficiency

This rare variant of congenital adrenal hyperplasia can have a wide range of phenotypes, and ranging from classical presentation (salt wasting, with a range of female external genitalia to ambiguous genitalia in genetic XY individuals) to non-classical (no salt wasting and later presentation). Rare defects in adrenal and gonadal 3β-hydroxysteroid dehydrogenase *(HSD3B2)* affect the synthesis of three major adrenal steroid hormones: cortisol, aldosterone, and testosterone synthesis. The resulting phenotype is adrenal insufficiency in XX and XY patients but genital ambiguity only in XY patients.

17α-Hydroxylase/17,20-Lyase Deficiency

Biosynthesis of adrenal and sex hormones require cytochrome P450 17α-hydroxylase/17,20-lyase (P450C17). Here, a single gene, *P450C17* catalyzes two steps in the steroidogenic pathway, which are essential for sex steroid and glucocorticoid synthesis. Mutations in this gene, ultimately result in decreased cortisol synthesis with shunting of steroidogenesis towards a mineralocorticoid precursor that has mineralcorticoid activity (Fig. 17.9). Isolated cases of 17,20-lyase deficiency are caused by mutations in Cytochrome b5 *(CYB5A)*, a gene that promotes the specific allosteric interaction between CYP17A1 and POR.[94,95]

Presentation and Diagnosis

Most patients present at puberty with primary amenorrhea, low-renin hypertension and hypokalemia (due to elevated mineralocorticoids). This autosomal recessive disorder results in ambiguous genitalia only in 46,XY patients. 46,XY affected individuals have phenotypes ranging from female external genitalia with no wolffian duct development to ambiguous genitalia with partial development of the male ductal system, depending on the severity of the enzymatic defect. All patients show müllerian structure regression, due to presence of functional Sertoli cells. In contrast to individuals with 3βHSD deficiency or Lipoid CAH, patients do not develop adrenal crisis due to the presence of glucocorticoid precursors that maintain some activity.[96] Serum diagnostic criteria are outlined in Table 17.4. Rare C*YP17A1* mutations which cause isolated 17,20-lyase deficiency[97] only affects sex steroid biosynthesis and results in variably feminized 46,XY individuals with normal levels of cortisol.

Management

Patients should be treated with physiological levels of glucocorticoids and appropriate sex steroid replacements to develop secondary sex characteristics.

P450 Oxidoreductase Deficiency

Cytochrome P450 oxidoreductase (POR) deficiency is a recently characterized cause of congenital adrenal hyperplasia, which affects the enzymatic activity of all microsomal P450 enzymes, including the steroidogenic enzymes P450c17, P450c21, and P450aro.[98-100] POR is a flavoprotein that is required for the action of all microsomal p450 enzymes. Unlike other genetic defects, which only affect one enzyme in the steroidogenesis pathway (Fig. 17.9), POR causes partial enzymatic activity in multiple enzymes. Therefore, POR deficiency presents with a wide range of phenotypes and is particularly difficult to diagnose using serum hormone levels. Partial enzymatic deficiencies in 21α-hydroxylase account for the CAH phenotype and virilization in XX females, while partial 17α-hydroxylase deficiency accounts for undervirlization of XY individuals. Aromatase deficiency secondary to POR deficiency is the likely cause of virilization of mothers carrying fetuses with POR deficiency.

In addition to the genital phenotype, some POR deficiency patients present with a complex skeletal phenotype known as Antley Bixler syndrome (ABS). These skeletal malformations, include but are not limited to craniosynostosis, radiohumeral synostosis, midface hypoplasia, and choanal stenosis.

Presentation and Diagnosis

The unique pathophysiology of POR deficiency results in a wide phenotypic spectrum, in which XX and XY patients present in the prenatal period, as newborns with ambiguous genitalia, or as XX adolescents with amenorrhea and poly cystic ovarian syndrome (PCOS). During prenatal screening, this diagnosis should be considered in the differential of a pregnant woman with low estriol levels but with a normal ultrasound exam and virilization. Endocrine changes are shown in Table 17.4. The majority of patients have compound heterozygous mutations in *POR*. The most common mutation is Ala287Pro in Caucasians,[101] and Arg457His in

Japanese patients.[102] More severe genital and skeletal malformations are associated with loss of function alleles, while missense mutations result in mild to moderate genital and skeletal malformations.[101]

17β-Hydroxysteroid Dehydrogenase Deficiency

The17β-HSD3 enzyme is a member of the 17β-HSD family, which catalyzes the conversion of Δ4A to testosterone in the testes (Fig. 17.9). However, isoenzymes 17β-HSD2 and 17β-HSD4 are widely expressed in peripheral tissues postnatally. This is a rare cause of 46,XY DSD, with less than 30 reported cases in the literature.

Presentation and Diagnosis

In XY individuals with female external genitalia, HSD17B3 deficiency may be difficult to recognize in infancy since they manifest no other significant clinical problems and near normal lab values. However, depending on the degree of residual enzyme activity, *HSD17B3* deficiency can cause a range of undervirilization of XY individuals at birth, from an empty bifid scrotum with perineoscrotal hypospadias to clitoromegaly and posterior labioscrotal fusion. Testes can be undescended or labial. Müllerian ducts are absent but interestingly, wolffian duct derivatives are present, despite the decreased testosterone production. During puberty, 46,XY patients may experience progressive adrenarche, including virilization, clitoral-phallic enlargement, muscle and breast development, testicular descent, and plasma T levels within the normal range of pubertal males.[103,104] To date, no affected 46,XX females have been reported. Biochemical changes in patients with *HSD17B3* mutations are outlined in Table 17.4.

Management

XY patients can be raised as females[96,98] or males. However, in one study, up to 40% of affected XY individuals who were raised as female successfully switched gender roles at puberty.[98] General management is covered below.

Defects in 5α-ReductaseType 2

Steroid 5α-reductase deficiency (SRD5A2) results from a defect in the enzyme converting testosterone to dihydrotestosterone (DHT). DHT is responsible for the differentiation of male external genitalia (Fig. 17.2). Peripheral conversion of testosterone to DHT is an irreversible reaction catalyzed by the two isoenzymes of 5α-reductase, SRD5A1 and SRD5A2. During puberty, the SRD5A1 isozyme is active in skin fibroblasts. During fetal development, SRD5A2 isozyme is expressed in the genital skin tissue and male accessory sex organs. Mutations in *SRD5A2* result in undermasculinized external genitalia (e.g., micropenis, perineal hypospadias). Examination of internal genitalia demonstrates normally differentiated wolffian ducts, with no müllerian structures, in addition to a blind vaginal pouch.[105,106] Adrenal and gonadal biosynthesis remains normal.

Presentation and Diagnosis

In 46,XY infants with mutations in *SRD5A2*, the degree of ambiguity ranges from isolated hypospadias to severe undermasculinization, complete with perineal hypospadias, micropenis, bifid scrotum, and hypoplastic prostate.[107] At puberty, 46,XY patients experience a surge of testosterone, which can cause development of male secondary sex characteristics, such as male body habitus, deepening of the voice, and penile enlargement. However, these individuals have unusually fine upper lip hair. Due to the underdevelopment of the prostate and seminal vesicles, their semen is highly viscous and their ejaculate volume is extremely low (<1 mL). Despite the low ejaculate volume, sperm counts are normal. Patients who are unambiguously female at birth often present in puberty with complaints of amenorrhea, a deepening voice, clitoromegaly, and increased musculature. In contrast to patients with 17β-HSD3 deficiency and androgen insensitivity syndrome, mutations in SRD5A2 do not develop gynecomastia during puberty. 46,XX females with SRD5A2 deficiency have normal sexual differentiation and fertility, but delayed puberty and sparse pubic hair.

Diagnosis of SRD5A2 deficiency during early infancy and puberty can be made based on an elevated ratio of testosterone to DHT (normal <30:1), either with or without human chorionic gonadotropin (hCG) stimulation.[105,108] DHT is low in infants but can reach near-normal levels during adolescence without treatment, presumably by peripheral SRD5A1 activity. If the diagnosis is suspected during pre-pubertal childhood or during adolescence, hCG stimulation is necessary to elicit the diagnostic hormone levels since the hypothalamo–pituitary–gonadal axis is inactive. Affected females are phenotypically normal, but have the same biochemical abnormalities (outlined in Table 17.4) as affected males.

Mutational analysis of the *SRD5A2* gene can be performed, and mutations have been reported in all five exons. 5-alpha reductase deficiency results from either homozygous or compound heterozygous mutations. To date, over 50 mutations in *SRD5A2* have been found, ranging from point mutations resulting in missense or nonsense mutations, deletions, and uniparental isodisomy.[109]

Management

This autosomal recessive disorder has been described in a number of consanguineous families in Turkey, New Guinea, Saudi Arabia, and the Dominican Republic. Many XY patients with this disorder are raised as female during childhood, but upon the onset of puberty identify with the male gender.[104,110,111] It is not clear why there is such a high prevalence of change in gender identity in this disorder, but it does not appear to be related to the degree of external genitalia masculinization at birth.[112] Topical DHT cream application in the pubic area promotes phallic growth prior to puberty and local application results in increased facial and body hair. These patients are not spontaneously fertile despite their normal sperm counts due to their highly viscous ejaculate. However, fertility is possible with surgical correction of the male ductal system or through assisted reproductive technology.

"Backdoor" Pathway to Fetal Testicular Androgen Production

An alternative pathway towards androgen synthesis has been described in familial and isolated human patients with 46,XY DSD. The severity of the disorder is dependent on the number of mutations in two genes that facilitate the conversion of 17-OH progesterone to DHT. The number of allelic mutations in both *AKR1C2* and *AKR1C4* determine the severity of the genital phenotype.[113]

Isolated 46,XY DSD with Apparently Normal Testosterone Levels

Mastermind-like domain containing 1 *(MAMLD1)* is expressed during fetal Leydig cell development and important in modulation of fetal testosterone synthesis. Nonsense mutations in *MAMLD1* have been identified in several patients with isolated 46,XY DSD with severe hypospadias and microphallus.[114,115] Mutations in *MAMLD1* result in a decrease in MAMLD1's transactivating properties[116] and a reduced testosterone and CYP17A1 expression *in vitro*.[117] Specific polymorphisms in *MAMLD1* have been associated with hypospadias.[118]

Defect in Androgen Activity

Complete and Partial Androgen Insensitivity Syndrome

In the presence of normal gonads and steroid biosynthesis, mutations in the steroid hormone receptors can also mute the effects of circulating steroid on specific tissues. Mutations in the androgen receptor (AR), which is located on the X-chromosome, result in androgen insensitivity syndrome (AIS). Both testosterone and DHT can bind to AR, and transcriptionally activate downstream genes. AR plays important roles in the differentiation of male internal and external genitalia and in the maintenance of spermatogenesis. Currently, there is an estimated incidence of 1:20,400 liveborn XY individuals, in whom more than 300 mutations have been identified.

AIS is characterized by a range of phenotypes in 46,XY individuals, from unambiguous XY female, termed complete AIS (CAIS) phenotypic XY males. In CAIS, the major reproductive manifestation is amenorrhea, typically presenting at puberty, and the associated infertility.[119,120] At the other extreme is minimal AIS (MAIS) in which phenotypically male patients often present with infertility, gynecomastia, or hypospadias. Patients who exhibit various degrees of XY ambiguity are referred to as partial AIS (PAIS). CAIS is generally associated with a complete absence of androgen binding and AR activation. Beyond this, there is almost no correlation between the percentage of residual AR activity and phenotype.[121] This suggests that there exist other genetic modifiers that modulate the phenotype. Even within families, the phenotype resulting from the same mutation can vary between PAIS and CAIS.[122,123]

The majority of mutations resulting in AIS are caused by single amino acid substitutions, which account for approximately 90% of the cases. Mutations are found throughout the gene, with the majority of mutations located within the ligand-binding domain. Exon 1 rarely possesses a causative mutation.[124] The majority of cases are inherited, but 30% of all AIS cases are *de novo* mutations which is consistent with other X-linked traits.[125]

In familial PAIS, and in both familial and sporadic CAIS, mutations in *AR* exonic sequences are found in 85% to 90% of cases.[122,124] In contrast, detectable mutations in *AR* account for only 10% to 15% of sporadic cases with hormonal profile and clinical presentation suggestive of *de novo* PAIS,[122] and the molecular defect in these cases is unknown. While the genotypes causing CAIS are consistent in phenotypic presentation, in PAIS there is phenotypic variability among affected individuals carrying the same mutation.

Presentation and Diagnosis

The phenotype of patients with PAIS is extremely heterogeneous. Patients present in infancy or childhood with variable degrees of virilization such as micropenis, cryptorchidism, and perineoscrotal hypospadias. Infants and children may present with unilateral or bilateral inguinal hernias. Testes are present and functional, producing high levels of testosterone and AMH resulting in variable wolffian derivative development and regression of müllerian structures in most patients.[126] In patients presenting at puberty, breast development and sparse pubic hair are suggestive of PAIS, and help differentiate it from a SRD5A2 deficiency.

CAIS usually presents at puberty with primary amenorrhea. Physical exam reveals a short, blind vagina, absent uterus, and sometimes palpable inguinal or labial testes. Testosterone dependent wolffian derivatives and prostate are absent or vestigial. Height, bone maturation, and breast development are normal but pubic and axillary hair, an androgen-mediated feature, is absent or sparse. Patients' identity and behavior are feminine without gender dysphoria.[127] Less commonly, CAIS may present in infancy with phenotypic female genitalia and inguinal or labial masses representing testes.

Diagnostic criteria for serum hormone levels are outlined in Table 17.4. In prepubertal children, basal luteinizing hormone (LH) and testosterone may be normal, but hCG stimulation elicits an exaggerated androgen response (a tripling instead of a doubling of testosterone and DHT). Exam and pelvic ultrasound reveals abdominal testes and the absence of müllerian structures.

Molecular genetic testing of the *AR* gene detects mutations in more than 95% of probands with CAIS. Molecular testing has been shown to be more consistent than biochemical functional assays of AR function which has been discredited based on high degree of variation due to biopsy site and testing laboratory.[128] PCR-based sequencing of *AR* exons 2 to 10 can be routinely performed,[129] as well as sequencing of the much longer exon 1, and some intronic and promoter regions. Prenatal testing by mutation analysis is available for families in which the AIS-causing allele has been identified in an affected family member.[122]

Luteinizing Hormone Receptor Defects

Leydig Cell Hypoplasia-Agenesis

Leyding Cell Hypoplasia (LCH) is a disorder caused by inactivating mutations of hCG/LH receptor (LHR) and characterized by impaired Leydig cell differentiation and testosterone production. HCG and LH activate a shared G protein-coupled hCG/LH receptor. *In utero*, placental hCG stimulates Leydig cells to produce testosterone, resulting in male internal and external genitalia. LH takes over during the third trimester of gestation and neonatal life to complete Leydig development and continue testosterone production.

Over 20 inactivating mutations of *LHR* have been identified scattered throughout the gene,[130,131] which cause variable degrees of loss of receptor activity. More severe mutations resulting in truncation, decreased surface expression, or decreased coupling efficiency are usually associated with unambiguous female phenotype. Partial inactivating mutations often result in an undervirilized phenotype such

as micropenis or hypospadias.[132] One specific mutation highlights the differential binding sites of hCG and LH to the same receptor. A splice variant resulting in the absence of exon 10 was described in an 18-year-old male who presented with normal male phenotype, pubertal delay, small testicles, and delayed bone age.[133] The receptor's response to hCG was normal, inferred from normal fetal sex development. However, LHR was not activated by LH resulting in delayed puberty and bone age.

Presentation and Diagnosis

Patients with severe inactivating mutations in *LHR* are often missed at birth and present in puberty with amenorrhea. Partially inactivating mutations present at birth, with undervirilization of the external genitals (e.g., micropenis, hypospadias, and cryptorchidism). In XX females, *LHR* mutations result in hypergonadotropic hypogonadism with primary amenorrhea or oligoamenorrhea, cystic ovaries, and infertility.[134-137] However, XX females will undergo spontaneous breast and pubic hair development during puberty.

Pelvic ultrasound demonstrates absence of uterus and fallopian tubes. To distinguish LCH from patients with XY gonadal dysgenesis, AMH is used as a marker of testicular Sertoli cell function and is normal-to-high in LCH patients and low-to-undetectable in XY GD patients.[138] Histological analysis of the testis shows normal Sertoli cells, hyalinized seminiferous tubules, without mature Leydig cells or spermatogenesis. Patients with partially inactivating mutations may show some early spermatogenesis, with arrest prior to spermiation. Therefore, patients have potential for fertility using assisted reproductive technology.

Biochemical changes are outlined in Table 17.4. Definitive diagnosis requires sequencing of the *LHR* gene for deletions, insertions, and point mutations.

Disorders of Anti-Müllerian Hormone or Anti-Müllerian Hormone Receptor

Persistent Müllerian Duct Syndrome

Unlike many of the conditions mentioned previously, there is no underdevelopment of the male internal genitalia, but rather the incomplete destruction of female precursors. Mutant *AMH* or AMH receptor type II (AMHR2) in XY individuals fail to cause müllerian duct regression, causing mixed (male and female) internal genitalia called persistent müllerian duct syndrome (PMDS). During the critical time in sex differentiation, between weeks 10 and 12 of gestation, testicular AMH causes the regression of the müllerian derivatives. However, inactivating mutations can prevent this regression, resulting in both male and female internal genitalia, but normal male external genitalia.

Presentation and Diagnosis

The diagnosis of PMDS is commonly made incidentally during abdominal imaging studies or surgical exploration of the abdomen.[139] XY patients with PMDS are phenotypically male. Aside from having a uterus and fallopian tubes in addition to the male external genitalia, they often have no clinical abnormalities. Therefore the true prevalence of PMDS is difficult to ascertain. The vast majority of cases of PMDS have mutations in the *AMH* or *AMHR2* in approximately equal proportions.[140,141] Inheritance is autosomal recessive for both loci.

Boys can also present with cryptorchidism (20%) or with an inguinal hernia[142] containing müllerian structures, but normal virilization.[141,143] The increased likelihood of abdominal testes in PMDS causes an increased incidence of gonadoblastoma.[144]

46,XX Disorders of Sex Development

46,XX Disorders of Gonadal Development

46,XX Testicular DSD

The spectrum of individuals who are XX and have a disorder of sex determination have a range of phenotypes, from phenotypic males to all degrees of genital ambiguity. Gonad histology ranges from immature testis to combinations of both testicular and ovarian tissue within an individual. Compared to XY DSD, the incidence of XX testicular DSD and XX ovotesticular DSD is estimated to be much less common, at 1:20,000. Eighty-five percent of all patients with XX testicular DSD are unambiguously male[145] and possess an Xp:Yp translocation including *SRY*. One-third of all recombination occurs at the *PRKX* locus hotspot on Xp22.3 and the Y-homologue *PRKY*.[146] In these cases, there is often skewed X-inactivation in favor of the X chromosome without Y-chromosome translocation,[145,147,148] cryptic mosaicism,[145,149] or duplications of the region of chromosome 17 with *SOX9*.[70,74,75,150] The rare families where XX DSD is inherited are typically *SRY*-negative, whose members can present with either 46,XX partial or complete testicular DSD, or ovotesticular DSD.[151]

Presentation and Diagnosis

In the classic medical literature, individuals with 46,XX DSD presenting with fully masculinized genitals have been referred to as *de la Chapelle syndrome*. While some characteristics of XX testicular DSD, such as small testes, azoospermia, and gynecomastia, and lack of secondary sex development are similar between 47,XXY (Klinefelter) and XX Testicular DSD,[148] these patients are neither short nor do they exhibit the same learning and behavioral issues. In XX Testicular DSD, phallus size is small to normal and patients exhibit normal sexual function.

The majority of cases are discovered during adolescence, because the majority of patients are unambiguously male. Occasionally, patients will present earlier with undervirilized genitalia. Typically, the presence of SRY is correlated with completely masculinized genitalia. The diagnosis of most XX testicular DSD patients is outlined below in 46,XX Testicular and Ovotesticular DSD.

46,XX Ovotesticular Disorder Sexual Development (DSD)

Ovotesticular DSD (previously known as *true hermaphrodites*) is defined by the presence of both testicular and ovarian gonadal tissue within a single individual. Testicular tissue is characterized by the presence of seminiferous tubules and ovarian tissue by the presence of follicles. The presence of both tissue types can be within the same gonad, which is known as an ovotestis. Within 46,XX Ovotesticular DSD, bilateral ovotestis occurs 20% of the time, an

ovotestis with a contralateral ovary or testis occurs in 50%, and an ovary and testis opposite each other occurs in 30% of the time.[152]

The predominant tissue within the individual gonad typically determines where in the abdominal or pelvic region the gonad can be found. Gonads with high percentage of testis material are typically found in the scrotal sac, while gonads with more ovarian tissue can be found in the abdomen.[152] Both side and the amount of testicular tissue determine the virilization of the internal and external genitalia. The degree of internal genitalia development correlates with the ipsilateral gonad and, like in cases of XY DSD, can be asymmetrical. The development of a uterus and fallopian tubes can occur in the presence of an ovary or ovotestis. However, the male wolffian structures require a well-formed testis to fully develop. External genitalia development is governed by the testis hormone production.

Patients with XX ovotesticular DSD lie on the spectrum with XX testicular DSD. Both of these can be caused by skewed X-inactivation of the *SRY* carrying X-chromosome,[147,153] or by cryptic mosaicism.[145,149,154] However, 90% of all XX ovotesticular DSD patients are not *SRY*-positive.

A fraction of ovotesticular DSD are found in patients with a 46,XY karyotype and in these, there have been two reports of mutations found in *SRY*.[49,152,154] Approximately one-third of ovotesticular DSD cases are mosaics, with one of the cell lines containing Yp material.[152,154] Some of these mosaics are 46,XX/46,XY and may actually represent chimeras.[155-159]

A form of syndromic ovotesticular DSD has been shown to be due to a single gene mutation in *RSPO1*[160] and associated with hyperkeratosis and squamous cell carcinoma.

Presentation

The majority of patients with ovotesticular DSD present in infancy due to underdeveloped genitalia. The phenotype of the internal and external genitalia is controlled by the gonad's hormonal status and often reflects the asymmetric mixture of ovarian and testicular tissue within the gonads. In all patients, müllerian duct regression is incomplete, yet the uterus remains immature, and a unicornate uterus is a common finding. However, more phenotypically female patients, the adnexa and vagina are generally better developed and approximately half of phenotypic females menstruate.[140,142]

Patients with a high degree of masculinization and male phenotype may have some uterine remnants, such as a prostatic utricle (prostatic vagina). Phenotypic males also tend to have bilateral palpable gonads, or at least one descended gonad. The undescended gonad may be an intra-abdominal ovary or an ovotestis and located at any point of the pathway of testicular descent.

In some rare cases, patients with unambiguous genitalia present with atypical secondary sex characteristic, opposite of their assigned gender. For example, at puberty, phenotypic female patients may experience clitoromegaly and deepening voice while phenotypic males may experience gynecomastia. In these cases, ultrasound may not fully demonstrate the testicular or gonadal nature of either gonad, but gonadal biopsy can definitively demonstrate the presence of both ovarian and testicular tissue.

Diagnosis of 46,XX Testicular and 46,XX Ovotesticular DSD

In males without genital ambiguity, a diagnosis is often made during an investigation of infertility or delayed puberty. Patients with XX Testicular DSD have hypergonadotropic hypogonadism with elevated follicle stimulating hormone (FSH) and LH, decreased T, DHT, and a less than twofold increase in response to the hCG-stimulation test. Unlike individuals with GD or XX Testicular DSD, the gonads of patients with ovotesticular DSD have some degree of function and therefore normal levels of FSH, LH, estradiol (E_2), T, and DHT. In children with 46,XX karyotype and genital ambiguity, the biochemical diagnostic criteria are the same as 46,XX testicular DSD. However, *SRY* is positive in only 10% of 46,XX ovotesticular DSD. Serum AMH levels are a good biochemical indicator of functioning testicular tissue, suggestive of XX testicular DSD or ovotesticular DSD.[138] Pelvic ultrasound shows absence of a uterus and semen analysis demonstrates normal semen volume with azoospermia. A combination of both male and female internal and external genitalia is suggestive of 46,XX ovotesticular DSD.

Genetic tests that should be performed are FISH for *SRY,* which is positive in 90% of phenotypically males with XX testicular DSD. The 10% of patients with XX testicular DSD who are negative for *SRY* should undergo further cytogenetic testing to explore the presence of *SOX9* promoter microduplication or microdeletion,[70,74,75] *SOX3* or *SOX3* promoter microduplication or microdeletion,[72] or cryptic mosaicism for Yp.

It is important to note that the only way to obtain definitive diagnosis of either ovotesticular or testicular DSD is by gonadal tissue confirmation in which both gonads are extensively examined for the presence of ovarian tissue. Complete absence of ovarian tissue can allow for definitive diagnosis of 46,XX Testicular DSD, while the slightest presence of ovarian tissue gives rise to 46,XX Ovotesticular DSD. If gonadal biopsy material is available, it should be tested for karyotype, cryptic mosaicism, and *SRY* mutations, as SRY expression in the gonads has been seen despite the lack of Yp in the karyotype.[145,149,161] Patients who are mosaic for 46,XX and 46,XY should be tested for potential chimerism particularly for those conceived with *in vitro* fertilization.

Management of 46,XX Testicular and Ovotesticular DSD

Of primary concern in patients with testicular or ovotesticular DSD are the surgical repair of issues, such as inguinal hernias, fistulas, and malignancies. Continuing management is determined by the gender assignment of these patients, which is discussed later in the chapter.

There is a 5% incidence of gonadal tumors in ovotesticular DSD,[162,163] which likely represents an increased risk from abdominal gonads possessing testicular tissue, as well as the increased risk of gonadoblastoma in dysgenic gonads with Yp material. Phenotypic male patients with XX testicular DSD, who are *SRY*-positive, are not at increased risk for gonadoblastoma, since they lack a complete Y chromosome. Some reports also indicate that the risk of breast cancer may be increased in 46,XX Testicular and Ovotesticular patients.[164,165]

Patients with 46,XX ovotesticular DSD with partially developed müllerian structures can also have ovarian follicles, with the potential to carry a biological pregnancy to

term.[152] There are eight reported pregnancies in patients with ovotesticular DSD. In all these patients, the karyotype was 46,XX, which contributes to the belief that patients with the Y chromosome cannot get pregnant. However, one case report describes a 20% 46,XX/80% 46,XY patient with ovotesticular DSD who carried a successful pregnancy to term.[163] Ovotesticular DSD males are generally azoospermic, but there have been infrequent cases of males with mature sperm,[152,166] and even more rare cases of ovotesticular DSD who have fathered children.[152]

Since the majority of individuals with XX testicular DSD carry the Xp:Yp translocation, paternal karyotype and FISH for *SRY* should also be performed to determine whether or not the father carries a balanced translocation, or if the translocation occurred in the germline, or *de novo*. Fathers who carry the balanced translocation can only produce offspring that are 46,XX *SRY*-positive testicular DSD or 46,XY *SRY*-negative complete gonadal dysgenesis offspring.

46,XX Gonadal Dysgenesis

The majority of cases of ovarian dysgenesis involve abnormal complements of X chromosomes in phenotypic females.[167,168] The most common of these is Turner syndrome, discussed earlier as a Sex Chromosome DSD. Impaired ovary development can occur with a normal female sex chromosome complement, referred to as 46,XX GD, which can occur as either an isolated entity or as part of a syndrome. Females possess normal female genitalia at birth, but remain sexually infantile and do not experience normal puberty. Ovarian failure with hypergonadotropic hypogonadism at puberty is highly suggestive of ovarian dysgenesis. Müllerian structures are normal, and gonadal histology reveals bilateral streaks. Patients often present with amenorrhea in adolescence of infertility in adulthood.

Several studies in familial ovarian dysgenesis have noted the frequent reports of consanguinity, suggesting that isolated 46,XX GD is inherited as an autosomal recessive condition.[167,169-171] Mutations in the gene for FSH receptor *(FSHR)* have been implicated in some cases,[170,172,173] but does not explain the phenotype in all populations studied.[174,175] Translocations involving the critical region Xq13.3-q26 have also been described.[176] The molecular basis for 46,XX GD remains unknown for the majority of patients.

46,XX Disorders of Sex Differentiation: Androgen Excess

High levels of intrauterine androgens or androgenic precursors result in masculinization of the 46,XX fetuses' internal and external genitalia. In these patients, development of the wolffian ducts and virilization of the external genitalia occur to varying degrees. Patients often present with clitoromegaly, "scrotalization" of the labia, urogenital sinus, and phallic urethra along with fully developed müllerian derivatives.

In XX infants with normally developed gonads and müllerian structures, the most common cause of genital ambiguity in newborns are the virilizing forms of CAH.[177] Inactivating mutations of any of the enzymes steroidogenic pathway, shown in Figure 17.9, are the most common cause of virilization in XX infants and are discussed later in the chapter. Rare mutations in *CYP11A1*, *STAR*, *HSD3B2*, and *HSD17B3* can result in CAH that can result in both 46,XY DSD or 46,XX DSD are described above in 46,XY DSD: Disorders in Androgen Biosynthesis.

There are three major sources of excess androgens *in utero:* fetal steroidogenesis, fetal-placental steroidogenesis, and maternal causes of excess androgens (i.e., iatrogenic, androgenic tumors).

Fetal Sources of Androgen Excess

Congenital Adrenal Hyperplasia

Congenital Adrenal Hyperplasia (CAH), depending on the enzyme implicated, can cause either 46,XY DSD or 46,XX DSD. 21-hydroxylase *(CYP21A2)* deficiency accounts for over 90% of CAH, and represents the most common etiology of ambiguous genitalia in the newborn.[177-179] CYP21A2 deficiency results in blockage of both aldosterone and cortisol biosynthetic pathways. The build-up of 17α-hydroxyprogesterone is shunted towards androgen biosynthesis, resulting in virilization of XX fetuses. The disease frequency in the general population is 1:15,000 people, but is much higher in certain ethnic groups, including Hispanics and Ashkenazi Jews.[180]

Mutations in 11β-hydroxylase *(CYP11B1)* account for another 5% of virilizing CAH.[177,181] Like in 21-hydroxylase deficiency, XX neonates are virilized, but XY neonates have normal genitalia. Blockage of CYP11B1 results in elevated levels of aldosterone and androgens, by shunting of cortisol precursors to biosynthesis of other hormones. Other adrenal causes of excess androgen, such as 3βHSD2 and P450 oxidoreductase defects, were discussed earlier in the chapter.

Presentation and Diagnosis

XX fetuses typically present at birth with ambiguous genitalia. XY fetuses do not exhibit any degree of undervirilization. However, there is a wide range of phenotypes, from the classical salt-wasting to simple virilizing to non-classical forms which present with hyperandrogenism and precocious puberty. There is a strong correlation between degree of enzymatic activity and the severity of clinical phenotype.[182] Figure 17.10 shows the wide-range of genital phenotypes in CAH.

XY individuals with 21-hydroxylase deficiencies are often not detected in the newborn period, unless accompanied by a salt-wasting crisis. XY patients often present with premature masculinization and accelerated physical development. If left untreated, there is premature fusion of epiphyses resulting in short stature.

Diagnosis of 21-hydroxylase deficiency can be initially ascertained by elevated 17-OH Progesterone. Biochemical changes are outlined in Table 17.4. Clinically 11-β-hydroxylase deficiency can be differentiated from 21-hydroxylase deficiency by the presence of hypertension with hypokalemic alkalosis. Molecular diagnosis can be ascertained by sequencing of the gene.

Management

Treatment for 21-hydroxylase defiency can begin *in utero* in order to prevent over virilization of XX fetuses. Due to the timing of genital development, oral treatment with dexamethasone begins at 4 weeks gestation, even before determination of sex and presence of disease genes in the fetus.

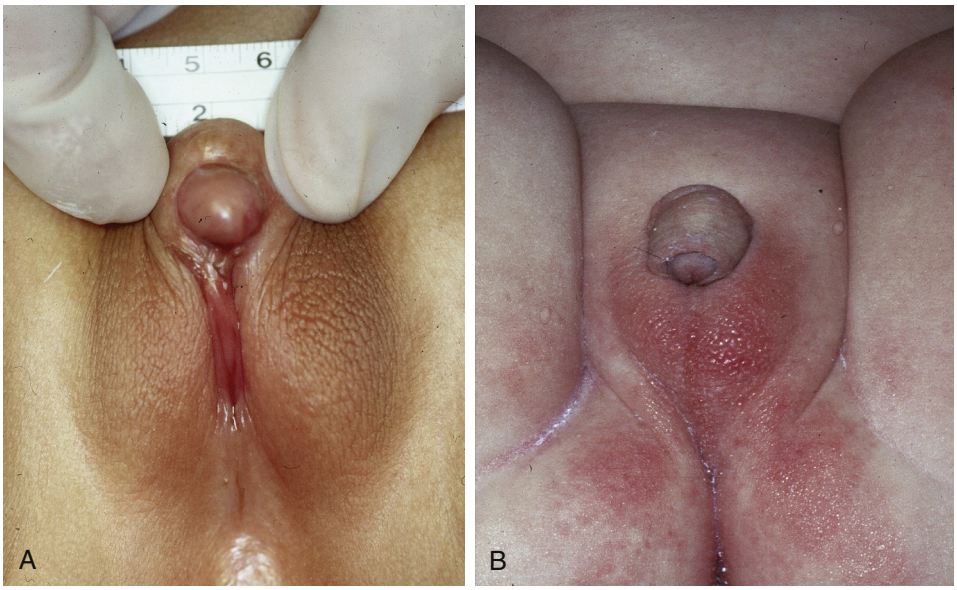


FIGURE 17.10 External genitalia of two CAH patients with CYP21A2 mutations with varying degree of virilization. **A**, The labia appear partially fused posteriorly, and show some rugae. The clitoris is moderately hypertrophic. **B**, The labia are completely fused and have a scrotal appearance. No gonads are palpated. The clitoris is very hypertrophic, and has a penis-like appearance.

Maternal dexamethasone can be continued until chorionic villous sampling or amniocentesis demonstrates XY karyotype or lack of homozygousity of the 21-hydroxylase mutation. If the fetus is XX and affected, treatment with oral dexamethasone continues until delivery.[183] This treatment remains controversial, because of a high likelihood of unnecessary treatment of unaffected fetuses, with little knowledge of long-term effects.

Newborns diagnosed with 21-hydroxylase deficiency should be monitored carefully for salt-wasting crises in early life. For newborns with either CYP21A2 or CYP11B1 deficiency, glucocorticoid replacement is essential to preventing adrenal crises and normalizing serum levels of steroidogenic precursors.

Fetal-Placental Causes of Androgen Excess

P450 Aromatase Deficiency

Aromatase *(CYP19A1)* catalyzes the conversion of androgens to estrogen. Mutations that affect aromatase activity have effects on both male and females. Estrogen is required for spermatogenesis in males and for the development of secondary sex characteristics in females.

Presentation and Diagnosis

The initial manifestation of aromatase deficiency is placental, with maternal virilization.[184-186] These mothers have elevated levels of androgenic precursors in their liver, causing their own virilization and that of their XX fetuses. Biochemical findings are outlined in Table 17.4. CAH is the major differential diagnosis considered and can be ruled out with ACTH-stimulation test showing increase in adrenal hormones and not androgens.

XX patients fail to undergo spontaneous puberty, and often have polycystic ovaries, increased virilization, and amenorrhea, without breast development.[184,187,188] XY males experience normal puberty but are infertile due to the lack of estrogen, which is essential to spermatogenesis.[186,189,190]

With regards to growth, both XX and XY patients do not experience a growth spurt during puberty and rather, experience linear growth. Patients end up tall but skeletal maturity is delayed and osteoporosis develops early.[186,191] Patients often complain of bone pain.[186] Refer to general management section later in the chapter for sex hormone replacement therapy at puberty.

P450 Oxidoreductase Deficiency

POR is a rare cause of ambiguous genitalia and 46,XX and 46,XY infants. Pathophysiology and presentation is described in detail in the 46,XY DSD section.

Maternal Etiologies of Androgen Excess

Maternal ingestion of androgens is a potential cause of virilization of XX newborns. However, present-day progestin containing oral contraceptives are not highly androgenic and this cause of XX virilization is rare.[192-194] Hormonally active tumors, such as luteomas are another uncommon causes of maternal virilization and ambiguous genitalia in an XX baby.[195] Rare cases of *hyperreactio luteinalis*, a cystic ovarian condition associated with virilization of the mother and fetus is associated with XX-virilization in 15% of cases.[195] While CAH causes the majority of cases of XX virilization, one should always consider oncological and iatrogenic causes of maternal and fetal virilization.

General Management for Patients with DSD

Patients with DSDs have grown up. What was viewed as a rare and unusual phenomenon, only seen in a few highly specialized academic centers, has become global. First, the issues generated by the management of DSDs have had wide exposure in the media, feeding societal debates on gender assignment and on patients' rights to be involved in medical decisions. The clinical issues that were limited to genital surgery have expanded to the management of low fertility, cancer, and psychosexual dysfunction in adulthood, thus informing knowledge of many other, more frequent conditions.

Multidisciplinary Approach

An essential aspect of care for children with DSDs is an experienced multidisciplinary team that typically includes an endocrinologist, a pediatric surgeon or urologist, a psychologist or psychiatrist, a gynecologist, a geneticist, a pediatrician or neonatologist, and, if available, a social worker, a nurse, and a medical ethicist.[196] Open communication between the team and the family, as well as participation in decision-making is essential. Support groups may have an important role in the delivery of care. Transition to adult care is also a key element of care. Fundamental concepts to initially establish with the family include the following: a child with a DSD has the potential to become a fully functional member of society; that DSD is not shameful; and that the situation is often complex and the best plan may not be obvious initially.

Diagnosis

Extraordinary progress has been made in the molecular and endocrine evaluation of patients with DSDs. After a thorough interview for family and prenatal history, the initial testing includes sex genotyping, which can be performed rapidly by FISH with X and Y probes, or by regular karyotype. It is followed by pelvic imaging (with ultrasound) increasingly being replaced by pelvic magnetic resonance imaging (MRI), and measurement of levels of 17-hydroxyprogesterone, testosterone, LH, FSH, AMH and electrolytes. Algorithms, such as the ones proposed in Figure 17.11, guide subsequent and more specialized investigation.

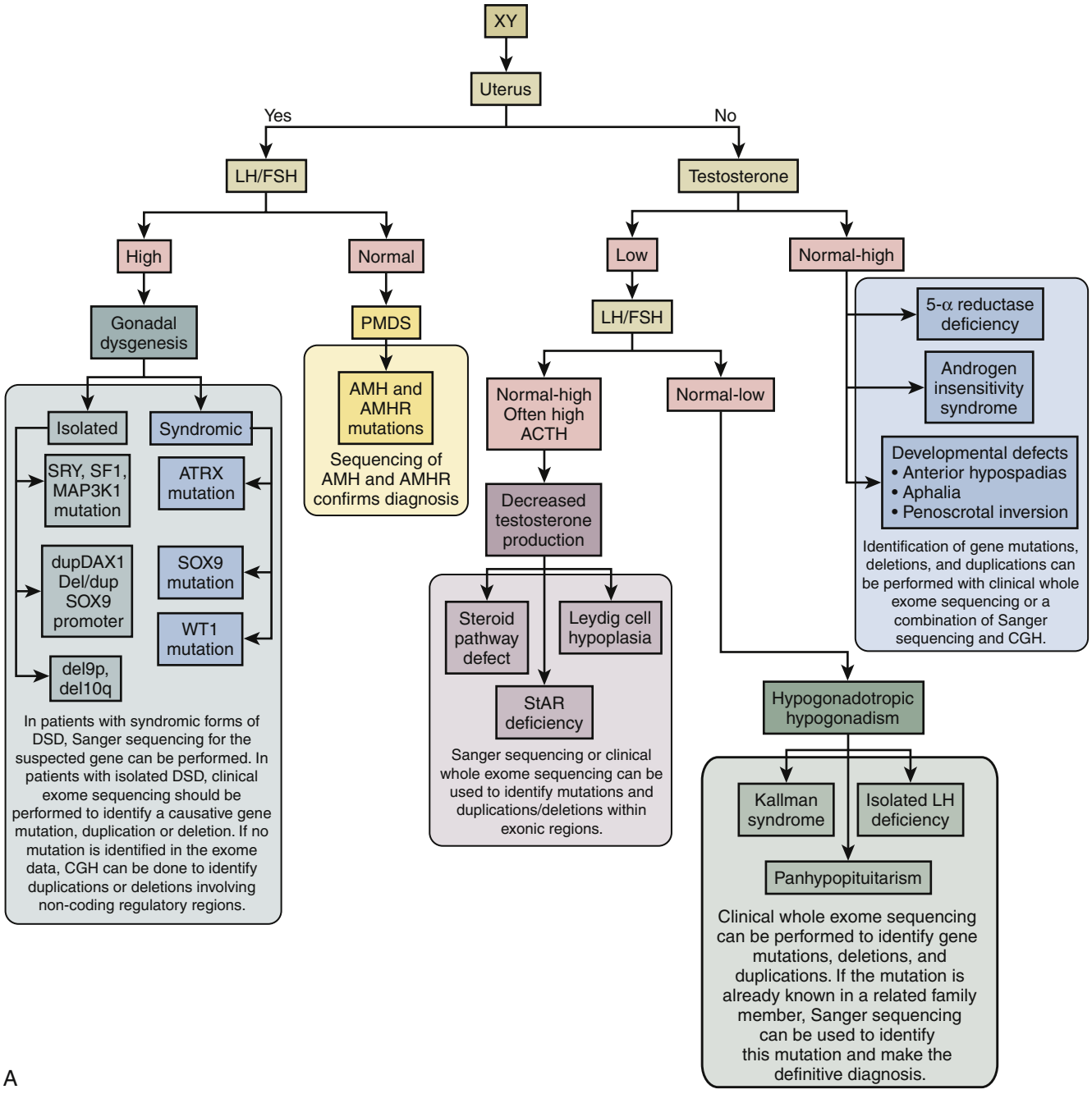
The clinical availability of next generation sequencing allows for the rapid sequencing of all known genes important in disorders of sex development. These large-scale approaches can be performed in a targeted manner, only sequencing the genes known to cause DSD.[197] Such approaches can be used to look for gene mutations, copy number variations, and sex chromosome copy number. Effectively, this high-throughput sequencing approach can combine the three major genetic tests performed in DSD patients: the sex chromosome karyotype, comparative genomic hybridization (CGH), and single gene sequencing. For practical purposes, karyotype and CGH are still performed as separate tests, but as turn around time and costs of whole exome and whole genome sequencing decrease, it is anticipated that high-throughput sequencing will be the primary test for diagnosis of DSD. In addition, high-throughput sequencing provides a more comprehensive and rapid diagnosis than single gene sequencing approaches. In the same vein, whole exome sequencing, which sequences nearly all the known exons within the human genome, can be performed and can identify mutations in known genes as well as novel mutations. In whole exome sequencing approaches, bioinformatics filtering can initially allow for the focus to remain on known genes that cause DSD. However, if no mutations are identified in known DSD genes, one can examine the entire genome in search of rare mutations in novel genes involved in DSD. The cost of whole exome approaches is comparable to the cost of sequencing a single gene. Genetic mutations underlying a clinically defined disorder, such as 46,XY gonadal dysgenesis (which can result from mutations in *SRY*, *SF1/NR5A1*, *MAP3K1*, or copy number variations in 9p and 10q) would require multiple tests, which is costly, time consuming, and inefficient. High throughput sequencing diagnostic approaches provide an accelerated time to diagnosis and decreases the risk of diagnostic uncertainty that often accompanies a clinical diagnosis of DSD.

Gender Assignment

Until the mid-1950s, the medical management of individuals with DSD was based on the assumption that the "true sex" was equivalent to the sex of the gonads. Human sexual anatomy was indeed classified in 5 categories in the 19th century: (1) male and (2) female (with typical feminine or masculine external genitalia, respectively; (3) male and (4) female pseudohermaphrodites (with ambiguous genitalia and presence of testes or ovaries, respectively); and (5) true hermaphrodites (with both testicular and ovarian tissue).[198] It was assumed that a person's identification as male or female would naturally conform to ''true sex.''

In the late 1950s, however, under the influence of psychologist John Money and pediatric endocrinologist Lawson Wilkins, gender assignment began to be influenced by the "optimal gender" principle. It became standard practice in the 1970s, and was only challenged recently, starting in the mid-1990s.

The "optimal gender" approach considered multiple aspects of outcome, giving greatest consideration to the potential for complete sexual functioning. It is based on the two assumptions that gender identity is not firmly established at birth, but rather is influenced by socialization and sex of rearing, and that stabilization of gender identity and favorable psychological outcome depends on the congruence between genital appearance and the assigned gender. The clinical consequences of the "optimal gender policy" are that gender should be assigned as early as possible, during a window of gender flexibility that ends at 18 to 24 months of age; reconstructive genital surgery should be performed equally early to match the chosen gender; the assignment should be to the gender that carries the best prognosis for reproductive and sexual function; and uncertainty for parents and health care providers about the gender of rearing should be minimized. Recent challenges of this paradigm view the determinant of gender identity and gender role as biological (with prenatal androgens as the best candidate),[199] and the appearance of genitals as a reflection of brain masculinization, and not the reverse. What was seen as an absolute requirement for gender assignment (a typical penis and scrotum for males, a typical clitoris and labia for females) was questioned by outcome studies of XY patients born with cloacal exstrophy, a complex abdominal malformation resulting in a *de facto* absence of functional penis, but normal testicular function. Only 5 out of 14 patients assigned female at birth had a clear female gender identity at follow-up in their teen years.[200] On balance, cases of ablatio penis in early childhood caused by botched circumcision have had diverse outcomes. Although the most famous example of the literature (the "John-Joan" case) which prompted the recent challenges to the optimal gender policy ended in a disastrous outcome (gender dysphoria, and ultimately suicide),[201] another case resulted in a clear, stable female gender identity in an otherwise bisexual woman with a history



A

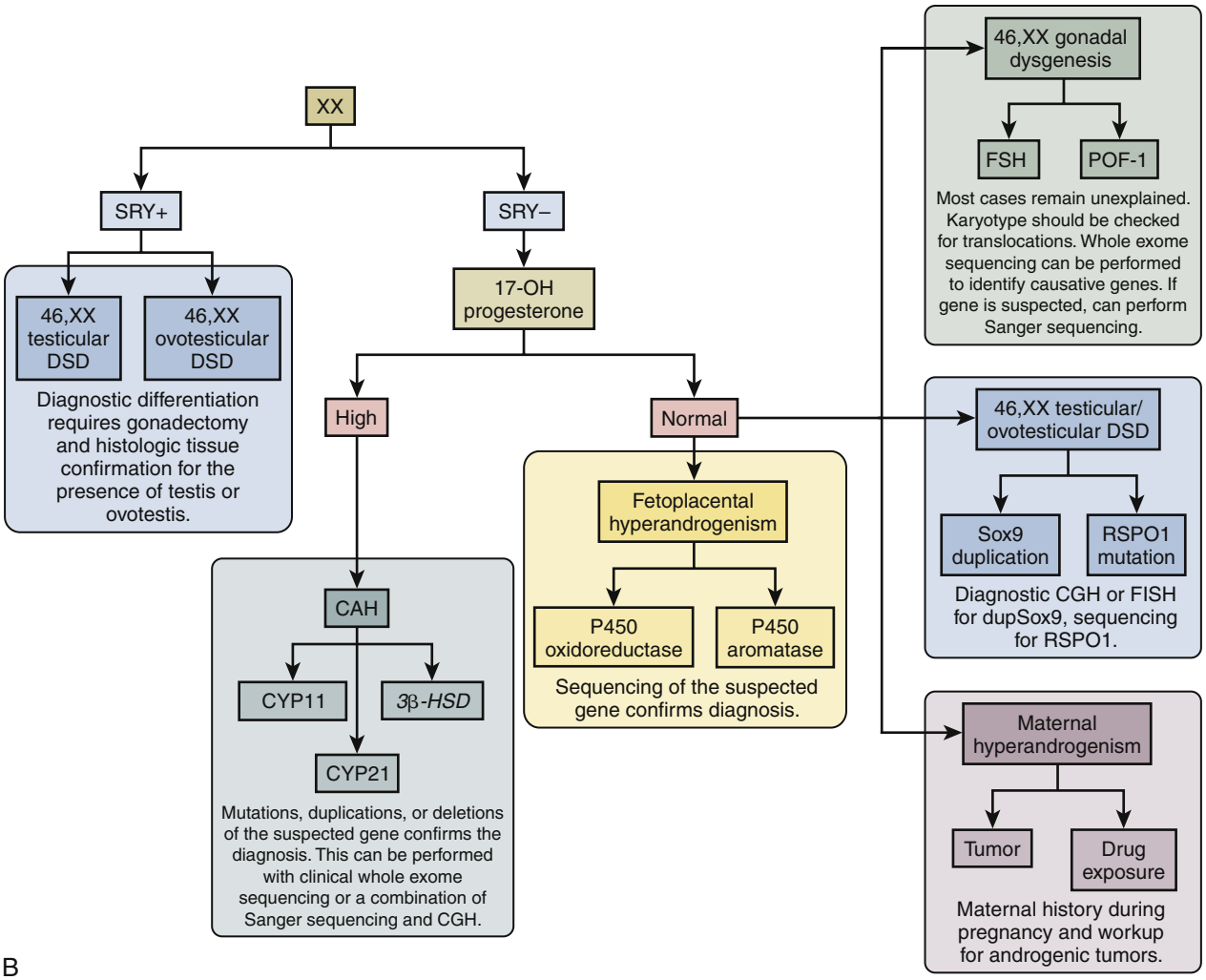
FIGURE 17.11 A, Diagnostic flowchart of the major disorders of sexual development in undervirilized XY individuals.

of "tomboyism" during childhood, who requested on two occasions (at ages 16 and 26) a vaginoplasty. This shows that female gender identity can develop in a genetic male.

So, what is the best way to assign gender in this post-"optimal gender" era? Just like before, based on the medical literature and outcome studies for specific diagnoses. Considerations of chromosomal constitution, hormonal levels, potential fertility, and sexual function, are, as always, quite important. The main difference is that early genital surgery is not necessarily directly linked to the gender assignment decision. One clear recommendation is that all individuals should receive a gender assignment early on.

Genital Surgery

As seen above, the traditional perspective, at least since the 1950s, has been to provide an anatomy that matches the sex of rearing. The goal has been to influence gender identity, provide a positive psychological adjustment and allow sexual intercourse. In this model, the decision-makers are the physician and the family, since the genital surgery has to happen during the early period of gender flexibility. This traditional paradigm has been challenged in the 1990s, initially by patients' advocacy groups, then by physicians,[202] arguing that the rationale for early genital surgery was the comfort of others, not necessarily the patient, and that there are negative consequences if the assigned gender does



B

FIGURE 17.11—cont'd **B**, Diagnostic flowchart of the major disorders of sexual development in XX individuals. *(Adapted from Fleming A, Vilain E. The Endless Quest for Sex Determination Genes.* Clin Genet *67:15–25, 2004.)*

not correspond to the ultimate gender identity, and if sexual function is impaired as a consequence of the surgery. In the challenging model, the decision-maker is the patient, when old enough. The 2005 consensus statement on DSD provides guidelines to a more cautious approach to surgery. The statement requires that the surgeons outline the consequences of surgery from infancy to adulthood, and that only surgeons with specific training in the care of DSD perform genital surgeries. It is also argued that clitoral surgery should only be considered in severe cases (Prader III to V), that no vaginoplasty in patients with short or absent vaginas be performed in infancy, and that no vaginal dilatation be performed before puberty.

Hormonal Therapy

Hormonal therapy may be used for several purposes. In the case of classic CAH, glucocorticoids and mineralocorticoids are used as a life-saving replacement therapy. In the cases of DSDs raised as males, testosterone may be used as a way to increase penile tissue growth, as well as to induce puberty. In the cases of DSD raised as females, estrogens are used for induction of puberty and bone protection.

Glucocorticoid Replacement Therapy

In CAH, it is in the form of hydrocortisone (10-20 mg/m^2 per 24 hours) given orally in two or three doses per day. The treatment needs to provide sufficient hormone to replace the adrenal steroids and suppress the adrenal androgens, and not too much to avoid iatrogenic Cushing's syndrome and its effect on growth rate and bone maturation. Daily doses are increased by two to three times in periods of stress (fever or surgery for instance).

More potent glucocorticoids (e.g., prednisone) can be used in adulthood, after growth is complete.

Mineralocorticoid Replacement Therapy

In the salt-wasting form of CAH, treatment with 9-fludrohydrocortisone (Florinef®) (0.05 to 0.3 mg/day PO) and sodium chloride (1-3 g/day in supplement) is often required in early infancy with maintenance doses slightly less. This is given in addition to the glucocorticoid therapy.

Testosterone Replacement Therapy

Management of individuals with testosterone deficiency (e.g., 46,XX testicular DSD or Klinefelter syndrome)

includes the initiation of low-dose testosterone therapy after age 14. Testosterone enanthate is given intramuscular (IM) every 3 to 4 weeks, starting at 100 mg and increasing by 50 mg every 6 months to 200 to 400 mg. Initial high doses of testosterone should be avoided to prevent priapism. The treatment should plateau, in adulthood, at the best possible dosage, typically between 50 and 400 mg every 2 to 4 weeks.

If an individual has short stature and is eligible for growth hormone therapy, testosterone therapy should be either

Table 17.5 Fertility in Patients with Disorders of Sexual Development

Disorder		Level of Fertility	Potential use of Assisted Reproductive Technology
Sex Chromosomes Disorder of Sex Development	Turner syndrome	Only 2%-5% of Turner patients are spontaneously fertile[205] There have been ~170 reported spontaneous pregnancies in patients with mosaic variants of Turner.[206] Recently 2 cases of spontaneous pregnancy in non-mosaic variants were reported.[207,208] 30%-60% pregnancy rates after oocyte donation; high risk of pregnancy complications (i.e., hypertension, pre-eclapmsia)[209]	Cryopreservation of follicles in youth Single embryo transfer from donor oocytes
	Klinefelter syndrome	3 case reports of spontaneous pregnancy with 47,XXY patients[210-212] 43 reports of pregnancy after ICSI leading to liveborn children[213]	Cryopreservation of sperm Testicular sperm extraction (TSE) ICSI
46,XY DSD: Testes Development	46,XY Gonadal Dysgenesis (pure, partial, mixed)	7 case reports of successful pregnancy after oocyte donation in patients with complete 46,XY GD[214-220] No reports in 46,XY partial or mixed GD. Patients with mixed GD are usually infertile.	Single embryo transfer from donor oocytes
	Testicular Regression syndrome	Infertile	None
46,XY DSD: Adrenal Synthesis and Action	Disorders of Androgen Biosynthesis	In XY patients with forms of CAH, infertility is often caused by testicular adrenal rest tumours.[221] 3B-HSD: XY: Lower levels of spermatogenesis; 2 case reports of severely affected male fathering 2 children. Fertility in XX females unknown[222,223]	Glucocorticoid therapy to optimize fertility; ICSI and *in vitro* fertilization
	Leydig Cell Hypoplasia	Untreated patients are azoospermic and infertile.[224]	Unknown
	5-α Reductase Deficiency	Fertility is possible with surgical reconstruction of the male ductal system.[225]	ICSI
	Complete/Partial Androgen Insensitivity syndrome	Male infertility; no uterus and therefore cannot carry pregnancy	
	Persistant Müllerian Duct syndrome	In patients with cryptorchidism, fertility is better preserved in adulthood if orchiopexy is done prior to age 2.[226]	ICSI
46,XX DSD: Ovarian Determination	XX Testicular DSD XX Ovotesticular DSD	Case report of patients with 46, ovotesticular DSD with 1 ovary and 1 ovotestis who became spontaneously pregnant after removal of ovotestis.[227,228] Fertility and pregnancy has been reported after removal of testicular portion of ovotestis removed.[228,229]	Potential for biological children using *in vitro* fertilization techniques or selective removal of only testicular tissue to stimulate spontaneous ovulation
46,XX DSD: Adrenal Excess	Congenital Adrenal hyperplasia 21-Hydroxylase 11-β Hydroxylase	In XX patients, excess adrenal steroids, ovarian hyperandrogenism, PCOS, ovarian adrenal rest tumors, neuroendocrine factors, genital surgery, and psychological factors contribute to decrease in fertility. Normalization of hormonal factors can increase chance for spontaneous pregnancy in these women.[221,231]	Long acting glucocorticoids can be used to regulate menstrual cycles and maximize fertility.[221] ICSI and *in vitro* for patients who cannot conceive spontaneously. Single embryo transfer in patients with stenotic vaginas due to genital surgery.
	P450 Aromatase P450 Oxidoreductase	XX patients have polycystic ovaries and do not undergo spontaneous puberty.[232] XY individuals undergo normal puberty but are sterile, due to the lack of estrogen. No reports of spontaneous pregnancy to date, however long-term studies of these patients have not been completed.	In theory, single embryo transfers (from biological or donor oocytes) for XX, DSD patients.
	Maternal Sources of DSD	No known effect on adult fertility. Fertility in XX individuals is affected by management of ambiguous genitalia, such as genital surgery, and psychological factors.	

Adapted from Lee PA, et al. Consensus statement on management of intersex disorders. International Consensus Conference on Intersex. *Pediatrics* **118**(2):e488–500, 2006.

delayed or given at lower doses initially in order to maximize the growth potential. Alternative delivery systems to injections that result in a more stable dosing include transdermal patches (scrotal and nonscrotal) and transdermal gels. Testosterone-containing gels have an increased risk of interpersonal transfer which can be reduced by the use of new hydroalcoholic gels.[203]

Estrogen Therapy

In order to develop and maintain secondary sexual characteristics in women who are hypogonadic (or in postgonadectomy CAIS patients) and to prevent osteoporosis, estrogens are started at the lowest available dose and progressively increased (starting, for instance, at 0.3 mg PO per day of conjugated estrogens and increasing to 0.625 mg/day). Other delivery systems include transdermal patches.

Disclosure

The traditional paradigm of the "optimal gender" policy calls to limit disclosure, based on the rationale that it prevents gender identity confusion, within the reasoning that nurture influences gender identity. The challenging view is full disclosure, in order to support patient autonomy, and with the rationale to prevent secrecy and shame. Studies of chronic medical disorders and of adoptees shows that disclosure is associated with enhanced psychosocial adaptation.[1] Although there is no rule about what age to fully disclose a condition, it is recommended to proceed gradually and recurrently with children, and adapt disclosure and cognitive and psychological development.[204]

Mental Health

Psychological care should be integral to the medical management of patients with DSD. Multidisciplinary teams caring for children with DSD should include a mental health professional, who should inform the decisions of gender assignment and re-assignment, and timing of surgery. Although atypical gender-role behavior is more common in children with DSD, it should not be taken as an indicator for gender re-assignment. The mental health professional should help parents cope with their child's condition, should help the process of full disclosure and, most importantly, should provide long-term behavioral support to the patient. This includes support for quality of life items such as falling in love, dating, intimacy, sexual functioning, ability to build relationships and raise children.

Fertility

Not long ago, infertility was seen as an immutable feature of DSD. Advances in assisted reproductive technologies (ART) have changed this view. Some patients with DSDs, who were previously infertile or subfertile have the opportunity to have biological children. If germ cells are present, sperm or oocytes can be biopsied from the gonads and used in intracytoplasmic sperm injection (ICSI) and *in vitro* fertilization. In Table 17.5, we have outlined the fertility levels and ART options for patients with various DSDs.

The ability to produce biological offspring in females is solely dependent on the presence of oocytes. Adolescent patients who undergo premature ovarian failure (i.e., Turner syndrome) can cryopreserve their oocytes for future use. Furthermore, phenotypic female patients with a uterus can undergo donor embryo transfer with a 30% rate of pregnancy. For phenotypic female patients, carrying a pregnancy to term is associated with higher risk of maternal complications and therefore should be followed closely by an obstetrician specializing in high-risk patients.

Cancer

DSD conditions are accompanied by variable risks of germ cell tumor, which should be taken into consideration in the long-term management of these patients. Higher risks of gonadal tumors have been shown in gonadal dysgenesis positive for the presence of TSPY (testis-specific protein Y encoded) on the Y chromosome, and for partial androgen insensitivity syndromes with intraabdominal gonads. Lower risks are observed in ovotesticular DSD and complete androgen insensitivity syndrome. A review of the risk of germ cell tumor according to the diagnosis is provided in Table 17.6.

Table 17.6 Gonadoblastoma Risk

Risk Group	Disorder	Malignancy Risk, %	Recommended Action	Patients, n	Studies, n
High	GD* (+Y)† intraabdominal	15-35	Gonadectomy	12	>350
	PAIS nonscrotal	50	Gonadectomy	2	24
	Fraisier	60	Gonadectomy	1	15
	Denys-Drash (+Y)	40	Gonadectomy	1	5
Intermediate	Turner (+Y)	12	Gonadectomy	11	43
	17β-hydroxysteroid	28	Watchful waiting	2	7
Low	CAIS	2	Biopsy and possible	2	55
	Ovotesticular DSD	3	gonadectomy	3	426
	Turner (-Y)	11	Testicular tissue removal	11	557
			None		
No (?)	5α RD2	1	Unresolved	1	3
	Leydig Cell Hypoplasia	1	Unresolved	1	2

Adapted from Lee PA, et al. Consensus statement on management of intersex disorders. International Consensus Conference on Intersex. *Pediatrics* **118**(2):e488–500, 2006.

*Gonadal dysgenesis (including not further specified 46,XY, 45,X/46,XY, mixed, partial, complete).

†GBY region positive, including the TSPY (testis-specific protein Y encoded) gene.

Patient-Centered Care

DSD conditions should be managed in a patient-centered model of care. In this model, open communication with patients and family should be promoted, especially at times of decision-making. Gender change should be supported by the multi-disciplinary team, as needed, in accord with patient's wishes. The consensus statement also calls for a limit on genital exams and on medical photography.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

Suggested Readings

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CHAPTER 18

Puberty: Gonadarche and Adrenarche

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Tony M. Plant

Puberty in humans is defined as the period of first becoming capable of reproducing, and is marked by maturation of the genital organs, development of secondary sex characteristics, acceleration in linear growth velocity, changes in affect and, in the female, the occurrence of menarche.[1] In humans, the transition into puberty is driven by two physiological processes: gonadarche and adrenarche. *Gonadarche* comprises growth and maturation of the gonads and is associated with increased secretion of sex steroids and with the initiation of folliculogenesis and ovulation in the female and spermatogenesis in the male. Gonadarche leads to thelarche and menarche in girls and testicular enlargement in boys.

Adrenarche, which typically precedes gonadarche,[2] comprises maturation of the adrenal cortex associated with increased secretion of adrenal androgens—namely, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), and androstenedione—and leads to the appearance of sexual hair (pubarche). Adrenarche occurring late in prepubertal development appears to be peculiar to our own species and to the great apes[3] and, in humans, the absence of adrenarche does not prevent gonadarche or the attainment of fertility.[4,5]

The age at onset of puberty and the tempo at which puberty progresses is dependent on many factors. In girls, increased ovarian and adrenal sex steroid secretion leads to the physical manifestations of puberty, thelarche, and pubarche. In general, these changes occur between 8 and 13 years of age. The mean age at menarche among multiple ethnic groups is between 12 and 13 years old.[6] In boys, the earliest physical manifestation of puberty is an increase in testicular volume, and this usually occurs between 9 and 14 years of age.

Traditionally, the diagnosis of precocious puberty is considered when signs of puberty develop prior to 8 years of age in girls and 9.5 years in boys, but these criteria should be used as guidelines to complement the evaluation of individual patients.[7] For girls, the absence of thelarche or menarche by age 13 and 16 years, respectively, is considered to be delayed puberty.[8] For boys, delayed puberty is defined as absence of testicular enlargement by age 14 years.

While the mechanism underlying the onset of adrenarche remains to be elucidated, it is now established that gonadarche results from the resurgence of activity in the hypothalamic-pituitary axis, which has been relatively quiescent since early childhood. The neuroendocrine regulation of gonadarche in man is similar to that observed in other higher primates,[5,9] and nonhuman primates (in particular the rhesus monkey) have been extensively employed as paradigms for the study of human puberty. Subsequently, our discussion of the control of the onset of gonadarche will be based on both the human and nonhuman primate literature.

Stages of Pubertal Development, Secular Trends, and Racial and Ethnic Differences

Pubertal Staging

For both sexes, the genital and pubic hair changes that unfold at puberty are classified into five stages: stage 1 is prepubertal and stage 5 is adult (Fig. 18.1; Table 18.1). These physical changes may either be the result of gonadarche (as in the case of breast or testicular enlargement) or adrenarche (as in the case of pubic hair development). Although the physical sequelae of gonadarche and adrenarche generally occur concomitantly a discordance of the two processes may also occur in normal development.

Increased estrogen secretion promotes breast development in girls. The development of breast buds with increased areolar diameter is considered to be stage 2; greater enlargement of the breasts occurs in stage 3, accompanied by increased pigmentation of the areolae and nipples. During stage 4, the areolae are mounded above the breast tissue. Recession of the areola to the general breast contour represents breast stage 5. Palpation of the breast is necessary to better differentiate between breast tissue and lipomastia. Additional effects of estrogen at this stage of development include cornification of the vaginal mucosa, uterine growth, and morphogenesis of an adult female body habitus.

Menarche follows an anovulatory cycle and generally occurs 2 to 3 years after the onset of breast development. Menstrual cycles during the first year after menarche are typically irregular and anovulatory, with most ranging in duration from 21 to 45 days. Within 5 years of menarche, most cycles are regular and range from 21 to 35 days. Although primordial and pre-antral follicles predominate during the prepubertal years, one should note that small antral follicles develop during this phase of maturation. During the early postmenarcheal period, ovarian morphology on ultrasound differs from that observed in older women. Specifically, multicystic ovaries and

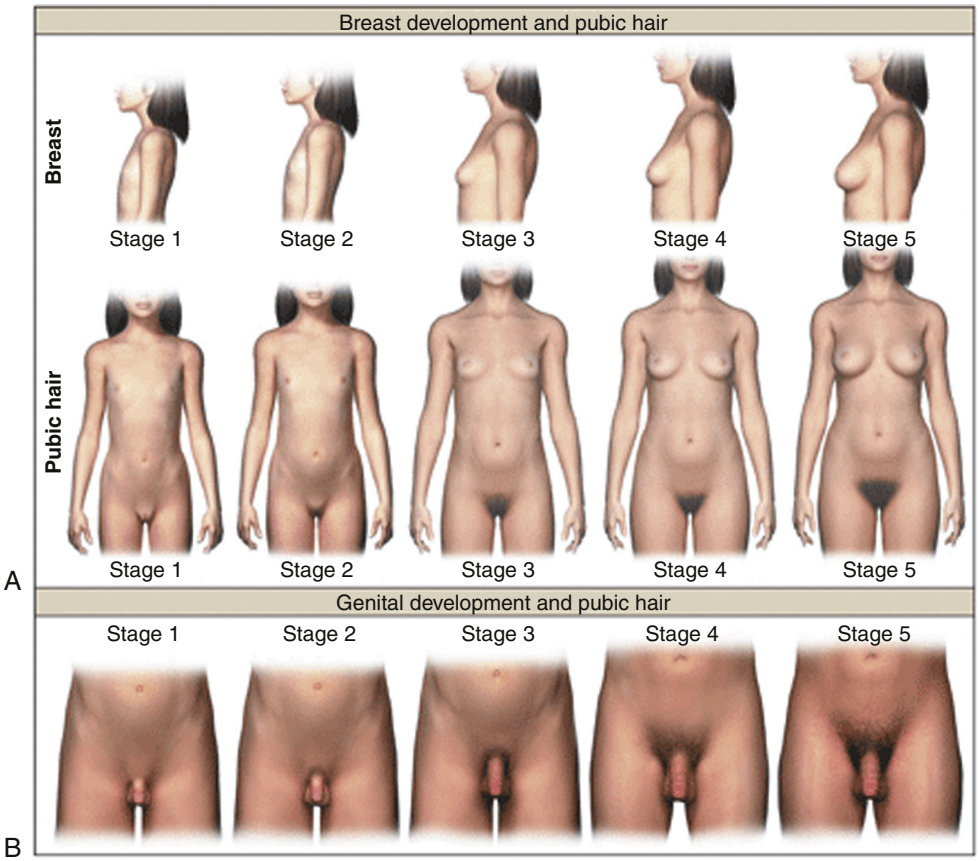


FIGURE 18.1 Pubertal Rating According to Tanner Stages. **A**, Breast development in girls is rated from 1 (prepubertal) to 5 (adult). Stage 2 breast development (appearance of breast bud) marks the onset of gonadarche. For girls, pubic hair stages are rated from 1 (prepubertal) to 5 (adult). Stage 2 marks the onset of adrenarche. **B**, Genital development in boys is rated from 1 (prepubertal) to 5 (adult). Stage 2 genital development, characterized by enlargement of the testes and scrotum accompanied by a change in the texture of the scrotal skin, marks the onset of gonadarche. Pubic hair development in boys is rated from stage 1 (prepubertal) to stage 5 (adult). Stage 2 represents the onset of pubarche, which can reflect adrenarche or gonadarche. Although pubic hair and genital or breast development are represented as synchronous in the figure, they do not necessarily take place simultaneously and should be scored separately. In normal boys, stage 2 pubic hair generally develops 1 to 1.5 years after stage 2 genital development. *(From Carel JC, Léger J. Clinical practice. Precocious puberty.* N Engl J Med. *358:2366;2008. Reproduced with permission of the New England Journal of Medicine.)*

Table 18.1 Stages of Pubertal Development (Tanner)

Girls

Stage	*Breast*	*Pubic Hair*
1	Prepubertal	No pigmented hair
2	Budding with larger areolae	Small amount of coarse, pigmented hair mostly along labia majora
3	Enlargement of breast and areolae	Spread of coarse, pigmented hair over mons pubis
4	Secondary mound of areolae	Almost adult pattern
5	Mature contour	Adult pattern

Boys

Stage	*Genitalia*	*Pubic Hair*	**Testicular Volume**
1	Prepubertal	No pigmented hair	<3 mL
2	Thinning and darkening of scrotum, increased size of penis	Small amount of coarse, pigmented hair at base of penis	3-8 mL
3	Increased diameter of penis	Coarse, pigmented hair extends above penis	10-15 mL
4	Increased diameter and length of penis	Almost adult pattern	15-20 mL
5	Adult size and shape	Adult pattern	>25 mL

increased ovarian volume have been observed using transabdominal ultrasound.[10,11]

In girls, increased adrenal androgen secretion is considered to be responsible for the development of darker hairs along the labia, which is classified as pubic hair stage 2. The hair becomes darker and coarser during pubic hair stage 3, spreading over the pubic symphysis with gradual progression to a full female escutcheon. Apocrine odor may precede or accompany the development of pubic hair. Associated findings include axillary hair, acne, and oiliness of skin and hair.

For boys, an increase in testicular volume and enlargement of the scrotum is considered genital stage 2. At stage 2, the testes are approximately 4 to 8 mL in volume with the longest axis being approximately 2.5 cm. The volume of the mature human testis is approximately 20 to 30 mL and represents increased growth of the seminiferous tubule due to Sertoli cell proliferation and differentiation, and initiation of spermatogenesis. At genital stage 3, further growth of the testes has occurred, and the length and diameter of the penis has increased. At genital stage 4, penile size has increased and the scrotal skin has become darkened. Palpation and use of orchidometer is preferable to inspection. Male pubic hair stage 2 consists of downy hair at the base of the penis. For pubic hair stage 3, the hair is longer, darker, and extends over the junction of the pubic bones. For pubic hair stage 4, the extent of hair has increased, but has not yet achieved the adult male escutcheon. Other secondary sexual characteristics in boys include axillary hair, increased size of the larynx, deepening of the voice, increased bone mass, and increased muscle strength. Approximately 3 years after the appearance of pubic hair, terminal hair appears in androgen-dependent regions on the face and trunk where it may develop for years thereafter. There is considerable variation in the distribution and density of beard, chest, abdominal, and back hair, presumably reflecting genetic differences. The appearance of spermatozoa in early morning urine specimens (spermaturia) occurs during genital stage 3. Gynecomastia is observed in 50% of boys.[12] Typically, this is most prominent in midpuberty when the ratio of circulating concentrations of estradiol to testosterone is relatively high. In most instances, gynecomastia resolves spontaneously by 16 years of age.

The pubertal growth spurt in girls occurs concurrently with the onset of breast development. Usually only 4 to 6 cm of growth occur after menarche. The pubertal growth spurt in boys, with an average height velocity of 9.5 cm per year, occurs around genital stages 3 and 4. In general, the age at peak height velocity shows an inverse relationship with the magnitude of the growth spurt.[13,14] Linear growth is approximately 99% complete for girls at a bone age of 15 years and for boys at a bone age of 17 years.[15]

Breast development in girls and testicular enlargement for boys generally precede pubic hair development. Yet, the tempo for pubic hair development is faster such that synchrony between genital and pubic hair development occurs during the later stages of puberty.[16] Schemata for the temporal development of the secondary sexual characteristic and their relationship to growth velocity is shown for girls and boys in Figures 18.2 and 18.3, respectively.

Secular Trends and Racial and Ethnic Differences in the Onset and Tempo of Puberty

Reports of secular changes in the onset of puberty have focused on girls. Available historical data indicate that there was a decline in the age of menarche in Europe and North America from the early nineteenth century (16 to 17 years of age) to the latter half of the twentieth century (13 years of age). This trend has been attributed to the improving socioeconomic conditions during this epoch.[4] Although the most recent data from North America, several European countries, and other regions of the industrialized world indicate that the trend to earlier menarche has been reduced or halted,[6,17-21] breast and pubic hair development are apparently occurring earlier than 50 years ago in both North America and Europe.[6,22] The biology underlying this

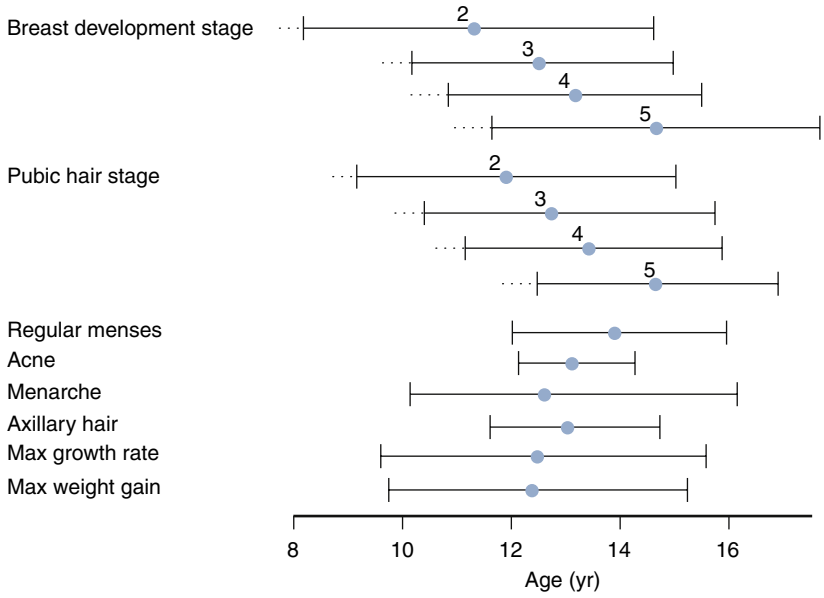


FIGURE 18.2 Mean ages *(dots)* and ranges *(horizontal lines)* of pubertal onset and development in girls. *Dashed lines* show the earlier limit for African-American girls. Max, maximum. *(From Lee PA. Puberty and its disorders. In Lifshitz F [ed].* Pediatric Endocrinology, *4th ed. New York: Marcel Dekker, 2003, p 212. Reproduced with permission of Dr. Peter Lee and Marcel Dekker.)*

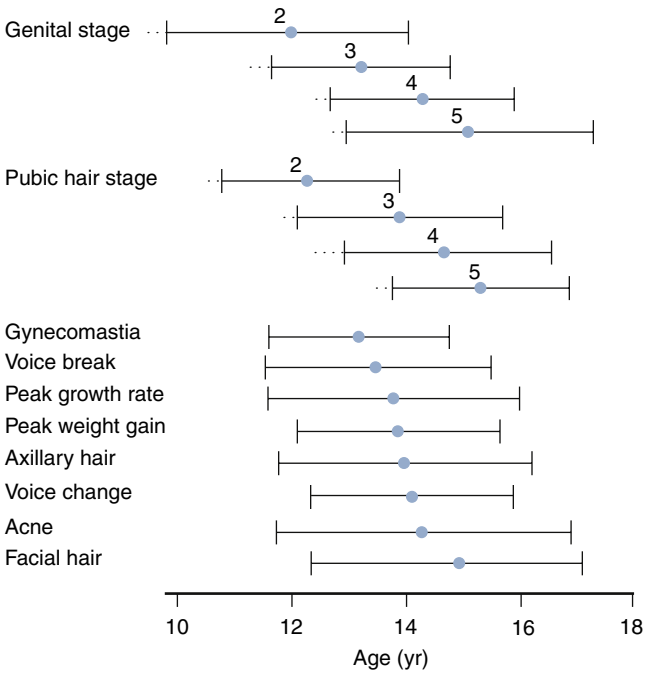


FIGURE 18.3 Mean ages *(dots)* and ranges *(horizontal lines)* of pubertal onset and development in boys. *Dashed lines* show the earlier limit for black boys. *(From Lee PA. Puberty and its disorders. In Lifshitz F [ed].* Pediatric Endocrinology, *4th ed. New York: Marcel Dekker, 2003, p 213. Reproduced with permission of Dr. Peter Lee and Marcel Dekker.)*

continued positive secular trend in sexual development in girls, which in some populations is loosely associated with a similar trend in growth, is unclear and may or may not involve an earlier onset of gonadarche or adrenarche. This earlier onset of breast development is not associated with increased gonadotropin or estradiol concentrations, suggesting that this represents a gonadotropin-independent event.[22] Analogous studies of boys are limited, but no striking sex differences in secular trends in puberty and growth are apparent. Using both genital staging and the orchidometer, the Copenhagen Puberty Study reported pubertal onset occurring 3 months earlier in Danish boys and that obesity advanced the onset of testicular enlargement.[23] However, other studies suggest that obesity delays onset of puberty in boys.[24]

It is important to also recognize that the age at onset of puberty varies between ethnic groups. Among American girls, mean ages for breast development, pubic hair development, and menarche were 9.5, 9.5, and 12.1 for African-American girls; 9.8, 10.3, and 12.2 for Mexican-American girls; and 10.3, 10.5, and 12.7 years for white girls.[25] Data obtained through the Third National Health and Nutrition Examination Survey (NHANES III) between 1988 and 1994 showed that black girls enter puberty first, followed by Mexican-American and white girls.[25,26] For boys in NHANES III, median estimated ages for genital and pubic hair stage 2 were 9.3 and 11.1 for African-American boys; 10.4 and 12.3 for Mexican-American boys; and 10.1 and 12.0 for white youths. For genital and pubic hair stage 5, median ages were 14.9 and 15.2 for African-American boys; 15.8 and 15.7 for Mexican-Americans; and 16.0 and 15.6 for white boys.[27]

Physiology of Puberty

Steroidogenesis

The biosynthetic pathways for gonadal and adrenal steroids are considered together because of their similarities and importance in understanding the physiology and pathophysiology of puberty (Fig. 18.4; also see Chapter 4). Synthesis of steroids from cholesterol requires expression of specific enzymes and other proteins in the adrenal cortex and the gonads. The adrenal cortex consists of three zones, the zona glomerulosa, zona fasciculata, and zona reticularis. The zona glomerulosa synthesizes aldosterone, a mineralocorticoid, and is primarily regulated by potassium concentrations and renin-angiotensin. The zona fasciculata synthesizes cortisol. Steroidogenesis in the zona fasciculata is primarily governed by adrenocorticotropin (ACTH). The zona reticularis synthesizes the so-called adrenal androgens, which are weak androgen-like hormones that do not bind to the androgen receptor (AR).

ACTH is a peptide derived following proteolytic cleavages of pro-opiomelanocortin (POMC) and has both acute and chronic effects on the adrenal cortex. Acutely, it promotes uptake of plasma low-density lipoproteins, stimulates cholesterol esterase activity, enhances synthesis and phosphorylation of steroidogenic acute regulatory protein (StAR), and promotes cortisol secretion. The chronic effects of ACTH involve stimulation of transcription and translation of steroidogenic enzyme genes. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) influence gonadal steroid biosynthesis. LH promotes ovarian theca cell and testicular Leydig cell steroidogenesis. FSH promotes ovarian granulosa cell estrogen biosynthesis. ACTH, LH, and FSH bind to cognate G-protein-coupled seven-transmembrane domain receptors that signal via cyclic adenosine monophosphate (cAMP) and protein kinase A.

Most enzymes involved in steroidogenesis are cytochrome P450s (CYPs) or hydroxysteroid dehydrogenases. The rate-limiting step of steroidogenesis is transport of cholesterol into mitochondria mediated by StAR. Within the mitochondria, cholesterol desmolase (also known as side-chain cleavage or P450scc) converts cholesterol into pregnenolone. One enzyme, 17 α-hydroxylase/17,20-lyase (P450c17), encoded by the *CYP17A1* gene, is the qualitative regulator of adrenal and gonadal steroidogenesis. This enzyme mediates 17α-hydroxylation to convert pregnenolone into 17α-hydroxypregnenolone. In the adrenal zona reticularis, ovarian theca, and Leydig cells, this same enzyme catalyzes scission of the C17–20 bond to produce DHEA. Although this one protein is capable of two distinct enzymatic reactions, these enzyme activities are differentially regulated. The three factors known to modulate 17,20-lyase activity include: (1) amount of P450 oxidoreductase; (2) expression of cytochrome b_5; and (3) phosphorylation of serine/threonine residues on P450c17.[28] P450 oxidoreductase (POR) is a protein that transfers electrons from nicotinamide adenine dinucleotide phosphate to microsomal cytochrome P450 enzymes such as P450c17, P450c21, and aromatase (P450aro). Cytochrome b_5 modulates adrenal androgen secretion by increasing the 17,20-lyase activity of (P450c17).[29-31]

The Δ^5-steroids are converted to the Δ^4-steroids by 3β-hydroxysteroid dehydrogenase type 2, the adrenal and

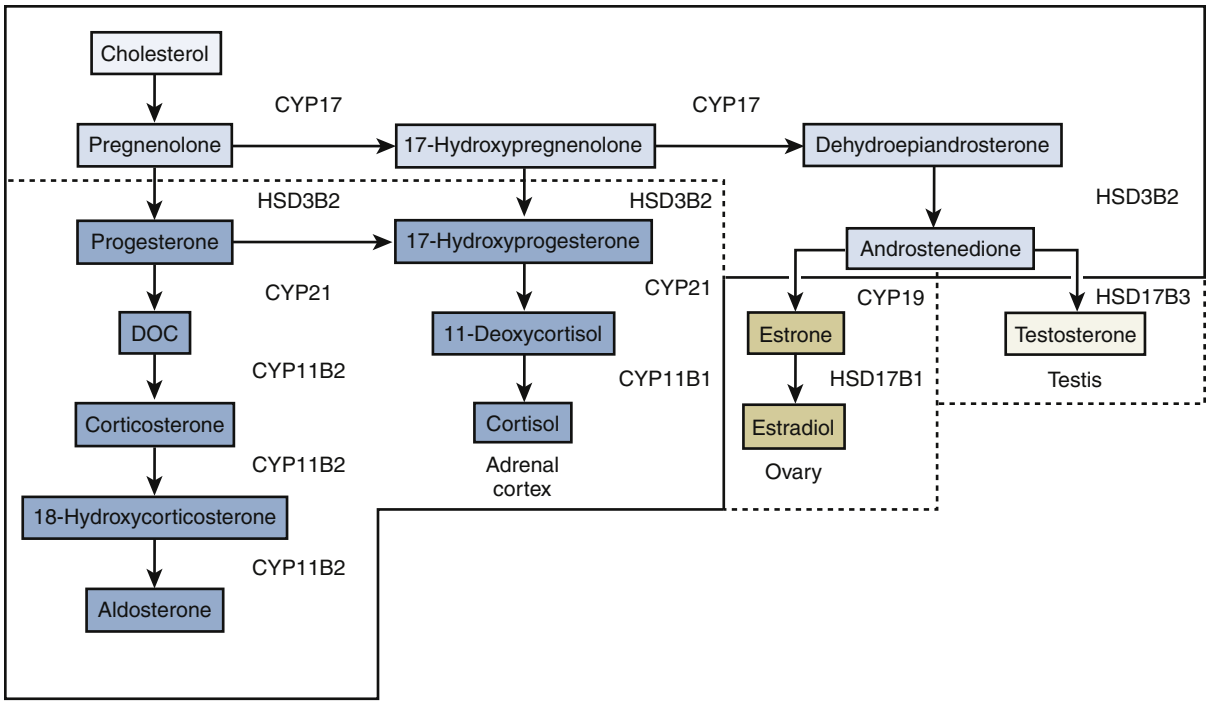


FIGURE 18.4 Steroidogenesis. This figure shows pathways of adrenal, ovarian, and testicular steroidogenesis. *Dark solid lines* indicate predominant pathways for adrenal steroidogenesis. *Dashed lines* indicate predominant pathways for gonadal steroidogenesis. Genes responsible for specific enzymatic steps are indicated. Enzymatic steps for which P450 oxidoreductase serves as a cofactor (encoded by POR) are indicated. *Arrow* indicates "backdoor pathway" of steroidogenesis. DOC, deoxycorticosterone. *(Adapted from Witchel SF, Lee PA. Ambiguous genitalia. In Sperling MA [ed].* Pediatric Endocrinology, *2nd ed. Philadelphia: WB Sounders, 2002, p 119.)*

gonadal isoform, resulting in conversion of DHEA to androstenedione. In the zona fasciculata, 17-hydroxypregnenolone is converted to 17-hydroxyprogesterone (17-OHP) by 3β-hydroxysteroid dehydrogenase. Subsequently, 17-OHP is converted to 11-deoxycortisol by 21-hydroxylase (P450c21) and to cortisol by 11β-hydroxylase (P450c11β).

The adrenals, ovaries, and testes synthesize sex steroids. In the zona reticularis of the adrenal cortex, DHEA sulfotransferase (SULT2A1) converts DHEA to DHEAS; this enzyme is also expressed in the liver. Sulfation of steroids by SULT2A1 requires a sulfate donor, 3′-phosphoadenosine 5′-phosphosulfate (PAPS), and the enzyme PAPS synthase.[32] In the ovary, androstenedione is synthesized in the theca cell and diffuses into the granulosa cell, where it is aromatized by aromatase (P450aro) to estrone and converted to estradiol by 17β-hydroxysteroid dehydrogenase type 1. In Leydig cells, androstenedione is converted to testosterone by 17β-hydroxysteroid dehydrogenase type 3. In many androgen target cells, such as those in the external genitalia and prostate, testosterone is converted to DHT by 5α-reductase type 2. In other androgen sensitive tissues, such as bone and adipose, testosterone is converted to estradiol by aromatase.

The 17β-hydroxysteroid dehydrogenase enzymes comprise a large family of enzymes involved in steroid biosynthesis and metabolism. At least 17 different enzymes have been described.[33] Differences in tissue distribution, substrate preferences, subcellular localization, and mechanisms of regulation lead to micro-environment adjustment of sex steroids.[34] The type 1 isozyme, 17βHSD1, is expressed in ovaries, placenta, endometrium, and liver where it favors conversion of estrone to estradiol. The type 3 isozyme, 17βHSD3, is expressed in the testis where it preferentially converts androstenedione to testosterone.[35] The type 5 enzyme, 17βHSD5, is an aldo-keto-reductase (AKR) enzyme (AKR1C3) which is expressed in steroidogenic and peripheral tissues; it can convert androstendione to testosterone.[36]

Through investigations of the tammar wallaby and patients with disordered steroidogenesis, the presence of another pathway leading to dihydrotestosterone (DHT) synthesis was uncovered.[37] In this "backdoor pathway," 17-OHP undergoes 3α- and 5α-reduction followed by 17,20-lyase, 17β-hydroxysteroid dehydrogenase, and 3α-oxidation steps to generate DHT in the absence of the intermediates, DHEA, androstenedione, and testosterone, requisite to the classical pathway. In human, since 17-OHP is not a favorable substrate for the 17,20-lyase reaction, this pathway acquires functional importance in disorders of steroidogenesis associated with increased 17-OHP concentrations such as congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency and oxidoreductase deficiency.

Once secreted, sex steroids circulate bound to sex hormone-binding globulin (SHBG) and to albumin.[38] The unbound or free hormone is considered to be the bioavailable form that diffuses passively into target cells and interacts with nuclear steroid receptors. These are ligand dependent transcription factors, comprising three functional domains: the N-terminal domain serves to modulate function; the DNA-binding domain mediates binding of the receptor to DNA; and the ligand-binding domain binds to steroids.[39] Steroid receptor activity is modulated by various tissue-specific cofactors, both coactivators and corepressors.

It should also be noted that nongenomic actions of steroids have been reported.[40] For example, testosterone can

activate phospholipase C leading to calcium influx into Sertoli cells and can activate the mitogen-activated protein kinase as well as other intracellular mediators.[41] Sex steroids can be metabolized to inactive forms by a variety of enzymes. Glucuronidation decreases the biological activity of steroid hormones and increases solubility to facilitate renal excretion. This process, catalyzed by UDP-glucuronyltransferase (UGT) enzymes, involves the transfer of glucuronic acid from uridine diphosphoglucuronic acid to steroid hormones. In humans, the UGT2B isoforms show greater specificity for C19 androgens.[42] A second mechanism is sulfoconjugation, in which DHEA sulfotransferase catalyzes conversion of DHEA to DHEAS and estrogen sulfotransferase converts estrogens to estrone sulfate. The inactive sulfated steroids can be hydrolyzed to active forms by steroid sulfatase.

Activation of Gonadarche

Increased gonadal steroidogenesis and the completion of gametogenesis during gonadarche are stimulated by enhanced secretion of the gonadotropins, LH and FSH. LH and FSH are heterodimeric proteins comprised of a common α-subunit and a unique β-subunit; all are glycosylated peptides (also see Chapter 2). The temporal increments in circulating LH and FSH concentrations at the time of puberty, and their relationships to those of the gonadal steroids, testosterone and estradiol, respectively, at this stage of development, have been well documented in both boys and girls.[4,5,43] The actions of LH and FSH are mediated by their cognate seven-transmembrane domain G-protein—coupled receptors: the LH receptor (LHR) and FSH receptor (FSHR), respectively (also see Chapter 2). In the female, LH stimulates androgen production by the thecal cells of the ovarian follicles and progesterone secretion from luteinized granulosa cells of the corpus luteum. FSH is critical for the process of follicular recruitment and selection.[44] In granulosa cells of the developing follicle, FSH induces expression of aromatase, which is responsible for aromatization of androgens secreted by the theca cells. FSH also induces LHR in granulosa cells of the dominant follicle, which selectively amplifies the effect of declining FSH concentrations on the dominant follicle.[33] In the male, LH regulates the secretion of testosterone from Leydig cells.

Recently, studies of mice have provided evidence that osteocalcin, an osteoblast-specific secreted hormone from bone, is a facilitator of testicular testosterone secretion.[45,46] In its undercarboxylated form, osteocalcin is biologically active signalling at its receptor, GPRC6A, which is expressed by Leydig cells.[45] Posttranslationally, osteocalcin is carboxylated on three glutamic acid residues, and the carboxyated form of the molecule is considered to be biologically inactive. Interestingly, increased osteocalcin concentrations are associated with the puberal rise in testosterone concentrations in boys.[47] Osteocalcin appears to have sexually dimorphic actions since it seems to modulate Leydig cell testosterone production, but not ovarian estrogen production.

FSH, together with testosterone, is responsible for initiating and maintaining spermatogenesis.[48] The action of FSH in this regard is indirect and exerted on the somatic Sertoli cell of the seminiferous tubule. While the actions of testosterone are also indirect, several somatic cell types in the testis (Sertoli, Leydig and peritubuar) express AR and are considered to be involved in the control of spermatogenesis.[49]

Activation of the ovary and testis during gonadarche also results in the increased secretion of the gonadal proteins, known as *inhibins*, which are members of the TGF-β superfamily. Mature inhibins are dimers composed of a common α-subunit covalently linked with one of two β-subunits (β_A and β_B). The α/β_A and α/β_B dimers are known as inhibin A and inhibin B, respectively.[50] The gonadal inhibins, like the gonadal steroids, play both an endocrine role in the regulation of gonadotropin secretion and a paracrine role within the gonads. Inhibin B, which is synthesized in part by the Sertoli cell, is the principal inhibin secreted by the testis.[51] In prepubertal boys, mean concentrations of circulating inhibin B are approximately 60 pg/mL, which compare with adult values that vary considerably about an average of 200 pg/mL.[52,53] The pubertal increase in inhibin B may be attributed to Sertoli cell proliferation and to the initiation of spermatogenesis, both of which reflect the increased gonadotropin drive to the testis at the time of gonadarche.[48]

In girls, circulating levels of inhibin A and B are low or undetectable prior to puberty.[54] Inhibin B begins to rise with the onset of puberty, as does inhibin A in breast stages 3 and 4. Adult levels are attained at approximately 14 to 15 years of age.[55] During the menstrual cycle, inhibin A levels are elevated in the luteal phase, while inhibin B predominates in the circulation of the follicular phase.[56]

Anti-müllerian hormone (AMH) or müllerian-inhibiting hormone (MIH) is also a member of the TGF-β superfamily. While AMH plays a critical role in testicular development and in the regression of the müllerian ducts during male fetal development, it continues to be secreted postnatally by the Sertoli cells of the testis and the granulosa cells of the ovary. In boys, AMH concentrations decline at puberty.[57] Although FSH stimulates AMH production, testosterone inhibits Sertoli cell AMH secretion. The decline in AMH concentrations appears to be closely coupled to rising inhibin B concentrations at the onset of puberty in boys,[58] the latter presumably reflecting androgen induced differentiation of the Sertoli cell. Both AMH and inhibin B concentrations are low in boys with bilateral anorchia or complete hypogonadotropic hypogonadism (complete HH).[59] Immunohistochemical studies of human ovaries showed no AMH staining in primordial follicles, highest expression in growing preantral and small antral follicles, and disappearance in larger follicles (greater than 8 mm).[60]

Insulin like factor-3 (INSL3), a peptide hormone secreted by Leydig cells, plays a major role in directing testicular descent during gestation. INSL3 concentrations are low during infancy, rise during puberty, and reflect Leydig cell function during adulthood.[61]

The pubertal drive to the pituitary-gonadal axis is generated by a diffusely distributed network of hypothalamic neurons expressing gonadotropin releasing hormone-1 (GnRH-I)[62] known as the "hypothalamic GnRH pulse generator" (discussed later in the chapter and also Chapters 1 and 8). As the name implies, the hypothalamic GnRH pulse generator produces an intermittent discharge of GnRH into the hypophysial portal circulation that is obligatory for gonadotropin synthesis and secretion by the pituitary gonadotrophs.[44] LH and FSH secretion is stimulated by GnRH acting through its receptor, GnRH-R1, located

on gonadotropin secreting cells (gonadotrophs) in the pituitary gland.[63,64] A unique feature of the human GnRHR, a 7-transmembrane domain G-protein coupled receptor, is its lack of a C-terminal cytoplasmic domain.[65]

A second GnRH gene *(GnRH-II)* is also expressed by neurons in the primate brain and a GnRH-II receptor has been cloned from the pituitary, but in the human the gene encoding for this receptor does not generate a functional protein.[66] The significance, if any, of this second GnRH system in the control of the human pituitary-gonadal axis has not been delineated. Similarly, a hypothalamic RFamide-related peptide (RFRP3) that is inhibitory to gonadotropin secretion in several species and considered to be the homolog of gonadotropin-inhibitory hormone (GnIH) in birds has been identified in mammalian species,[67] but its neuroendocrine role in man is unclear.

In conditions under which pulsatile GnRH release is compromised, such as occurs in anorexia nervosa and during periods of strenuous physical training especially in young women, gonadotropin secretion is attenuated and pubertal development is arrested. Thus, the pituitary-gonadal axis in both males and females may be viewed as being a slave to the hypothalamic GnRH pulse generator, and this analogy should be held in mind when considering the mechanisms triggering the onset of gonadarche.

In the pubertal and postpubertal individual, the ovaries and testes are governed by feedback control systems.[68] GnRH and the gonadotropins comprise the feed-forward components from hypothalamus to pituitary and from pituitary to gonad, respectively. Steroid and protein hormones from the gonads, in turn, provide the feedback signals that regulate the secretion of LH and FSH. The feedback actions of these gonadal hormones, which involve both negative and stimulatory (positive) actions, may be exerted directly at the level of the pituitary gonadotrophs to modulate expression of the genes encoding LHβ and FSHβ (*LHB* and *FSHB*, respectively).[63] Feedback may also be exerted indirectly at the level of the hypothalamus to regulate the release of GnRH (see Chapter 1).

In the male, a negative feedback action of testosterone and inhibin B are the major regulators of LH and FSH secretion, respectively.[48,68,69] The action of testosterone is predominantly exerted at the hypothalamic level, while that of inhibin appears to occur directly at the pituitary. The role of aromatization of testosterone to estradiol in mediating the negative feedback action of this androgen on LH secretion continues to be an area of active investigation.[70-72] The feedback control of LH and FSH throughout the menstrual cycle is complicated and involves both negative and positive feedback actions of ovarian steroids at both the hypothalamic and pituitary levels[44] (see also Chapter 8). The maintenance of normal ratios of circulating LH and FSH concentrations is important for gonadal function, particularly for folliculogenesis and ovulation. The role of ovarian inhibins in regulating gonadotropin secretion in the pubertal and premenopausal women remains to be fully elucidated.

The Hypothalamic GnRH Pulse Generator

In the human hypothalamus there are some 1000 to 2000 diffusely distributed GnRH neurons, many of which send their projections to the median eminence where they synchronously and intermittently discharge their peptide into the primary plexus of the hypophysial portal circulation thereby providing the pituitary gonadotrophs with the pulsatile stimulation that is essential for maintaining gonadotropin secretion.[44,62] While the neurobiological mechanisms that underlie GnRH pulse generation remain controversial, compelling evidence indicates the fundamental role of KNDy neurons in the arcuate nucleus (aka infundibular nucleus).[73-76] These hypothalamic neurons, named because they co-express kisspeptin, neurokinin B (NKB), and dynorphin,[77] project their axons to the median eminence where they mingle intimately with GnRH fibers en route to the portal vessels.[78] It is proposed that GnRH pulse generation is achieved by reciprocating stimulatory (NKB) and inhibitory (dynorphin) connections within the arcuate nucleus, while the output of the pulse generator is relayed to GnRH fibers projecting to the median eminence by an intermittent kisspeptin signal. Thus, these neurons may represent the anatomic site of the GnRH pulse generator. Loss of function mutations in the genes encoding for kisspeptin *(KISS1)* or the kisspeptin receptor, also called GPR54, *(KISS1R)* and for NKB *(TAC3)* or the NKB receptor *(TACR3)* in humans lead to a profound hypogonadotropic state that is typically manifest at puberty.[79-82] The importance of these neurons has also been demonstrated by the finding that selective ablation of KNDy neurons in the postpubertal rat results in a loss in hypothalamic drive to LH secretion.[83] A contemporary model for the GnRH pulse generator is shown in Figure 1.15.

Neurobiology of Gonadarche

Fetal Development of the GnRH Pulse Generator

The 1000 to 2000 GnRH neurons in the postnatal hypothalamus are born in the olfactory placode and migrate during early fetal development from the nose through the forebrain to the hypothalamus.[84] The fetal ontogeny of the GnRH neurons can be categorized into several stages each with distinct regulatory mechanisms; (1) differentiation of GnRH neurons; (2) migration along with axons of the vomeronasal nerve and guidance into the forebrain; (3) localization in the hypothalamus and development of processes to the median eminence; and (4) attainment of final location and functionality.[84] Specific mutations in families with disorders of puberty and studies of transgenic mice have established some of the factors involved in GnRH neuron migration. Precise spatiotemporal expression of these factors, which include cell matrix or adhesion proteins, neurotransmitters, growth factors, hormone receptors, and transcription factors is essential to normal GnRH pulse generator activity and therefore pituitary-gonadal function.

GnRH neurons are derived from a heterogenous stem cell population in the embryonic olfactory placode. Factors involved in the differentiation of GnRH neurons include fibroblast growth factor receptor-1 (FGFR1), fibroblast growth factor-8 (FGF8), heparan sulfate 6-O-sulfotransferase 1 (HS6ST1); chromodomain helicase DNA-binding protein 7 (CHD7), and nasal embryonic LH-releasing hormone (NELF). The FGFR1 is a tyrosine kinase receptor composed of three extracellular immunoglobulin domains, a transmembrane domain, and a cytoplasmic tyrosine kinase domain. The extracellular domain interacts with heparan

sulphate proteoaminoglycan, its coreceptor. Heparan sulfates are cell membrane and matrix-associated proteoglycans involved in neural development. These polysaccharides undergo nonrandom modifications of the sugar moieties to facilitate cell to cell communication. HS6ST1 introduces a sulfate at the 6-O position within heparan sulfate. This action appears to be necessary for FGFR1 function. CHD7 is a large protein that participates in chromatin remodeling and transcription.

Identification of a missense mutation in the FGF8 gene in families with HH confirmed that FGF8 is the cognate ligand for FGFR1.[85] Upon ligand binding, FGFR1 and its co-receptor dimerize leading to autophosphorylation, and protein kinase activity.[86] Expression studies in human fetuses demonstrated FGF8 is detected in the telencephalon and ventral diencephalon, but not in Rathke's pouch. FGF8 plays a role in the development of the neuroendocrine system; loss of function *FGF8* mutations have been associated with holoprosencephaly indicating its role in the development of the hypothalamus and pituitary.[87]

Around the 6th week of human gestation, GnRH neurons begin their migration along vomeronasal nerves to the cribiform plate and eventually find their way to the hypothalamus. Several adhesion and guidance molecules have been implicated in this process, including anosmin-1, ephrins, fibroblast growth factor 1, and prokineticin 2 (PROK2).[84]

The gene encoding anosmin-1, *KAL1*, is located at Xp22.3 in the pseudoautosomal region of the X chromosome and encodes anosmin-1.[88-90] Anosmin-1 is an extracellular matrix glycoprotein that contains motifs found in other molecules involved in neuronal guidance activity, including fibronectin III repeats and a whey-acidic protein domain. The precise function of anosmin-1 is unclear. However, putative functions include a guidance platform for migrating GnRH neurons, chemoattractant for olfactory axon pathfinding, and/or interactions with FGFR1.[84]

NELF is hypothesized to play a role in neuronal migration.[91] Semaphorins comprise a large and diverse family of secreted and membrane-associated proteins that influence navigation of growing axons. Semaphorin-3A (SEMA3A) is a secreted protein with repulsive effects on primary olfactory axons expressing the coreceptor neuropilin-1 (Nrp1), which may influence the migration of GnRH neurons. PROK2 and its receptor (PROKR2), a G-protein-coupled receptor, appear to modulate GnRH neuron migration.

Upon arrival in the hypothalamus, the GnRH neurons then extend projections to the median eminence to form a network, which can secrete GnRH into the primary plexus of the hypophysial portal circulation. LH and FSH reach detectable levels by the 10th week of gestation in the human pituitary, peak in midgestation, and are higher in female fetuses than male fetuses.[4,5] Although the hypothalamic control of the fetal pituitary gonadal axis has not been extensively studied in higher primates, the GnRH pulse generator is clearly driving the gonadotrophs of the fetal pituitary around the 15th week of gestation.[5,62] Functional activity of this hypothalamic-pituitary system is essential for fetal testicular testosterone synthesis by the Leydig cell and normal male sex development. In contrast to the fetal testis, the ovary at this stage of development is relatively quiescent, and the absence of gonadal feedback signals likely account for the higher gonadotropin levels in the female fetus. As gestation progresses, the secretion of estradiol and other steroids by the fetoplacental unit increases dramatically, and suppresses gonadotropin secretion from the fetal pituitary by exerting an inhibitory action either directly at the pituitary or indirectly on the hypothalamus to restrain GnRH release.

Postnatal Development of GnRH Pulsatility

Following birth, GnRH pulse generator activity is robustly expressed presumably due to the the loss of placental steroids, and the pituitary gonadotrophs of the infant respond with LH and FSH secretion.[5] At the hypothalamic level, kisspeptin and NKB are found in the arcuate nucleus at this stage of development,[92,93] and a loss of function mutation in *KISS1* has been associated with HH during infancy.[94] Moreover, in the case of the infant boy, the Leydig cells of the testis are stimulated such that circulating testosterone levels are similar to those observed in adult men.[95] Peak testosterone concentrations occur at approximately 2 to 3 months of age and typically decline by 6 months of age. Among preterm male infants, LH and testosterone concentrations are higher than among full term infants; phallic growth was positively correlated with urinary testosterone levels and testicular growth was positively correlated with urinary FSH levels.[96] Despite the "adult-like" endocrine milieu of increased gonadotropin secretion and elevated testosterone concentrations during the first few months of life, sexual hair does not develop and gametogenesis is not initiated presumably due to limited androgen receptor signalling in skin and the immature Sertoli cell.[97,98] The hypothalamic-pituitary-ovarian axis is active in term and preterm girls and is associated with a transient increase in antral follicles.[99]

Full hormonal responsivity of the gonad is acquired during childhood but by this stage of development the GnRH pulse generator has been brought into check resulting in the hypogonadotropic state that guarantees continued gonadal quiescence until the prepubertal phase of development is terminated by a resurgence of GnRH pulse generator activity.[100,101] During childhood and juvenile development neither the GnRH neurons, pituitary gonadotrophs, nor the cells of the gonads are limiting to the onset of gonadarche.[5] Accordingly, during these stages of development when the primate hypothalamus or pituitary is provided with pulsatile neurochemical (N-methyl-D-aspartate [NMDA], a glutamate receptor agonist)[102,103] or GnRH stimulation, respectively, or when the ovary and testis are stimulated directly with LH and FSH, gonadarche may be readily elicited. This phenomenon is graphically illustrated in children with GnRH-dependent precocious puberty, a disorder discussed later in this chapter. That the GnRH neuronal network in the juvenile monkey hypothalamus is able upon NMDA stimulation to immediately elicit an adult hypophysiotropic GnRH drive is consistent with the finding that hypothalamic GnRH gene expression and peptide content are maintained throughout this phase of prepubertal development.[102] Thus, transcriptional regulation of the gene encoding GnRH from birth to puberty appears to be minimal, and the locus of the developmental control of GnRH release must lie upstream to the GnRH neuronal network.

Because GnRH is secreted in only picogram quantities into the hypophysial portal circulation, changes in the concentration of this neuropeptide in the peripheral circulation

do not reflect hypothalamic activity. Therefore studies of the dynamics of the pubertal resurgence of GnRH pulse generator activity in man and other higher primates have generally utilized the high-fidelity relationship that exists between the frequencies of pulsatile GnRH release and episodic LH secretion.[104] The latter may be tracked with relative ease by measuring moment-to-moment changes in LH concentrations in the peripheral circulation.[68] Although the pubertal increase in hypothalamic GnRH drive to the gonadotroph probably involves both frequency and amplitude modulation of the GnRH pulse generator, the relationship between GnRH and LH pulse amplitude is more complex than the relationship between frequency; amplitude modulation of LH release may not reflect changes in GnRH pulse amplitude. During initiation of gonadarche in boys and girls, an acceleration in LH pulse frequency and an increased LH pulse amplitude is observed in association with an amplification of a pre-existing sleep-related diurnal pattern in release.[5,105-109] This change in neuroendocrine activity may occur before the physical changes of gonadarche are manifest. In boys in particular, LH pulse frequency appears to decline later in pubertal development, probably due to a negative feedback action of rising testosterone concentrations.[110] A longitudinal study of the agonadal monkey[111] suggests that, as in humans, the pubertal acceleration of pulsatile GnRH release is an early neurobiological event in the initiation of gonadarche, and that it is a rapidly completed process. Thus, the slow tempo of the overall progression of puberty probably results from mechanisms downstream from the hypothalamus, and particularly at the level of the pituitary. A model illustrating the ontogeny of GnRH pulse generator activity from fetal to pubertal development in the male and female is shown in Figure 18.5.

The control system that dictates the up-down-up pattern of GnRH pulse generator activity from birth until puberty may be viewed as a neurobiological "brake" (or central restraint, as it has been previously described in the pediatric literature[4]) that holds GnRH neuronal activity in check during the greater part of prepubertal development.[102] Here it is important to recognize that the notion of a brake is conceptual, i.e., the pubertal resurgence in robust GnRH pulsatility could be occasioned either from the removal of an inhibitory input or by the application of a stimulatory signal to the GnRH pulse generator, or a combination of the two. A similar argument may be applied to the earlier transition between infancy and childhood when GnRH pulsatility is markedly diminished. The neurobiological brake on pulsatile GnRH release throughout childhood and juvenile development is imposed in the absence of the ovary or testis. Consequently, the characteristic pattern of gonadotropin secretion observed during postnatal development in humans—with robust gonadotropin secretion during infancy and puberty, separated by a prolonged hiatus in LH and FSH secretion—is maintained in the agonadal situation (Fig. 18.6).[112,113] Similarly, in male infants with partial androgen insensitivity, LH concentrations are generally elevated and this is associated with higher testosterone levels. Yet, infants with complete androgen insensitivity often fail to demonstrate a postnatal rise in LH and testosterone secretion.[114] The later finding is counterintuitive, and an understanding of the molecular basis of this phenomenon may well reveal fundamental insights into the ontogeny of GnRH pulse generation. In agonadal children, the degree of the prepubertal suppression of gonadotropin release is less than that observed in eugonadal individuals.[4] Interestingly, in agonadal children circulating gonadotropin levels are higher in girls than boys indicating that the intensity of the neurobiological brake imposed on the GnRH pulse generator during prepubertal development is less in the female than in the male. As a result, the gonadotropin drive to the prepubertal ovary stimulates a low level of estradiol secretion,[43] which, through negative feedback action on LH and FSH release, amplifies the relatively weaker neurobiological brake restraining gonadotropin secretion in the prepubertal girl. This sex difference in the strength of the neurobiological brake on prepubertal GnRH release is associated

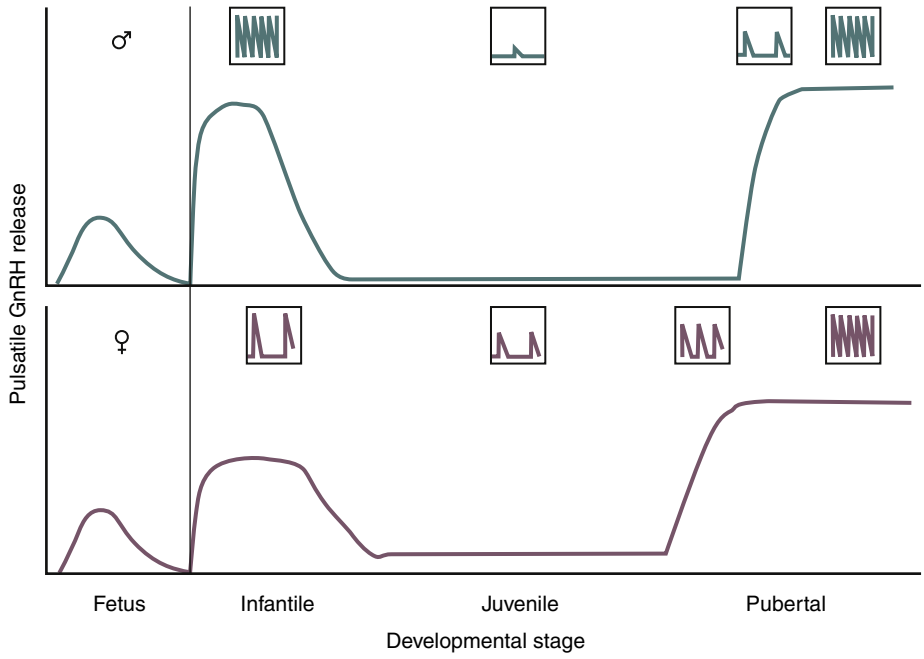


FIGURE 18.5 A schematic of the pattern of pulsatile gonadotropin-releasing hormone (GnRH) release during juvenile development (including childhood) in boys *(above)* and girls *(below)*. Insets indicate frequency of pulsatile GnRH release at respective stages of development. *(From Plant TM. Control of onset of puberty in primates.* Topic Endocrinol *20:1, 2002.)*

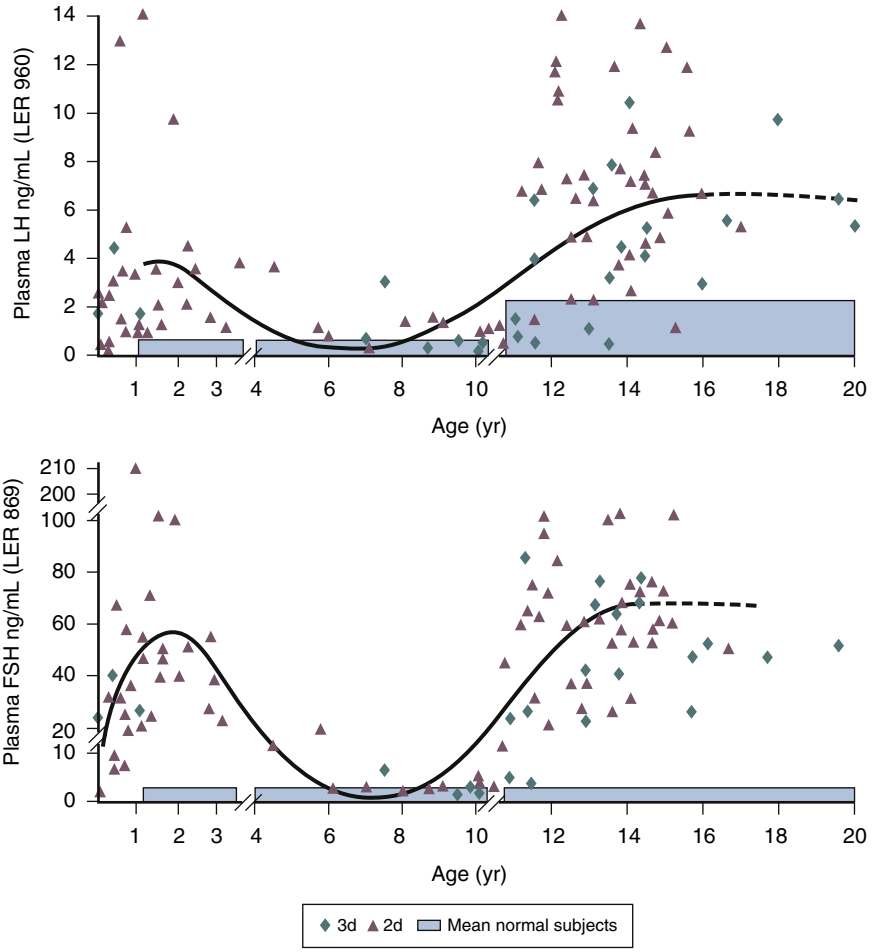


FIGURE 18.6 The neurobiological restraint that holds the gonadotropin-releasing hormone (GnRH) pulse generator in check during childhood is imposed in the absence of the gonads, as reflected by the time courses of circulating concentrations of luteinizing hormone (LH; *top*) and follicle-stimulating hormone (FSH; *bottom*) in 58 patients with gonadal dysgenesis. *Closed triangles*, 45,X karyotype; *circles*, X chromosome mosaicism or structural abnormalities; cross-hatching, mean plasma gonadotropin concentrations in normal girls. *Bold curves* represent polynomial regressions of gonadotropin concentration with age. *(From Conte FA, Crumbach MM, Kaplan SL. A diphasic pattern of gonadotropin secretion in patients with the syndrome of gonadal dysgenesis.* J Clin Endocrinol Metab *40:670, 1975.)*

with a shorter duration of the brake in girls, which probably accounts for the relatively earlier age of gonadarche in the female. These and other sex differences in the developmental control of the GnRH pulse generator are presumed to result from greater exposure of the fetal male hypothalamus to testosterone.[5]

The Nature of the Neurobiological Brake

The discovery in 2003 that loss of function mutations in *KISS1R* in humans were associated with HH and delayed or absent puberty,[79,80] demonstrated the critical role of kisspeptin in regulating GnRH secretion. A multitude of subsequent studies in several different experimental models supported this finding.[76,81,115-118] These data led to the proposal in the previous edition of this book that a major component of the neurobiological brake imposed upon pulsatile GnRH release during the greater part of prepubertal development is comprised by a hiatus in a stimulatory kisspeptin input to the GnRH neuronal network. This proposal was based on findings in the monkey, that hypothalamic expression of *KISS1* and release of kisspeptin in the region of the median eminence increase at the time of the pubertal resurgence in GnRH pulsatility.[119,120] Additionally, intermittent administration of kisspeptin at hourly intervals during juvenile development elicits a precocious and sustained adult-like pulsatile pattern of GnRH,[121] and the pubertal increase in GnRH release may be suppressed by administration of a KISS1R receptor antagonist directly to the median eminence.[122]

The recent finding in humans, however, that loss of function mutations in the NKB signalling pathway are associated with a phenotype similar to that reported earlier for inactivating mutations in *KISS1R*,[82] together with the fascinating observation that these two neuropeptides are expressed in the same neurons (KNDy neurons) in the arcuate nucleus,[77] have led to the emergence of the idea that these neurons are responsible for the generation of GnRH pulsatility (described earlier in this chapter). Thus, the current data justify revision of the earlier model. While kisspeptin signalling remains obligatory for puberty in the revised model, this is simply because the kisspeptin expressing KNDy neurons in the arcuate nucleus comprise a critical component of the GnRH pulse generator.[76] In the absence of an intact kisspeptin signalling pathway, the output of the GnRH pulse generator will be abrogated and pulsatile GnRH release would be compromised resulting in a delay or absence of puberty. According to this model, KNDy neurons in the arcuate nucleus play no "regulatory" role in controlling the timing of puberty: rather, as a component of the GnRH pulse generator they are subservient to upstream regulatory mechanisms that govern the developmental pattern of pulsatile GnRH release and the onset of puberty (Fig. 18.7).

The nature of the upstream pathways that comprise the neurobiological brake on the GnRH pulse generator during childhood and juvenile development remain poorly understood. Studies of the female rhesus monkey provide evidence that γ-aminobutyric acid (GABA), the major

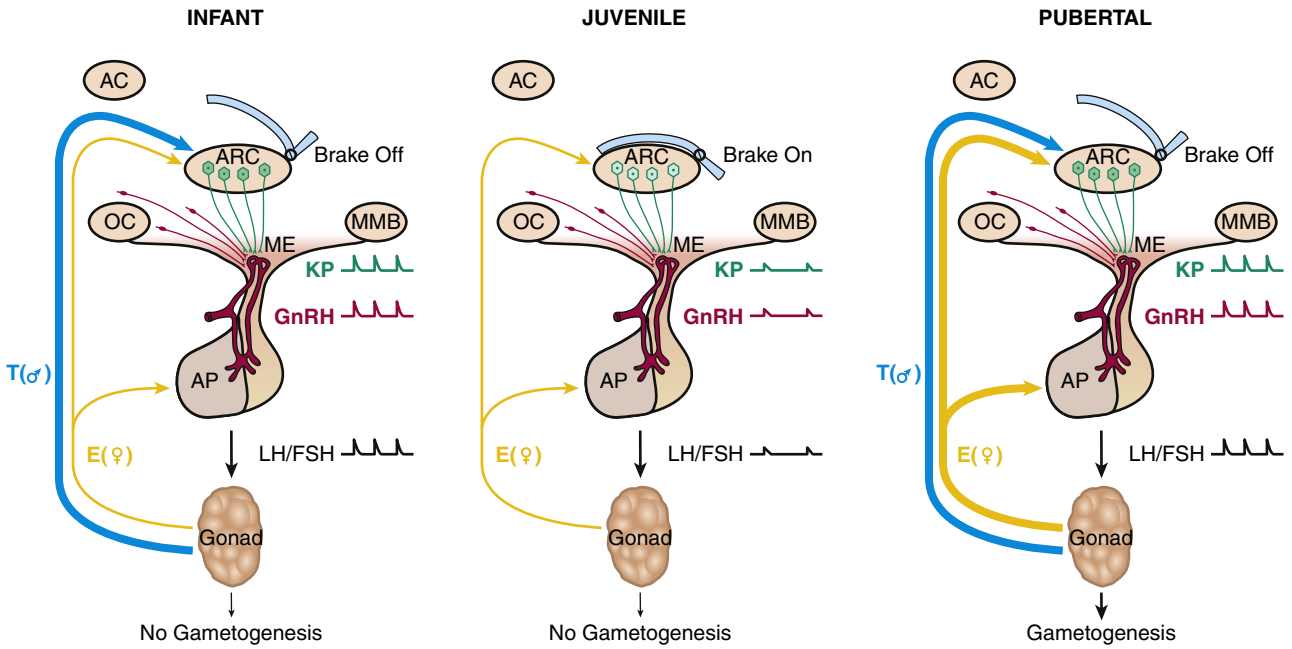


FIGURE 18.7 A model for the control of the timing of puberty. The role of kisspeptin signaling is posited to be a critical component of the neural machinery that generates pulsatile gonadotropin-releasing hormone (GnRH) release from the hypothalamus (the hypothalamic GnRH pulse generator). In this model, the hypothalamic GnRH pulse generator resides in the arcuate nucleus (ARC) and the output of the pulse generator is relayed to GnRH terminals *(red)* in the median eminence (ME) by kisspeptin projections arising from KNDy neurons in the ARC *(green)*. During infancy *(left panel)*, GnRH pulse generator activity is robust leading to intermittent release of kisspeptin in the ME, resulting in a corresponding pattern of GnRH release into the portal circulation. This, in turn, drives pulsatile gonadotropin secretion. In the transition from infancy to the juvenile phase of development *(middle panel)*, a neurobiological brake (central inhibition) restrains the GnRH pulse generator and pulsatile release of kisspeptin in the ME is markedly suppressed. This leads to reduced GnRH release and to a hypogonadotropic state in the juvenile period. Puberty is triggered when the neural brake is released and GnRH pulse generator activity with robust intermittent release of kisspeptin in the ME is reactivated *(right panel)*. According to this model, the mystery of primate puberty lies in the nature of the neurobiological brake and in the mechanism that times its application during infancy and its release at the end of the juvenile phase of development. It should be noted that the ability of the postnatal gonad to respond fully to gonadotropin stimulation is not acquired until the juvenile stage of development, by which time luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion is low as a result of the GnRH pulse generator being brought into check. The thickness of the *blue* (T, testosterone) and *gold* (E, estradiol) *arrows* indicating negative feedback by the testis and ovary, respectively, reflect the degree of gonadal steroid inhibition exerted on LH secretion at these three stages of development. AC, anterior commissure; AP, anterior pituitary gland, ARC, arcuate nucleus; OC, optic chiasm; ME, median eminence; MMB, mammillary body. *(From Terasawa E, Guerrier KA, Plant TM. Kisspeptin and puberty in mammals. In Kauffman AS, Smith JT, (eds).* Kisspeptin Signaling in Reproductive Biology, *Springer, New York, p 253, 2013.)*

inhibitory neurotransmitter in the brain, is upregulated during juvenile development and inhibition of GABA tone in the hypothalamus of the prepubertal monkey leads to precocious menarche and ovulation.[9,123] Interestingly, infusion of the GABA antagonist, bicuculline, into the median eminence of prepubertal female monkeys stimulates release of kisspeptin-54 into this region of the hypothalamus in association with that of GnRH, and the bicuculline-induced GnRH release is blocked by simultaneous infusion of a kisspeptin antagonist.[124] It is unclear, however, what reduces GABA inhibition prior to puberty and how GABA signalling interacts with the GnRH pulse generator.

Other transynaptic signals implicated in the regulation of the pubertal resurgence of GnRH pulse generator activity include glutamate and neuropeptide Y (NPY). Glutamate is the major excitatory neurotransmitter in the brain and, in contrast to GABA, hypothalamic release of this amino acid is increased at the time of puberty in the female monkey,[123] and as discussed earlier in the chapter, repetitive activation of glutamate receptors in the juvenile monkey leads rapidly to the onset of precocious gonadarche.[103] Neuropeptide Y (NPY) neurons are found in the arcuate nucleus and, in the male rhesus monkey, NPY gene expression in the hypothalamus is inversely related to the up-down-up pattern of GnRH pulse generator activity from birth to puberty.[125] NPY receptors are inhibitory G-protein receptors, and their activation leads to hyperpolarization and inhibition of neural activity.[126,127]

While neuroglia have classically been regarded as subserving only a "supporting role" in the central nervous system (CNS), contemporary views hold that these non-neuronal cells play important functional roles within the brain.[128] Moreover, in the context of the hypothalamus, secretion of transforming growth factor-α (TGFα) by astroglia has been postulated to provide the GnRH neuronal network with a stimulatory input at the time of puberty.[129,130]

As discussed earlier in this chapter, attempts to elucidate the neural mechanism dictating the postnatal pattern of GnRH pulse generation have traditionally led investigators to focus in relative isolation on a "favorite" signalling pathway. Recently, however, systems biology has been applied to the problem. With this contemporary approach, global gene discovery is combined with computational *(in silico)* biology to identify functional linked networks of hypothalamic

genes that are found to be associated with changes in GnRH pulse generator activity.[131,132] To date this has been conducted without regard to the phenotype of the cells in which the respective genes are expressed and gene networks are operating. Accordingly, it has been proposed that developmental changes in a network of genes encoding for a cohort of transcriptional factors lie, in a molecular sense, upstream of the *KISS1* gene and therefore presumable in a functional sense upstream to GnRH pulse generation. It is argued that such a network of genes serve as a governing hierarchy to orchestrate the resurgence of pubertal GnRH release and therefore the timing of puberty. Since such networks of genes are further proposed to function in the absence of signals derived from the periphery, conceptually they may be viewed at a systems level as comprising a pubertal clock (discussed later in the chapter). Until information is forthcoming on whether the "governing" gene network is expressed in KNDy neurons or by other cells in the hypothalamus or both it will be difficult to integrate the contemporary ideas with those that have evolved from more classical approaches.

Nevertheless, it is interesting to note that one of these transcriptional regulators, enhanced at puberty 1 (EAP1), has been studied in the classical manner, and shown to be expressed in kisspeptin neurons in the arcuate nucleus of the monkey.[133] Moreover, its expression in the hypothalamus increases at the time of puberty in the female monkey, and knock down of *EAP1* using a lentivirus approach interrupts menstrual cyclicity in the adult female.[134] Additionally a single nucleotide polymorphism (SNP) upstream of the *EAP1* gene has been associated with irregular menses in the monkey.[135]

Regardless of the components of the neurobiological brake, which dictate the up-down-up pattern of pulsatile GnRH release from birth until puberty, it is to be anticipated that application and withdrawal of the brake will be associated with a corresponding structural remodelling in those hypothalamic neuronal and glial circuits involved.[136,137] In this regard, the hypothalamus of the postnatal brain retains its capacity for plasticity, as reflected by the expression of polysialic acid-NCAM.[138]

Putative Physiological Control Systems Governing the Timing of Gonadarche

The physiological control systems that dictate the timing of puberty have intrigued investigators for decades, but the governance of this fundamental developmental event in humans remains largely a mystery. Two basic schemata, however, may be proposed.[139] In the first, a cue to reawaken the GnRH pulse generator is provided by the attainment of a particular state of somatic maturation. According to this hypothesis, the brain receives this information by way of a signal in the circulation that is tracked by a somatometer resident within the CNS. In the second schema, a pubertal clock (presumably resident in the CNS) generates the signal.

In the case of the somatometer hypothesis, the attainment of a particular proportion of body fat has long been argued to be necessary for the onset of gonadarche.[140] Interest in this hypothesis was rekindled with the discovery of leptin and its receptor. Leptin, encoded by the leptin *(LEP)* gene, is primarily secreted by adipocytes and regulates feeding behavior and body weight by providing the hypothalamus with information on fat mass and energy status.[141-143] Leptin action is mediated by leptin receptors, which are single transmembrane domain receptors. Six isoforms of the leptin receptor have been described; all represent alternatively spliced products derived from a single gene *(LEPR)* located at chromosome 1p31.3.[144]

In girls, it is generally recognized that plasma leptin levels increase progressively through breast stages 1 to 5.[145-147] In boys, plasma leptin levels also increase during early puberty to reach peak levels between 10 and 12 years of age, but subsequently decline as blood levels of testosterone rise into the adult range.[145,148,149] The finding that circulating concentrations of the soluble form of the leptin receptor progressively decrease during childhood until approximately 11 years of age suggests that the increase in bioavailable leptin during early puberty may be greater than that reflected in total leptin concentrations.[149,150]

Individuals with mutations in leptin signalling, resulting from loss of function mutations in either *LEP* or *LEPR*, have failed to progress through puberty.[151-153] The phenotype of *LEPR* mutations included morbid obesity, abnormal eating behaviors, and lack of spontaneous pubertal development.[153] Over the last 15 years, leptin replacement has been administered to several patients with leptin deficiency due to *LEP* mutations. When administered at an age appropriate for puberty, leptin promoted pubertal development. Importantly, there has been no evidence of premature puberty in younger children following replacement treatment.[152,154,155] Moreover, among children with GnRH-dependent precocious puberty, leptin concentrations correlated with body mass index and not pubertal status.[156] These findings provide compelling evidence that the action of leptin, albeit obligatory for the onset of puberty, is nevertheless permissive.[157-160] This action of leptin may require only low circulating levels of the adipocyte hormone because pubertal development in both male and female subjects with various lipodystrophies has been reported to be normal despite low leptin concentrations.[160-162]

The neuronal targets for leptin's action in the hypothalamus to permit puberty to unfold is an area of considerable contemporary interest. While it is generally recognized that GnRH neurons do not express *LEPR*, data obtained in rodents and sheep for the KNDy neurons are inconsistent and the site of action of leptin may be upstream to the GnRH pulse generator.[163]

Before leaving the subject of leptin and obesity it should be noted that although overweight girls tend to "mature" earlier,[164,165] sleep related increments in LH secretion, and therefore presumable GnRH pulse generator activity, have recently been reported to be blunted in healthy obese premenarcheal girls.[166]

Other somatic factors have been proposed to serve as signals to the somatometer. The hypothesis that such a factor is skeletal in origin[139] is based on the finding that, in children with an accelerated or retarded maturational tempo, menarche and testicular enlargement correlate better with skeletal age than with chronological age.[167,168] Skeletal age, also known as "bone age," is a surrogate marker of biological maturation and may be determined by comparing a radiograph of the left hand to gender-specific standards

obtained at various chronological ages. Several caveats are relevant when assessing skeletal maturity. The degree of skeletal maturation at various sites, such as hands, elbows, and knees, may differ. Skeletal maturation of the carpal bones, distal radius, and distal ulna often lag behind the metacarpals and phalanges.[169] Nevertheless, the association between bone age and the onset of gonadarche is maintained in disorders of growth. In children with constitutional delay of growth and with true isolated growth hormone (GH) deficiency, gonadarche occurs at a late chronological age but at a normal skeletal age.[170,171] On the other hand, when skeletal maturation is advanced, as may occur in association with congenital adrenal hyperplasia (CAH) or familial testotoxicosis, secondary GnRH-dependent precocious puberty may develop.[171] Although proteins synthesized in bone enter the vascular compartment,[172,173] the ability of osteocalcin and other bone proteins to modulate the activity of the GnRH pulse generator has not been addressed.

GH secretion during childhood is relatively stable, but GH release is amplified up to threefold in boys and girls with the initiation of gonadarche and the rise in circulating levels of sex steroids.[174] The pubertal increase in GH, in combination with the increased circulating concentrations of insulin-like growth factor-1 (IGF-I),[175] estrogens, and androgens, contribute to the adolescent growth spurt. The increased secretion of GH at the time of gonadarche is not sustained; by late puberty, GH levels begin to decline.[174] Because the increases in GH and IGF-I appear to be in response to the initiation of gonadarche and particularly to increased gonadal steroid secretion at this time,[174] neither GH nor IGF-I represent compelling candidates for the signal responsible for the resurgence of GnRH release.[5]

At the time of gonadarche, insulin resistance increases. This insulin resistance is greatest among children in Tanner stages 2 and 3 when compared with prepubertal children and adults.[176] Manifestations of insulin resistance appear to be limited to effects on carbohydrate metabolism and are associated with compensatory hyperinsulinemia. Using euglycemic-hyperinsulinemic clamp studies in conjunction with investigation of substrate utilization, one longitudinal study found that puberty was associated with decreased insulin sensitivity, increased insulin secretion, increased total body lipolysis, decreased glucose oxidation, and increased IGF-1 concentrations.[177] In a longitudinal prospective cohort study involving healthy children, insulin sensitivity decreased before the onset of physical features of puberty.[178] The ability of insulin to suppress hepatic glucose production was noted to be preserved during puberty[179] indicating that decreased insulin sensitivity is limited to peripheral glucose uptake. Although cross-sectional studies suggest that the magnitude of insulin resistance is influenced by body mass index (BMI; body weight/height^2 [kg/m^2]), gender, and ethnic background, these associations have not been consistently noted in longitudinal studies. IGF-1 concentrations during puberty have been reported to mirror those in insulin sensitivity.[180] Mean 24-hour serum GH and IGF-I concentrations positively correlate with the degree of insulin resistance during puberty, and the pubertal changes in insulin sensitivity may be partially mediated by increased GH and IGF-I concentrations.[177] Yet, while it is generally recognized that increased gonadal steroid levels are responsible for activation of the GH/IGF1 axis at puberty (discussed earlier in the chapter), a causal relationship between testosterone or estradiol and insulin resistance has not been demonstrated.[181]

Treatment of nonobese Catalunyan girls with premature adrenarche and advanced skeletal maturation with the insulin sensitzer, metformin, was associated with later onset of breast development and menarche.[182] Moreover, in this relatively homogenous ethnic population, metformin treatment for 3 years slowed pubertal tempo among low birth weight girls who are at risk for early puberty and shorter adult stature.[183,184] Taken together, the foregoing considerations raise the possibility that decreased insulin sensitivity may represent a component of the cue that times the onset of gonadarche.

Ghrelin is a small peptide, secreted predominantly by the stomach, which circulates in two forms. The active form is acetylated and promotes GH secretion.[185] Circulating ghrelin concentrations are higher during fasting and decrease after food intake, suggesting that ghrelin signals energy deficient states and modulates appetite and carbohydrate metabolism. Ghrelin concentrations peak during the first 2 years of life and decrease during puberty.[186] The ghrelin receptor, GH secretagogue receptor-1a (GHSR-1a) is expressed in human hypothalamus, pituitary, testis, and ovaries.[187]

Ghrelin and leptin appear to act as reciprocal regulators of energy homeostasis exerting opposing influences on the hypothalamic-pituitary-gonadal axis.[181,188] Resistin and adiponectin are two recently described hormones secreted by adipocytes, which appear to provide metabolical and nutritional status information.[189,190] The respective roles, if any, of these hormones on gonadarche or adrenarche remain to be determined.

Activation and Timing of Adrenarche

Adrenarche is characterized by development of the zona reticularis in the adrenal cortex and increased DHEA, DHEAS, and androsenedione secretion. DHEA and DHEAS are not bioactive androgens; these hormones serve as precursors for more potent sex steroids. Prior to birth, the fetal zone of the adrenal cortex produces large amounts of DHEAS, which serves as the precursor for placental estrogen synthesis. Following involution of the fetal zone after birth, DHEAS concentrations remain low until 6 to 7 years of age at which time they begin to increase. This elevation in DHEAS secretion is the earliest hormonal manifestation of adrenarche; it is not associated with changes in pituitary ACTH secretion or changes in cortisol secretion.

The onset of adrenarche is associated with increased 17,20-lyase activity and decreased 3β-hydroxysteroid dehydrogenase activity.[191] Available data suggest that, at the time of adrenarche, changes in expression of cytochrome b_5 (CYB5A), DHEA sulfotransferase (SULT2A1), and 3β-hydroxysteroid dehydrogenase (HSD3B2) play essential roles in DHEA and DHEAS production.[192] Histologically, increased thickness of the zona reticularis occurs concurrently with the increase in DHEAS concentration. Using more sensitive methodology such as urinary GCMS, studies indicate that concentrations of DHEA and its metabolites show a continuous rise beginning at 3 to 4 years old. These data contradict conventional concepts and imply that

adrenarche is a gradual process beginning earlier than previously considered.[193] DHEAS concentrations continue to rise and peak between 20 and 25 years of age, followed by progressive decline. Although the findings have been inconsistent, clinical studies suggest that insulin, IGF-I, and GH concentrations influence the timing, onset, and progression of adrenarche.[194] Comparison of IGF-I concentrations among prepubertal children have shown higher concentrations in African-American children.[195] Whether these ethnic differences contribute to the earlier onset of adrenarche or increased incidence of premature pubarche in African-American girls is unknown. Clinical observations regarding associations between adrenarche and body size and fatness have been inconsistent; one longitudinal study showed that DHEAS concentrations increased commensurate with the largest increase in BMI, whereas another longitudinal study found no association between DHEAS and weight, BMI, or body surface area.[196,197]

Despite numerous hypotheses, the primary signal for adrenarche remains to be elucidated.[198] Hypothalamic releasing factors, such as corticotropin-releasing hormone (CRH) and vasopressin (ADH), play important roles in governing hypothalamic-pituitary-adrenal axis function. In contrast to the role of hypothalamic GnRH in gonadarche, however, CRH and ADH do not appear to trigger the onset of adrenarche. No specific adrenal androgen-stimulating factors have been isolated from the pituitary.[199]

Moreover, adrenarche occurs independently of developmental changes in the hypothalamic-pituitary-gonadal axis. For example, children with gonadal dysgenesis experience normal adrenarche and pubarche, whereas children with primary adrenal insufficiency may have normal gonadarche.[200] Nevertheless, despite the long-established notion that gonadarche and adrenarche are independent events, recent data hint that adrenal androgens may influence pubertal timing. Specifically, using methodology with lower limits of detection, these data show that higher pre-pubertal urinary androgen excretion correlated with an earlier onset of breast development and penile growth respectively and with a shorter duration of pubertal growth spurt.[201]

Genetics and Puberty Genes

The observations that (1) the temporal correlation in somatic maturation and attainment of pubertal stages in monozygotic twins is more robust than that in dizygotic twins, (2) the age of menarche in mothers and daughters is correlated, (3) parents of children with constitutional delay of puberty (discussed later in the chapter) recall a later age of menarche and the adolescent growth spurt than those of a control group of children,[202-204] (4) precocious gonadarche in girls may be familial and transmitted in an autosomal dominant mode,[205] and (5) the age of menarche varies with racial group, underline the major influence of genetic factors for timing the onset of puberty. However, it remains unclear whether the genetic factors directly dictate the timing of the pubertal resurgence of GnRH release (perhaps a network of clock genes in analogy to circadian time keeping)[206] or simply control permissive factors that are required for expression of pubertal mechanisms.

We suggest that the term "puberty" gene should therefore be restricted to those genes that are specifically involved in the timing of either adrenarche or gonadarche.[5] For gonadarche, such genes would determine the age of the pubertal resurgence of pulsatile GnRH release by regulating the timing of the application or withdrawal of the neurobiological brake to the GnRH pulse generator of the juvenile hypothalamus. Puberty genes could time the resurgence of pulsatile GnRH release not only by triggering a hypothalamic signal at puberty but also potentially by determining the duration of the prepubertal brake on pulsatile GnRH release or by timing the "turn off" of the GnRH pulse generator during infancy. In the case of adrenarche, the developmental increase in adrenal androgen secretion would presumably be timed by genes that are responsible for the changes in adrenal steroidogenesis manifested by increased zona reticularis activity.

Despite the accelerating pace of elucidation into the genetic causes of disorders of pubertal development,[207] the identity of specific puberty genes that dictate the timing of the resumption of pulsatile GnRH release (and therefore determine the age of gonadarche) and those that regulate the developmental increase in adrenal androgen secretion that determine the age of pubarche have not been identified. Association studies have shown no significant associations between common variants in several genes (*GNRH1*, *GNRHR*, *KISS1R*, *LEP*, *LEPR*, *FGFR1*, *KAL1*, *PROK2*, and *PROKR2*) and age at menarche.[208] Thus, rather than being the master genes responsible for triggering the pubertal resumption of pulsatile GnRH secretion, these genes are critical for GnRH release at all stages of development, and therefore do not meet the criteria of a puberty gene. The whole genome can be interrogated by linkage and genome wide association (GWA) analyses without a priori hypotheses regarding specific candidate genes. However, these approaches have largely been unsuccessful.[209] Two loci located at chromosome 9q31.2 or chromosome 6q21were significantly associated with age at menarche in normal women.[210,211] In addition to *LIN28B* at chromosome 6q21, meta-analyses have identified additional loci associated with age at menarche.[212]

Genetic information is also transmitted by DNA (CpG) methylation, chromatin packaging, and small RNA or microRNA effects on transcription. Whereas most autosomal genes are expressed from the alleles of both parents, a number of growth regulatory genes show parent-of-origin effects due to genomic imprinting.[213] Environmental factors such as nutrition, hormone and chemical exposures, and physical factors can alter gene expression through these epigenetic modifications.[214,215] Maternal nutritional status can influence fetal gene expression through epigenetic mechanisms; animal studies suggest that this effect may be transmitted to subsequent generations.[216] Developmental disorders such as Prader-Willi syndrome, in which the timing and tempo of puberty is altered (discussed later in the chapter), are associated with abnormal expression of imprinted genes.[213] These disorders may represent the "tip of the iceberg" for epigenetic influences on the genes that dictate or modulate the timing of puberty.

Factors Modulating the Timing of Puberty

In general, the majority of studies that have examined the influence of various parameters on the onset and tempo of

puberty have focused on the process of gonadarche. Considerably less attention has been paid to the timing and progression of adrenarche. Additionally, many factors that influence the onset and tempo of puberty (for example, nutritional status) also modulate hypothalamic GnRH pulse generator activity in the majority of adults. Therefore, a modulator of puberty is not necessarily a component of the mechanism that initiates this developmental event. Rather, it is more likely that such factors play permissive, albeit in some cases obligatory, roles to allow the process of puberty to unfold once the signal responsible for the resurgence in pulsatile GnRH secretion has been activated.

Nutrition and Diet

Adequate energy stores are essential for the accelerated linear growth and achievement of reproductive competence at puberty. Nutritional state modulates the onset of gonadarche in girls,[217] as reflected by the findings that menarche is delayed in malnourished girls,[218] and menarche tends to occur at a particular or "critical" body weight rather than at a set age.[219] Obesity is associated with early breast development and menarche.[220] In addition, the timing of adrenarche may also be influenced by nutritional status. In the female rhesus monkey, high calorie diets were associated with a precocious increase in nipple volume, sex-skin swelling, and menarche in association with increased concentrations of leptin and IGF-1 concentrations, and an increased BMI.[221] While the impact of undernutrition on gonadarche in boys has received less attention, there is no reason to suspect that the male axis is unaffected in this regard.

Leptin and osteocalcin appear to communicate energy status to several tissues including adipose tissue, bone, CNS, and gonads.[222] In its undercarboxylated (active) form, osteocalcin promotes insulin secretion, improves insulin sensitivity, induces β-cell proliferation, increases adiponectin secretion, and decreases lipolysis.[223] In osteoblasts, insulin signalling increases bone formation, bone resorption, and secretion of active osteocalcin production. Whereas insulin acts to promote bone formation and secretion of active osteocalcin, leptin acts indirectly through the hypothalamus to decrease bone formation.[224] Rapid weight gain during the first year of life is associated with obesity and early menarche in a prospective UK cohort study.[225]

Differences unrelated to racial or ethnic background in the timing and tempo of gonadarche have been reported even among well-nourished girls. Researchers have suggested that diet may account for such variations.[226-228] Attention has been drawn to subtle relationships between diets high in animal protein and early menarche, and between diets high in vegetable grains and delayed onset or tempo of gonadarche. Moderate to vigorous exercise in the absence of weight restriction appears to have a negligible impact on the timing and tempo of puberty in either sex. However, breast development, menarche, and skeletal maturation can be delayed in girls involved in strenuous physical training.[229] The association between extreme energy expenditure and delayed gonadarche in the female is particularly marked in ballerinas, long-distance runners, and figure skaters, who must maintain their body weight within strict limits. Such girls have amenorrhea secondary to hypothalamic hypogonadism (HH). The factors responsible for compromising pulsatile GnRH release in pubertal children who exercise vigorously are probably similar to those resulting from undernutrition. The life of the young female dancer or athlete is stressful and, therefore, stress may also represent a contributing factor underlying exercise-induced delayed gonadarche. Because certain physical and psychological characteristics are necessary for outstanding athletic performances, there may be a significant contribution of self-selection involved in the decision of girls to participate at such an exceptional level. In a study of young female gymnasts and their parents, menarche in the mothers was found to be delayed relative to that of mothers of sedentary girls.[229] Therefore, the possibility of a genetic contribution to this phenomenon cannot be totally excluded. The effect of physical training on the timing of pubarche in girls has been less studied and the available data are contradictory.[230,231]

Although the relationship between strenuous physical training and male puberty has received less attention, this developmental process is apparently less susceptible in boys than it is in girls.[232] This may be related, in part, to sex differences in the age at which training is initiated, and to the intensity of the exercise. Nevertheless, in sports like wrestling that require weight control achieved with a combination of strenuous exercise and dietary restriction, impaired testosterone secretion may occur.[232]

Environmental Disruptors

Increased attention has been focused on endocrine disrupting chemicals (EDCs) as modulators of pubertal timing.[233] Most EDCs have estrogenic or anti-androgenic activities. Most occur as mixtures rather than single agents. Sexually dimorphic actions can occur. Dioxins, polychlorinated biphenyl (PCB), polybrominated biphenyl (PBB), bisphenol A (BPA), plasticizers (phthalates), pesticides, fungicides (vinclozolin), and pharmaceutical agents (diethylstilbestrol) are considered to be EDCs. Phytoestrogens, which can function as selective estrogen receptor modulators (SERMS), are found in soybeans, flaxseed, peanuts, and some vegetables. Most are diphenolic compounds with structural features common to estrogenic steroid agonists and antagonists. EDCs can affect peripheral reproductive systems such as inducing breast development. These chemicals may also exert neuroendocrine effects to influence hypothalamic and pituitary function. EDCs can act through hormone receptors and by affecting enzymatic processes.[234] Further investigation into EDCs, defining mechanisms of action and consequences, is necessary.[235]

Prenatal Influences

The role of prenatal influences and environment on postnatal development including puberty are increasingly recognized.[236,237] Children with intrauterine growth retardation (IUGR) or born small for gestational age (SGA) have increased risks to develop insulin resistance, hypertension, diabetes mellitus, metabolical syndrome, and coronary artery disease in adulthood.[238] Increased weight gain during the first few years of life exaggerates these risks. Increased adrenal androgen levels and in some cases precocious or exaggerated adrenarche has been reported in both boys and girls born SGA.[239-241] Some, but not all, girls with premature adrenarche have an increased risk to develop polycystic ovary syndrome (PCOS) during adolescence.[239,240,242] Despite inconsistent reports, the majority of studies indicate that

the timing of menarche in girls born SGA does not appear to be markedly different from girls born at an appropriate size for gestational age.[243-246] The timing of puberty in boys born SGA appears to be normal,[245] although sub-fertility has been reported after they reached adulthood.[247]

Adoption or Migration From Developing to Developed Countries

Several European countries have reported precocious gonadarche in a relatively dramatic proportion of children—particularly girls—adopted from developing world regions like Asia and South America.[248,249] This precocity is preceded by increased endocrine activity in the pituitary-ovarian axis prior to the onset of physical manifestations of puberty, indicating a central origin to the condition.[250] While it was initially argued that improved nutritional and social conditions were in some way responsible for this phenomenon, the cause of the precocity appears to be complex. Precocious gonadarche is also seen in immigrant girls arriving with their parents and without evidence of earlier compromised growth and nutrition.[251] Moreover, ethnic background does not appear to be involved. A study of immigrant girls in Belgium with premature gonadarche found an association with previous exposure to organochlorine pesticides.[251] A study in Spain found an increased relative risk for precocious gonadarche in adopted girls (both national and international), but not in immigrant girls.[252]

Disorders of Puberty

The terminology used to describe disorders of puberty has evolved as the pathophysiology and molecular etiologies of these disorders have been clarified (Box18.1; Table 18.2). The term "central precocious puberty" or GnRH-dependent precocious puberty refers to premature resurgence of GnRH pulse generator activity, which we have labeled as *GnRH-dependent precocious gonadarche*. The terms "partial," "incomplete," "peripheral," "pseudo," and "GnRH-independent precocious puberty" have been used to describe other etiologies of premature sexual development. We refer to these disorders as *GnRH-independent precocious puberty*. *Isosexual* refers to the development of sexual characteristics typical for the patient's gender. *Heterosexual* refers to the development of sexual characteristics typical of the other gender (e.g., feminizing tumors in males). Delayed puberty has been categorized as being either *hypogonadotropic* (low gonadotropin concentrations) or *hypergonadotropic* (elevated gonadotropin concentrations). With expanding knowledge of the functional genomics of the pubertal process and the molecular genetics underlying its pathophysiology, the classification of the etiologies of disorders of puberty will continue to evolve.

Disorders of Early Puberty

GnRH-Dependent Precocious Pubertal Development

Progressive Precocious Gonadarche or Central Precocious Puberty

Although this disorder is labeled GnRH-dependent precocious puberty or central precocious puberty (CPP), it represents precocious gonadarche due to either premature

Box 18.1 Etiologies of Precocious Puberty

GnRH-DEPENDENT GONADARCHE
- Idiopathic: Progressive, nonprogressive
- Congenital CNS lesions: Hypothalamic hamartoma, septo-optic dysplasia, arachnoid cysts, suprasellar cysts, *KISS1R* activating mutation
- Acquired CNS disorders: Postinflammatory, postradiation therapy, abscess, hydrocephalus, trauma, tumors
- Chronic exposure to androgens: Congenital adrenal hyperplasia, familial male limited precocious puberty
- Other conditions: Williams-Beuren syndrome, maternal uniparental disomy of chromosome 14, histiocytosis X

GnRH-INDEPENDENT GONADARCHE
- McCune-Albright syndrome
- Feminizing disorders, estrogen secreting tumors: Ovarian (granulosa cell, Peutz-Jeghers syndrome, gonadoblastoma/dysgerminoma, carcinoma, cystadenoma, theca cell, lipoid) and adrenal
- Feminizing disorders, estrogen secretion unrelated to tumors: Aromatase mutation
- Isolated premature menarche: Estrogen-secreting cyst, tumor, McCune-Albright syndrome, primary hypothyroidism
- Premature thelarche: Variant of normal development, Rubinstein-Taybi syndrome, Kabuki make-up syndrome
- Primary hypothyroidism
- Exposure to exogenous sex steroids or endocrine disruptors

VIRILIZING DISORDERS
- Premature adrenarche
- Congenital adrenal hyperplasias (21-hydroxylase deficiency, 3β-hydroxysteroid dehydrogenase deficiency, 11 β-hydroxylase deficiency)
- Other disorders affecting steroidogenesis (oxidoreductase deficiency, apparent cortisone reductase deficiency, apparent DHEA sulfotransferase deficiency)
- Inherited glucocorticoid resistance
- Familial male-limited precocious puberty (testotoxicosis)
- Androgen-secreting tumors (adrenal sex steroid-secreting tumors like adenoma and carcinoma; ovarian tumors like arrhenoblastoma; testicular Leydig cell), hCG-secreting tumors (hepatoblastoma/hepatoma, dysgerminoma, teratoma, choriocarcinoma)
- Cushing syndrome (Cushing disease associated with increased ACTH secretion, adrenal disease, ectopic secretion of CRH or ACTH)

Table 18.2 Monogenic Disorders Associated with Precocious Puberty

Gene	*Locus*	*Phenotype*
GNAS1	20ql3.2	McCune-Albright syndrome
STK11/LKB1	19pl3.3	Peutz-Jeghers syndrome
CYP21A2	6p21	CAH due to 21-hydroxylase deficiency
HSD3B2	Ipl3.1	CAH due to 3β-hydroxysteroid dehydrogenase deficiency
CYP11B1	8q21	CAH due to llβ-hydroxylase deficiency
LHR	2p21	Familial male-limited precocious puberty
CYP19A1	15q21.1	Precocious puberty/gynecomastia
GRL	5q31	Inherited glucocorticoid resistance
KISS1R	19p13.3	Precocious puberty associated with activating mutations

CAH, congenital adrenal hyperplasia.

resurgence or incomplete suppression of the hypothalamic GnRH pulse generator. It occurs more often in girls than in boys. This sex difference is probably related to the lesser prepubertal suppression of the GnRH pulse generator in girls than boys. The sequence of pubertal development is typical of a normal puberty including adrenarche in some cases, but it begins at an earlier-than-normal age.

Useful laboratory studies include x-ray assessment of skeletal maturation (bone age), and gonadotropin, IGF-1, and sex steroid concentrations. A GnRH (Factrel or a GnRH-R agonist [GnRH-Ra] such as leuprolide) stimulation test is important to demonstrate a pubertal pattern of gonadotropin responses. Generally, a stimulated LH predominant response is noted which confirms the increased GnRH pulse generator activity. In girls, when gonadotropins are measured using third-generation monoclonal fluorometric assays, basal and GnRH-stimulated LH concentrations greater than 0.6 and 6.9 U/L, respectively, are 70% and 92% sensitive for the diagnosis of GnRH-dependent precocious gonadarche.[253] Comparison of two third generation LH assays, Architect and Delfia, to assess for precocious puberty in girls indicated that basal LH values may be adequate to diagnose CPP in most instances.[254] Girls with CPP had higher IGF-1, fasting insulin, and oral glucose-stimulated insulin concentrations compared to healthy girls; these differences persisted after adjustment for chronological age, skeletal age, and pubertal stage.[255] The IGF-1 concentrations do not decrease to pre-pubertal values despite adequate treatment.

Since affected children appear older than their chronological age, parents and teachers may have inappropriate expectations regarding psychosocial development and abilities. Cognitive and emotional development, however, are normal for chronological age. Thus, the guilelessness and naiveté of such children exposes them to an increased risk of sexual abuse, with affected girls at risk of becoming pregnant.

Although GnRH-dependent precocious puberty is considered to be idiopathic in girls, the recognition of genes involved in GnRH and gonadotropin secretion has allowed for identification of specific gene mutations in patients with CPP. A heterozygous missense mutation, Arg386Pro, in the *KISS1R* gene was associated with precocious puberty in a girl.[256] The mechanism of action of this mutation may be related to delayed degradation of the receptor yielding prolonged duration of action.[257]

Magnetic resonance imaging (MRI) and computerized tomograpghy (CT) imaging are helpful to identify anatomical lesions such as hypothalamic hamartomas.[258] Hypothalamic hamartomas, congenital malformations composed of a heterotropic gray matter, neurons, and glial cells usually located on the floor of the third ventricle or attached to the tuber cinereum, are a common etiology of precocious gonadarche. The tumors can be classified as *parahypothalamic*, attached or suspended from the floor of the third ventricle, or as *intrahypothalamic*, in which the mass is enveloped by the hypothalamus and distorts the third ventricle. The lesions do not grow over time and do not metastasize. Extreme precocity or the absence of circulating tumor markers such as β-human chorionic gonadotropin-(β-hCG) and α-fetoprotein[259,260] suggests a hamartoma. Although gelastic or laughing seizures can be associated with precocious puberty due to hypothalamic hamartomas, the majority of patients with hypothalamic hamartomas do not exhibit neurological symptoms.[261]

Hypothalamic hamartomas are generally sporadic, but may occur as a feature of Pallister-Hall Syndrome. Pallister-Hall Syndrome is an autosomal dominant disorder associated with mutations in the *GLI3* gene located at chromosome 7p13. Additional features can include pituitary anomalies, hypopituitarism, imperforate anus, and polydactyly.[262]

On CT or MRI, hamartomas appear as an isodense, abnormal fullness. Imaging with MRI is superior to CT, but the lesions do not enhance with contrast material. Histological examination of hypothalamic hamartoma tissue has shown immunoreactivity for GnRH and for astroglial-derived factors such as TGFα.[263] Two potential mechanisms have been hypothesized, with one being increased GnRH secretion from tissue emancipated from suppression by the prepubertal brake. The other possibility is that factors such as TGFα provide an ectopic drive to GnRH neurons with a normal distribution in the hypothalamus. In one series, hypothalamic hamartomas associated with precocious puberty tended to be larger and were more likely to contact the infundibulum or tuber cinereum than those without precocious puberty.[264] Gene expression profiling of hypothalamic harmartomas associated with precocity may provide clues regarding genes, proteins, and regulatory pathways associated with the timing of puberty.[265]

In addition to hypothalamic hamartomas, optic gliomas, suprasellar cysts, arachnoid cysts, previous head trauma, static cerebral encephalopathy, CNS infections, CNS radiation, hydrocephalus, meningomyelocele, and neurodevelopmental disabilities may also be associated with progressive precocious gonadarche.[266-268] For hypothalamic-pituitary disorders, the endocrine symptoms of precocious puberty may precede neurological symptoms.[269] Three predictors associated with CNS lesions in girls are: (1) age younger than 6 years; (2) absence of pubic hair; and (3) estradiol concentrations greater than 110 pmol/L.[270] The type of CNS lesion influences the presentation of GnRH-dependent precocious puberty presumably due to differences in the mechanisms inducing puberty and to the hypothalamic-pituitary deficiencies associated with the initial lesion or its treatment.[271] Optic gliomas are associated with neurofibromatosis type 1 (NF1), an autosomal dominant disorder that is diagnosed based on clinical features that include size and number of cafe-au-lait spots, macrocephaly, and family history of the disorder. The mechanism of action has been presumed to be a mass effect.[272] Pineal tumors can be associated with precocious puberty due to tumor-induced hydrocephalus or germ cell tumors.[273] CNS radiation used to treat intracranial tumors or used prophylactically for malignancies can induce precocious gonadarche, perhaps as a result of an astroglial response with increased TGFα production.[274] The Childhood Cancer Study Group noted that higher radiation doses and younger age, less than 4 years old, conferred a greater risk for premature menarche.[275] In this situation, it is important to note that simultaneous GH deficiency may be masked by an accelerated growth velocity due to precocious gonadarche.

Precocious gonadarche, which appears to be GnRH-dependent, has been reported in the Williams-Beuren syndrome, histiocytosis X,[276,277] and maternal uniparental disomy for chromosome 14.[278] An imprinted domain located

at chromosome 14q32 appears to be the critical region for the phenotype associated with maternal uniparental disomy. In addition to precocious puberty, clinical features include prenatal and postnatal growth retardation, developmental delay, hypotonia, and joint laxity. Deletion of the paternal allele and imprinting defects involving this region can result in a similar phenotype.[279] Other situations associated with progressive precocious gonadarche include children with virilizing disorders (such as CAH) and familial male-limited precocious puberty (testotoxicosis) in whom skeletal maturation is usually markedly advanced. In these situations, the precocious gonadarche is considered to be secondary to the virilizing disorder, but the mechanism through which the GnRH pulse generator is prematurely activated is unclear.

The treatment of choice for children with progressive precocious gonadarche is a GnRH receptor agonist (GnRH-Ra). In situations of coexisting GH deficiency, combined treatment with recombinant human GH may be helpful to preserve height potential. GnRH-Ras are modifications of the native GnRH decapeptide, have greater resistance to degradation, and possess increased affinity for the GnRH-R. They are, therefore, perceived by the pituitary as a continuous GnRH stimulation, which induces down-regulation of GnRH-R function and leads to decreased gonadotropin secretion. GnRH-Ras are available as daily injections or depot forms that are currently administered every 28 days. The depot formulation of leuprolide acetate is commonly used in the United States; the recommended dose is 0.3 mg/kg administered every 28 days. Another GnRH-Ra, histrelin, has been formulated as a hydrogel subdermal implant that is surgically inserted into the inner aspect of the upper arm.[280] Over the course of 1 year, histrelin diffuses from the 50 mg implant. Studies indicate excellent gonadotropin suppression.[281,282] When the progressive nature of the disorder is equivocal, serial evaluations are necessary to detect a sustained acceleration in tempo of pubertal development, including skeletal maturation (and therefore loss of height potential), before initiating therapy. This strategy is necessary because children with nonprogressive precocious puberty do not benefit from treatment. The major goals of treatment are to prevent further pubertal progression until appropriate for chronological age and to achieve improved adult height.

Clinically, the cessation of pubertal progression is apparent within 3 months of initiating treatment. Signs related to adrenarche neither regress nor are prevented, and may progress. In girls, breast size typically decreases, but may not completely regress. Vaginal bleeding secondary to estrogen withdrawal, and acne may occur during the first month of treatment. Subsequently, there should be no further vaginal bleeding even if menarche occurred prior to initiation of therapy. The ovaries and uterus decrease in size. In boys, testicular volume decreases. Linear growth velocity and the rate of bone mineral accretion decrease. Longitudinal evaluation of a cohort of children with precocious gonadarche showed increased lumbar bone mineral density and BMI standard deviation (SD) scores at initiation of therapy. Two years after cessation of therapy in these children, bone mass, bone turnover, and percent body fat were normal, suggesting that peak bone mass will be appropriate.[283]

Adequacy of treatment is judged by prepubertal estradiol/testosterone concentrations and prepubertal gonadotropin response to GnRH stimulation. Monitoring to confirm efficacy of therapy includes interim history, physical examination to ascertain height, weight, and stage of pubertal development, and bone age x-rays. In addition, GnRH stimulation tests should be repeated at regular intervals (3 to 12 months) to document gonadotropin suppression. This monitoring is necessary because bone maturation may progress despite regression of the clinical features associated with gonadarche. Urinary gonadotropin determinations do not provide adequate sensitivity to judge efficacy of therapy.[284]

The duration of GnRH-Ra therapy should be individualized with the decision to discontinue therapy based on chronological age, skeletal maturation, projected adult height, and psychosocial readiness for resumption of puberty. For girls, menstruation usually occurs 9 to 15 months after discontinuation of GnRH-Ra therapy, with earlier onset in those who had experienced menarche prior to treatment. Studies indicate that final height is improved over initial predicted height at diagnosis but is still less than that based on midparental height. Rapidly progressive pubertal development, advanced skeletal maturation, predicted compromise of adult height, and psychosocial considerations justify treatment.[285,286]

Among the adverse effects of treatment, parents may note increased emotional lability and moodiness just prior to the GnRH-Ra injection. Local or systemic allergic reactions or sterile abscesses may occur, but are uncommon. Some children show increased weight gain disproportionate to their linear growth. Intermittent therapy, often due to poor compliance, may have the deleterious effect of increasing gonadotropin and gonadal steroid secretion, leading to progressive skeletal maturation and further compromise of adult height.

Long-term experience (more than 20 years) has now accrued for GnRH-Ras. Pregnancies with normal offspring have been observed.[287] Although no major adverse effects on reproductive function have been noted, some girls appear to have an increased risk to develop PCOS.[288-290]

Nonprogressive Precocious Gonadarche

Some children experience a nonprogressive (or slowly progressing) form of precocious gonadarche attributed to a premature but intermittent or transient activation of the hypothalamic GnRH pulse generator.[291] Among this latter group of children, basal gonadotropin concentrations and gonadotropin responses to GnRH stimulation may be normal for chronological age but can overlap values observed among children with progressive precocious gonadarche. Because the physical signs of pubertal development do not always correlate with GnRH-stimulated gonadotropin responses, physical findings alone cannot differentiate between progressive and non-progressive precocious gonadarche.[292] In general, children with this nonprogressive form of precocious gonadarche show no evidence of pubertal responsiveness to GnRH stimulation, no loss of height potential, and do not usually benefit from GnRH-Ra therapy.[293]

GnRH-Independent Precocious Pubertal Development

Precocious pubertal development may occur independently of pulsatile GnRH secretion. In these situations of

peripheral precocious puberty, inappropriate gonadal or adrenal steroid secretion or exposure to exogenous steroids induces the physical signs of puberty. In most instances, the pubertal development is incomplete and fertility is not attained.

McCune-Albright Syndrome

The classical clinical triad of McCune-Albright syndrome (MAS) is precocious pubertal development, cafe-au-lait spots, and bony fibrous dysplasia. The café-au-lait lesions are generally large, do not cross the midline, and have irregular "coast of Maine" margins, but exceptions to the classic features can occur. Precocious pubertal development is not observed in all cases and appears to be more common among girls than boys. This disorder is due to constitutive activation of the $G_{s\alpha}$ protein that is coupled to membrane-bound glycoprotein hormone receptors and is associated with autonomous function of endocrine glands. The syndrome is due to postzygotic somatic cell mutations in the *GNAS1* gene; missense mutations Arg201His and Arg-201Cys are among the most common.[294] Partial or atypical forms are increasingly recognized; among 113 children with 1 to 3 features typical of MAS, a PCR-based mutation analysis protocol identified a missense mutation at codon 201 in 90% when an affected tissue, e.g., ovarian, bone, adrenal tissue, etc was analyzed.[295] These gain-of-function mutations in the $G_{s\alpha}$ gene lead to constitutive activation of gonadotropin receptors, and subsequent increased autonomous ovarian estrogen and testicular testosterone secretion in affected girls and boys, respectively.[296]

Vaginal bleeding, with or without breast development, is the typical presentation in prepubertal girls. The vaginal bleeding represents estrogen withdrawal following resolution of an estrogen secreting cyst. Estrogen concentrations may be elevated and pelvic ultrasound may confirm a unilateral ovarian cyst. The frequency of recurrent episodes of ovarian cysts and vaginal bleeding is variable. Because the precocious pubertal development is GnRH-independent, GnRH-Ra treatments are ineffective. Treatment with aromatase inhibitors, estrogen receptor modulators, and medroxyprogesterone acetate have been tried.[297,298] Although safe, treatment with anastrozole for 1 year did not halt vaginal bleeding or attenuate rates of skeletal maturation and linear growth in girls with MAS suggesting that aromatase inhibitors may not be useful in the treatment of this form of precosity.[299] Letrozole, another aromatase inhibitor, has some efficacy, but was associated with ovarian torsion in one girl.[300] A partial estrogen antagonist, tamoxifen, has been helpful. Preliminary results using Fulvestrant, a pure estrogen blocker, are promising to decrease vaginal bleeding and slow bone maturation.[301] One outcome study showed variable gonadal function in affected adult women, with some having regular menses and fertility while others had persistent autonomous gonadal function associated with irregular menses and infertility.[302]

Among boys with MAS, precocious puberty is less common. In a cohort of 54 males, 11 (21%) presented with precocious puberty. Gonadal involvement occurred in approximately 80% and one patient had a testicular embryonal carcinoma.[303] Macroorchidism may be noted on examination. Ultrasound features can include macroorchidism, hyperechoic lesions, hypoechoic lesions, and testicular microlithiasis. Leydig cell hyperplasia can occur in postpubertal men, but is rare in boys. AR blockers, aromatase inhibitors, and blockers of sex steroid synthesis have been used to treat the precocious puberty. A conservative approach to MAS-associated testicular lesions has been endorsed with emphasis on serial imaging and testicular preservation.[303]

The typical bone lesion is polyostotic fibrous dysplasia. Lesions tend to be asymmetric and can affect any bone, including the skull. Skeletal disease is generally apparent by 10 years of age. The cystic bone lesions can lead to pathological fractures and deformities. Pseudoarthrosis can occur. With involvement of the skull, hyperproliferation of the pre-osteoblastic cells results in impingement into cranial foramina leading to compression of cranial nerves. Blindness, deafness, facial asymmetry, or ptosis can result. Rather than being associated with a single abnormality of bone, the specific histopathology varies depending on anatomical location: axial or appendicular skeleton, cranial bones, or gnathic bones.[304] Bone lesions may be apparent on bone scans before they are visible on x-rays. Hypermetabolical bone disease is indicated by increased serum osteocalcin and alkaline phosphatase concentrations, as well as increased urinary hydroxyproline concentrations. Short-term studies suggest that bisphosphonate therapy may benefit the bone disease. In one small series, bone pain decreased without apparent change in the bone lesions by radiography or scintigraphy.[305] However, other patients showed increased bone mineral density and radiographic evidence of bone healing with pamidronate treatment.[306]

Hypophosphatemic rickets can occur secondary to increased fibroblast growth factor 23 (FGF23) production by the bone lesions. There is a positive relationship between disease activity and FGF23 concentrations. Treatment of the hypophosphatemia includes phosphate and vitamin D. Other endocrine manifestations include nodular thyroid hyperplasia with hyperthyroidism, multiple pituitary adenomas associated with gigantism, acromegaly or hyperprolactinemia, and parathyroid adenoma or hyperplasia with hyperparathyroidism. Cushing syndrome is extremely rare and almost always occurs during infancy.[307]

Feminizing Disorders

Estrogen-Secreting Tumors

Estrogen-secreting tumors are a rare cause of premature or abnormal pubertal development. Types of tumors include granulosa cell, gonadal stromal cell, ovarian sex cord stromal, and theca cell tumors.[308,309] The majority of juvenile granulosa cell tumors can be palpated on bimanual examination. Estrogen concentrations may be very elevated, and circulating tumor markers such as α-fetoprotein or hCG may be detected. Rarely, gonadoblastomas in streak gonads, lipoid tumors, cystadenomas, and ovarian carcinomas can secrete estrogens, androgens, or both. Elevated serum inhibin and AMH concentrations, and the finding of AMH immunoreactivity in the tumor, indicate that the tumor cells are of granulosa or Sertoli cell origin.[310-312] Expression of *FOXL2*, *SOX9*, and other specific genes can be helpful to discriminate between gonadoblastomas and intratubular germ cell neoplasias; *FOXL2* expression suggests that a gonadoblastoma has a granulosa cell component.[313,314] If originally positive, these markers may be useful to recognize recurrence.

Sex cord tumors with annular tubules are common in patients with Peutz-Jeghers syndrome (PJS), an autosomal-dominant disorder characterized by mucocutaneous pigmentation and gastrointestinal polyposis. These tumors, which are multifocal and bilateral, may differentiate into granulosa cell or large cell calcifying Sertoli tumors with the potential to secrete estrogen. Thus, affected males may present with gynecomastia.[315] Although usually benign, granulosa or Sertoli cell tumors can undergo malignant changes.[316] Affected individuals have an increased risk for colon, stomach, small intestinal, breast, and pancreatic cancers. The Sertoli cell tumors have been found in association with both PJS and the Carney Complex (CNC).[317] PJS is associated with mutations in the *STK11/LKB1* gene, which encodes a serine-threonine kinase.[318] CNC is often associated with mutations in the protein kinase, c-AMP dependent, regulatory, type 1, alpha *(PRKAR1A)* gene which encodes the regulatory subunit type 1 of protein kinase A. Among boys, adrenal, testicular, or hepatocellular tumors can express aromatase, leading to secretion of estradiol and estrone.[319-321] In instances where gynecomastia is excessive, prolonged, and apparent at a time other than midpuberty (around stage 3), further evaluation may be warranted.[322] The evaluation should include testosterone, estradiol, hCG, LH, FSH, thyroid stimulating hormone (TSH), and DHEAS measurements. In addition to tumors, the differential diagnosis of gynecomastia includes Klinefelter syndrome, impaired testosterone biosynthesis, androgen insensitivity, and hyperprolactinemia.

Estrogen Secretion Unrelated to Tumors

In one family, autosomal-dominant familial gynecomastia was due to aberrant transcription of the aromatase gene; the affected girl developed GnRH-independent precocious pubertal development.[323] Autosomal dominant aromatase excess syndrome is characterized by high systemic estrogen levels, short stature, prepubertal gynecomastia and testicular failure in males, and premature breast development, macromastia, and uterine pathology in females. Small chromosomal arrangements in the promoter region of the aromatase gene appear to be associated with increased promoter activity resulting in increased aromatase activity.[324] Novel cryptic deletions and duplications were identified; phenotypic severity in this gain of function disorder is primarily determined by the tissue expression pattern of *CYP19A1*.[325]

Isolated Premature Menarche

Isolated premature menarche refers to vaginal bleeding at an inappropriately early age in the absence of other signs of puberty. The duration of bleeding is usually limited to a few days. The most common endocrine etiology is spontaneous resolution of an estrogen-secreting ovarian cyst. Often, the ultrasound shows no abnormality because the cyst has resolved by the time the study is obtained. Most instances are self-limited, remit spontaneously, and are associated with normal pubertal development. Typically, isolated menarche due to an estrogen-secreting cyst is a sporadic event that usually occurs once. However, such cysts and episodes of vaginal bleeding may recur. The bloody vaginal discharge noted in female infants during the first week of life is a physiological event secondary to estrogen withdrawal.

Tumors or trauma usually do not cause cyclic bleeding. The other major differential diagnoses of isolated vaginal bleeding include sexual abuse, vaginal foreign bodies, vaginal infection, MAS, and primary hypothyroidism. Neoplasms such as rhabdomyosarcoma and scleroing stromal tumors can present with isolated vaginal bleeding.[326]

Premature Thelarche

Premature thelarche is defined as isolated breast development without other signs of pubertal maturation. Typically, the parents or pediatrician note breast development, either unilateral or bilateral, between 9 and 18 months of age. No significant nipple development or pigmentation occurs, and the vaginal mucosa remains pink and shiny.

Breast ultrasound can help distinguish breast tissue from cysts, fibroadenomas, neurofibromas, or other less common lesions, but is usually not needed.[327] Pelvic ultrasound may show a bilateral increase in the number of ovarian follicular cysts. Using a recombinant cell bioassay with increased sensitivity, estradiol concentrations are higher among girls with premature thelarche than healthy controls. However, estradiol concentrations in such patients are still low and remain below assay detection limit for most radioimmunoassays.[328] FSH concentrations may be increased for chronological age, but LH concentrations and LH responses to GnRH stimulation are prepubertal. No acceleration in linear growth velocity or skeletal maturation occurs, and the breast development usually regresses spontaneously over time.[329] Although premature thelarche prior to 2 years of age is often transient, closer observation is indicated if growth velocity is accelerated or if the basal LH concentration is greater than 0.3 IU/L.[330] In most instances, onset of puberty, adult height, and adult reproductive function are normal.[331] Premature thelarche can usually be considered as a normal variant. Nevertheless, longitudinal evaluation is helpful to ensure the nonprogressive nature of this disorder.[332]

Premature thelarche has been described in Rubinstein-Taybi syndrome (RTS), an autosomal-dominant disorder characterized by short stature, psychosocial retardation, a characteristic fades, broad thumbs and halluces, and increased risk for neoplasia. Premature breast development is also observed in approximately 23% of female infants with the Kabuki make-up syndrome, which is characterized by a peculiar facies with eyes reminiscent of Kabuki actors, mental retardation, and decreased growth velocity.[333]

Hypothyroidism

Girls with primary hypothyroidism can, on rare occasions, present with breast development or isolated vaginal bleeding.[334] This constellation of clinical features was first described by Van Wyk and Grumbach in 1960.[335] On ultrasound, enlarged multi-cystic ovaries may be noted.[336,337] Additional features may include delayed bone age, ascites, and pleural and pericardial effusions.[338] This is the only etiology for precocious puberty associated with delayed bone age. Thyroid hormone replacement therapy is associated with regression and resolution of the cysts; surgical treatment is not indicated.[339] The mechanism underlying the ovarian stimulation is unclear. One possibility is that the excessively elevated TSH concentrations cross-react with the FSHR to promote estrogen secretion.[340] Another

explanation is that increased FSH secretion observed in the hypothyroid state is responsible.

Hypothyroid boys may show increased testicular volume (macroorchidism) for age. Interestingly, after thyroxine replacement to a cohort of such boys and the subsequent attainment of stage 5, testicular volume was found to be considerably greater than that in controls.[341] The macroorchidism associated with prepubertal hypothyroidism is probably the result of an expanded population of undifferentiated Sertoli cells resulting from increased FSH signaling in response to elevated concentrations of either FSH or TSH.[341-344]

It should be noted that at the hypothalamic-pituitary level, hypothyroidism leads to a delay in the pubertal resurgence of LH secretion,[345] which presumably accounts for the delayed puberty that is generally associated with chronic hypothyroidism (discussed later in the chapter).

Exogenous Hormone Exposures

Exposure to exogenous estrogenic steroids or estrogen receptor agonists can induce pubertal development.[21,346] Potential sources of estrogenic steroids include oral contraceptives, creams, shampoos, and various lotions.[347] In addition, phytoestrogens found in a variety of foods, and phthalate esters present in plastics are environmental endocrine disruptors with estrogen agonist activity.

Epidemics of premature breast development reported in Puerto Rico and Italy have been attributed to increased exposure to estrogenic steroids, phthalates, phytoestrogens, or estrogenic mycotoxins.[348] Mycotoxins are naturally occurring substances that can be found as environmental contaminants in cereals, corn, and nuts.[349] In the Puerto Rico epidemic, 69% of samples from girls with premature thelarche contained phthalates compared with 3% of control children.[346] It has been suggested that prenatal exposure to endocrine disruptors may influence fetal programming of the endocrine system and, therefore, may influence the timing and tempo of puberty. Certain drugs such as marijuana, isoniazid, spironolactone, ketoconazole, and cimetidine can induce gynecomastia by a variety of mechanisms. The use of lavender and tea tree oils has been associated with gynecomastia.[350]

Testosterone gels are used to treat male hypogonadism. Several case reports have been published reporting precocious puberty and virilization in children accidentally exposed to these gels.[351]

Virilizing Disorders

Premature pubarche is defined as the development of pubic hair, axillary hair, and apocrine body odor prior to age 8 years in girls and age 9.5 years in boys. The differential diagnosis includes premature adrenarche, CAH, and androgen-secreting tumors.

Premature Adrenarche

While the age of adrenarche varies considerably among ethnic groups, it is generally considered premature when it occurs prior to age 8 years in girls and age 9.5 years in boys. As in normal adrenarche, pubic hair, axillary hair, adult type apocrine odor, and acne may develop, whereas skeletal maturation may be appropriate for chronological age or slightly advanced. Clitoromegaly and marked phallic enlargement are unusual findings in patients with premature adrenarche. Usually, gonadarche occurs at an appropriate chronological age and subsequent pubertal development proceeds normally. More girls tend to be referred for evaluation than boys. Despite BMI comparable with controls, one recent study reported that girls with premature pubarche appear to have increased total and central fat mass.[352] In one study of Spanish and Italian girls, mean age at menarche for girls with documented premature pubarche was 6 months earlier than healthy controls.[353]

Typically, androgen concentrations are elevated for chronological age but within normal limits for the stage of pubic hair development. However, a subset of girls with premature adrenarche show persistent hyperandrogenism upon gonadarche.[354,355] Insulin resistance, hyperinsulinemia, and dyslipidemia have been described in these girls. These girls may develop chronic anovulation, hirsutism, insulin resistance, hyperinsulinemia, severe acne, and an increased LH:FSH ratio. These features are suggestive of incipient PCOS. Subsequently, adolescent girls with PCOS have an increased risk to develop impaired glucose tolerance and type 2 diabetes mellitus.[356] In some populations, the frequency of heterozygosity for mutations in the 21-hydroxylase *(CYP21A2)* gene is higher among children with premature pubarche and adolescent girls with incipient PCOS.[357]

Premature pubarche due to premature adrenarche is a diagnosis of exclusion. The majority of children with premature adrenarche require no pharmacological intervention. However, at this time, the ability to predict outcome and risk for PCOS is imperfect. Because lifestyle interventions involving food choices and exercise programs decrease the progression from impaired glucose tolerance to diabetes mellitus among adults,[358,359] it seems prudent to counsel children with premature pubarche to adopt healthy lifestyles. The interested reader is referred to recent reviews regarding premature adrenarche and polycystic ovary syndrome.[360,361]

Disorders of Steroidogenesis

Virilizing Congenital Adrenal Hyperplasias

The virilizing CAHs are a group of autosomal-recessive disorders in which cortisol synthesis is impaired due to decreased 21-hydroxylase, 3β-hydroxysteroid dehydrogenase, or llβ-hydroxylase activity (see also Chapters 4 and 17). The specific pattern of circulating steroid hormone concentrations reflects which steroid enzyme is involved. Approximately 90% to 95% of cases are due to 21-hydroxylase deficiency, which is due to mutations in the *CYP21A2* gene.[362-364] Mutations in 3β-hydroxysteroid dehydrogenase type 2 *(HSD3B2)* and 11β-hydroxylase *(CYP11B1)* genes account for the remaining 5% to 10% of patients with virilizing CAH.[365]

In all cases, decreased cortisol concentrations lead to loss of negative feedback inhibition, increased ACTH secretion, and increased adrenal androgen biosynthesis. The clinical spectrum for these disorders ranges from complete loss of function mutations, which typically present during infancy with genital ambiguity, to partial loss of function mutation, which may present in childhood, adolescence, or adulthood. Here, discussion is limited to the milder, so-called

non-classical or late-onset forms of CAH where the major symptoms are secondary to hyperandrogenism, rather than to adrenal cortical insufficiency, as in the classical forms of this disease.[366]

During childhood, boys or girls with milder forms of CAH can present with premature pubic hair, adult type apocrine odor, increased growth velocity, and tall stature. In contrast to premature adrenarche, clitoromegaly or phallic enlargement and advanced skeletal maturation are more common. In female patients, the symptoms of late-onset CAH are similar to those of PCOS. Chronic hyperandrogenism associated with mutations involving any of the three steroidogenic enzymes can be associated with increased LH: FSH ratios and polycystic ovaries on ultrasound, but such patients typically lack the magnitude of insulin resistance associated with PCOS. The late onset of hyperandrogenic signs and symptoms of the milder forms of CAH (e.g., hirsutism and amenorrhea) result in an ascertainment bias such that affected males are generally not recognized unless sisters with CAH have been detected.

21-Hydroxylase Deficiency. The milder form (non-classical or late onset) of this disorder is reported to affect 1 in 1000 patients, whereas the reported incidence of the more severe forms is approximately 1 in 14,000. In 21-hydroxylase deficiency, decreased 21-hydroxylase activity leads to increased concentrations of 17-OHP, 17-OH-hydroxypregnenolone, DHEA, androstenedione, and testosterone. The gene coding for 21-hydroxylase, *CYP21A2*, maps to chromosome 6p21. A highly homologous nonfunctional pseudogene, *CYP21A1P*, is located in close proximity to the functional gene. The *CYP21A2* and *CYP21A1P* genes show greater than 95% homology within coding regions.[367] The majority of mutations associated with 21-hydroxylase deficiency represent gene conversion events in which the functional gene has acquired deleterious nucleotide sequences from the pseudogene. To date, over 100 mutations have been reported, but approximately 10 mutations account for the majority of affected alleles.[368] Functional analysis of mutations associated with non-classical CAH generally indicates a 50% to 80% loss of enzymatic (21-hydroxylase) function. Most affected individuals are compound heterozygotes and carry different *CYP21A2* mutations on each allele (Fig. 18.8). For individuals with non-classical CAH, the missense mutation, V281L, accounts for at least one of the *CYP21A2* alleles for most patients with non-classical CAH. Phenotype—genotype correlations are fairly consistent, with the phenotype usually representing the least severe mutation.[369-372] However, phenotypic heterogeneity does occur and is most commonly identified with either the intron 2 splicing mutation or I172N.[373-375] A single *CYP21A2* allele can carry multiple mutations; two mutations on the same allele can act synergistically to impair enzyme activity to a greater extent than would be anticipated for each mutation individually.[376,377]

Although random 17-OHP concentrations can be diagnostic (especially in the more severe forms), ACTH-stimulation tests may be necessary to confirm the diagnosis of milder forms. Despite the availability of *CYP21A2* genotype analyses, the complexity of this locus precludes routine use of molecular diagnostics; therefore, ACTH-stimulation tests may be necessary to confirm CAH. Techniques that

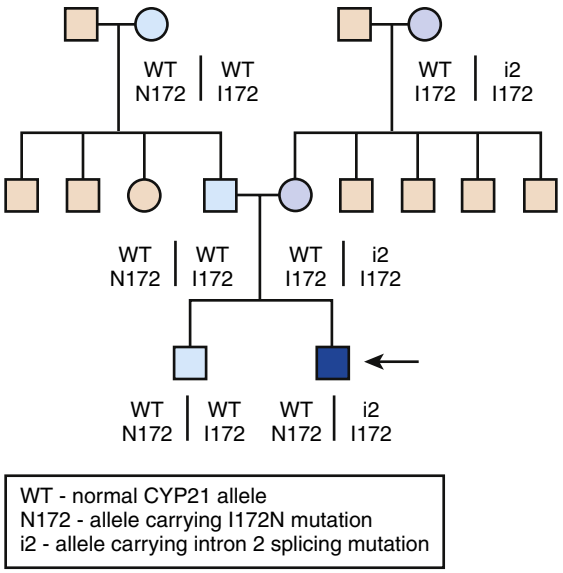


FIGURE 18.8 Molecular genetics of congenital adrenal hyperplasia. The proband *(arrow)* presented with premature pubarche at age 5 years, 9 months. His height and weight were greater than the 95th percentile. He had Tanner 3 pubic hair, phallic enlargement, prepubertal testes, and a small amount of axillary hair. His bone age was 13 years. Laboratory findings showed elevated 17-OHP (10,735 ng/dL), androstenedione (1372 ng/dL), and testosterone (220 ng/dL). Molecular genotype analysis showed that he was a compound heterozygote with I172N on his paternal allele and the intron 2 splicing mutation on his maternal allele. This paradigm is applicable for other autosomal recessive disorders.

genotype each allele or inclusion of parental DNA samples to segregate the alleles are helpful.

3β-Hydroxysteroid Dehydrogenase Deficiency. In this form of virilizing CAH, decreased activity of the adrenal and gonadal specific form of 3β-hydroxysteroid dehydrogenase leads to increased concentrations of 17α-hydroxypregnenolone and DHEA.[378] This disorder is due to mutations in the gene coding for 3β-hydroxysteroid dehydrogenase type 2, *HSD3B2*. Patients with classical 3β-hydroxysteroid dehydrogenase deficiency have been found to have mutations in the *HSD3B2* gene.[379-381] However, non-classical CAH due to *HSD3B2* mutations is extremely rare.[382-384] Thus, molecular genetics have shown that non-classical 3β-hydroxysteroid dehydrogenase deficiency is not a common allelic variant. Consequently, correlation of molecular genotype data with hormonal responses has led to adoption of more stringent criteria for the diagnosis of 3β-HSD deficiency. In general, ACTH-stimulated 17-OH-hydroxypregnenolone and DHEA responses are elevated.

11β-Hydroxylase Deficiency. The clinical features associated with 11β-hydroxylase deficiency are similar to 21-hydroxylase deficiency. Patients with this type of virilizing adrenal hyperplasia may develop hypertension attributed to increased deoxycorticosterone secretion. This disorder is due to mutations in the *CYP11B1* gene located on chromosome 8. Non-classical CAH due to *CYP11B1* mutations is extremely rare. The incidence of 11β-hydroxylase deficiency has been estimated to be 1:100,000 among Caucasians,

but the incidence among Israeli Jews of Moroccan origin is reported to be as high as 1:7000.[385,386] Elevated basal and ACTH-stimulated 11-deoxycortisol concentrations are typically found in this form of CAH. Serum 17-OHP, androstenedione, and testosterone concentrations may be mildly elevated. Plasma renin activity concentrations are low or suppressed.

Other Disorders Affecting Steroidogenesis

Oxidoreductase Deficiency

This autosomal recessive disorder of steroidogenesis is characterized by a steroid profile suggesting combined 17α-hydroxylase and 21-hydroxylase deficiencies.[387,388] The more severe phenotype is characterized by ambiguous genitalia, adrenal insufficiency, and skeletal anomalies and is known as the Antley-Bixler syndrome. The skeletal abnormalities can include craniosynostosis, midface hypoplasia, choanal atresia, low set ears, pear-shaped nose, arachnodactyly, clinodactyly, and radiohumeral synostosis. This disorder is due to mutations in the P450 oxidoreductase *(POR)* gene located at chromosome 7q11.2.[389] The protein encoded by the *POR* gene functions as an electron donor to cytochrome P450 enzymes. *POR* loss of function mutations influence the activities of 21-hydroxylase, 17α-hydroxylase, and aromatase enzymes.

Prenatal virilization of female fetuses occurs, but there is minimal postnatal virilization. Maternal virilization during pregnancy may occur and has been attributed to the aromatase deficiency. Curiously, some patients, both male and female, present with delayed puberty; ovarian cysts can occur in adolescent females.[390] One woman was found to be a compound heterozygote with loss of function mutations on both alleles; she presented with breast development, primary amenorrhea, and cystic ovaries.[391] Basal and ACTH-stimulated steroid profiles are variable because oxidoreductase deficiency affects multiple steroidogenic enzymes; serum 17-OHP concentrations tend to be elevated. Urinary analysis of steroid excretion using gas chromatography and mass spectroscopy can provide hormonal confirmation of the diagnosis. Cortisol deficiency may occur; affected individuals may benefit from glucocorticoid replacement therapy.[388] Mutations in the *FGFR2* gene are associated with similar skeletal anomalies, but have normal steroidogenesis and normal external genitalia.[392]

Apparent Cortisone Reductase Deficiency (ACRD)

Cortisol is the active glucocorticoid secreted by the adrenal zona fasciculata. Concentrations of cortisol and its inactive metabolite, cortisone, are modulated by the enzymes, 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1) and 11β-hydroxysteroid dehydrogenase type 2.[393] ACRD is due to hexose-6-phosphate dehydrogenase deficiency and has been associated with premature adrenarche. This disorder is due to loss of function mutations in the hexose-6-phosphate dehydrogenase *(H6PD)* gene which interferes with HSD11B1 oxo-reductase activity and prevents local conversion of cortisone to cortisol.[394] The net result is accelerated peripheral clearance of cortisol resulting in decreased negative feedback inhibition of the HPA axis, increased ACTH secretion, and increased ACTH-mediated adrenal androgen secretion. DHEAS, androstenedione, and testosterone concentrations are increased. Urinary steroid metabolite profiling by combined gas chromatography and mass spectrometry (GC/MS) may be helpful in the differential diagnosis of ACRD.[395]

Apparent DHEA Sulphotransferase Deficiency (PAPSS2). In the adrenal cortex, DHEA can be converted to DHEAS by the enzyme DHEA sulphotransferase (SULT2A1). The catalytic activity of SULT2A1 depends on availability of sulphate donor 3′-phosphoadenosine-5′-phosphosulphate (PAPS). The isoform predominantly expressed in the adrenals and liver is *PAPSS2*. Sequencing of the *PAPSS2* gene in a girl with premature adrenarche who subsequently developed a PCOS-like phenotype revealed compound heterozygous mutations.[396]

Diagnosis and Treatment of CAH and Other Disorders of Steroidogenesis. Steroid hormone responses to ACTH stimulation help to differentiate between premature adrenarche and CAH. To perform an ACTH-stimulation test, a pharmacological dose of synthetic ACTH (0.25 mg cosyntropin) is administered after a basal blood sample has been obtained. A second sample is collected 30 to 60 minutes later. Basal and stimulated steroid hormone concentrations and hormone ratios provide important information. Stimulated 17-OHP responses of less than 500 ng/dL at 30 minutes are within normal limits. Responses greater than 1500 ng/dL are consistent with 21-hydroxylase deficiency.[397] Intermediate responses, 500 to 1500 ng/dL, are consistent with heterozygosity for 21-hydroxylase deficiency. Children with premature pubarche due to *HSD3B2* mutations have had ACTH-stimulated 17-OH-hydroxypregnenolone values greater than 9000 ng/dL.[398] Genetic testing is necessary to confirm the molecular diagnosis.[399]

Treatment of the virilizing CAHs involves hormone replacement therapy with glucocorticoids.[400] The goal of treatment is suppression of excessive ACTH and adrenal androgen secretion without hypercortisolism. Hydrocortisone or a synthetic glucocorticoid, such as prednisone or dexamethasone, can be used. During childhood, hydrocortisone is generally the preferred glucocorticoid because linear growth in the growing child is extremely sensitive to glucocorticoid levels. Longer-acting glucocorticoids such as prednisone and dexamethasone may interfere with linear growth velocity. Typically, hydrocortisone dosages range from 7 to 15 mg/m^2/day and must be administered three times daily because of the hormone's short duration of action. This relatively short duration of action is the major disadvantage of hydrocortisone because patients may have significant variation in serum hormone concentrations between doses.

Some suggest the use of a reverse diurnal dosing such that the highest dose is administered at night whereas other suggest that the highest dose should be administered in the morning.[401] Older adolescents and young adults may like the convenience of fewer daily doses and use prednisone (5.0 to 7.5 mg divided into two daily doses) or dexamethasone (0.25-0.5 mg daily). The growth suppressive potencies of prednisone and dexamethasone are greater than their anti-inflammatory potencies.[402] It is also important to remember that the mineralocorticoid activity of these glucocorticoids varies with prednisolone having less mineralocorticoid

activity than hydrocortisone and dexamethasone having no mineralocorticoid activity.

Long-term management involves interim medical history, physical examination with assessment of growth velocity, and hormone measurements to assess for adequate adrenal suppression.[402,403] Glucocorticoid therapy alone is generally sufficient for children with milder forms of CAH. For patients with oxidoreductase deficiency, ACRD, and PAPSS2, the decision to initiate glucocorticoid replacement therapy needs to be individualized and based on clinical features, ACTH-stimulated cortisol response, and skeletal maturation.

Stress doses are indicated for fever, persistent vomiting, serious injuries, and surgery. Families should have injectable hydrocortisone readily available for situations in which oral medications are not tolerated. General guidelines for oral stress doses are 3 to 4 times the maintainance doses or approximately 50 mg/m^2/day. Parenteral stress dosing varies by age.[364] All affected individuals should wear a MedicAlert identification badge to alert emergency health care providers to their disorder.

Children with CAH who develop a secondary GnRH-dependent precocious gonadarche may benefit from GnRH-Ra treatment. Children with overt salt loss, as well as those with simple virilizing forms, benefit from receiving mineralocorticoid replacement therapy.

Now that increased numbers of children with CAH or other disorders of sexual differentiation survive into adulthood, the medical and psychosocial aspects of their health care are undergoing re-evaluation. Thus, interest in the determinants of gender identity, approach to genital reconstructive surgery, and outcome has increased.[404] Hence, issues related to gender identity, surgery, sexuality, and outcome should be discussed when evaluating patients with these disorders.

Because CAH is one of the most common disorders associated with aberrant external genital differentiation, most of the literature in this area relates to outcome in women with classical CAH. One study reported that adult women with CAH tended to be unmarried, have fewer children than healthy controls, and have negative self-images. In this study, the frequency of homosexuality was not increased.[405] Girls with CAH tend to have female gender identity with preferences for male-type play and male career choices.[406] Gender identity in adolescent girls with CAH was not associated with the extent of prenatal androgen exposure, magnitude of virilization of the external genitalia, or age at reconstructive surgery.[407] Preliminary data from a limited number of outcome studies indicates that honesty, extensive education, and counseling benefit these patients with disorders of sexual differentiation and their families.[408,409]

Testicular adrenal rest tumors (TARTS) may develop in the testes of boys with CAH.[410] These testicular tumors are more common in those boys who are undertreated or poorly compliant. The tumors tend to be benign, bilateral, and are believed to arise from aberrant ACTH-responsive adrenal cells. Due to their location in the mediastinum testis, obstruction of the seminiferous tubules leading to gonadal dysfunction and infertility can occur.

Inherited Glucocorticoid Resistance

Familial glucocorticoid resistance is an autosomal-dominant disorder due to loss of function mutations in the glucocorticoid receptor (*GRL* or *NR3C1*) gene. Consequently, in the face of normal or elevated cortisol concentrations, there is loss of negative feedback inhibition leading to increased ACTH secretion and adrenal androgen biosynthesis, similar to that seen in CAH patients. The predominant symptoms of this disorder are due to excessive androgen and mineralocorticoid secretion rather than glucocorticoid deficiency. Affected children may present with premature pubarche, hypertension, fatigue, or hypokalemia.[411] Laboratory investigation shows elevated plasma concentrations of cortisol, adrenal androgens, aldosterone, and ACTH; these levels recede after administration of a high dose of dexamethasone. Treatment involves high-dose therapy with a potent glucocorticoid (i.e., dexamethasone), leading to suppression of ACTH release and decreased adrenal androgen secretion.

Familial Male-Limited Precocious Puberty

This autosomal-dominant disorder is due to constitutive activating mutations of the *LHCGR* gene[412,413] that alter the tertiary confirmation of the receptor protein, leading to increased cAMP signaling in the absence of ligand.[414] Clinical manifestations are limited to male patients and include phallic enlargement, increased testicular volume, pubic hair, body odor, accelerated growth velocity, and advanced skeletal maturation. These characteristics may present within the first few years of life. Testosterone concentrations are high, while gonadotropin concentrations are low. Short-term treatment with steroid synthesis inhibitors like testolactone, spironolactone, or ketoconazole can be used. If a secondary GnRH-dependent gonadarche develops, GnRH-Ra treatment can improve height prediction.[415] Long-term treatment with cyproterone acetate or ketoconazole revealed similar outcomes without major side effects in 10 boys with testotoxicosis; effects on final height outcome were variable.[416,417] The lack of a female phenotype[418] is a mystery; perhaps the most attractive possibility is that expression of *LHCGR* by the prepubertal ovary is low.

Androgen-Secreting Tumors

Leydig cell tumors secrete testosterone, leading to precocious pubertal development in boys. Testicular volume may be asymmetric because the tumors are often unilateral. Because some tumors are too small to be palpated, ultrasonography may be helpful to localize the tumor. The majority of Leydig cell tumors are benign. Malignant tumors tend to be larger, show greater cell atypia, and tend to infiltrate beyond the testis. In rare instances, the tumor can secrete large quantities of other steroids like 17-OHP, which may confound the diagnosis.[419] A novel somatic cell-activating mutation of the *LHCGR* gene has been found in some adenomas.[420] The D578H mutation associated with familial male-limited precocious puberty has been found in association with Leydig cell adenomas and nodular hyperplasia of Leydig cells in prepubertal boys.[421] Alterations in other factors, such as AMH, inhibin, and other growth factors, may also contribute to the development of such tumors. Hence, the etiologies of tumors at the molecular level are heterogeneous.[422] If possible, the tumor should be excised without removing the testis. Ovarian androgen-secreting tumors are a rare cause of virilization in girls. Such tumors can occur at

an anatomical location distinct from the ovary such as the round ligament.[423]

hCG-Secreting Tumors

In boys, hCG-secreting tumors induce testicular testosterone secretion, leading to precocious pubertal development. These tumors are frequently hepatic in origin. In girls, hCG-secreting tumors in the absence of pubertal levels of FSH are not usually associated with precocious pubertal development. Although exceptions have been described.[424] this finding is consistent with the idea that *LHCGR* expression in the prepubertal ovary is low.

Cushing Syndrome

Cushing syndrome is characterized by excessive glucocorticoid concentrations, whether endogenous or exogenous in origin. Although the predominant features of glucocorticoid excess in children and adolescents are arrested pubertal development and growth failure, precocious virilization may occur when the hypercortisolism is accompanied by hyperandrogenism. This occurs in Cushing disease, in cases of ectopic secretion of ACTH or CRH, and in rare cases of adrenal tumors. Individuals with chronic conditions like rheumatoid arthritis, steroid dependent nephropathies, and asthma requiring high-dose steroid treatment may develop signs and symptoms due to glucocorticoid excess. Intranasal steroids can cause iatrogenic Cushing syndrome and adrenal suppression.[425]

Among children younger than 8 years of age, adrenal tumors are the most common cause of Cushing syndrome. Such tumors are often malignant. The combination of rapid pubertal development and hypercortisolism suggests an adrenal tumor. Such tumors, which are rare, can secrete a mixture of steroid hormones. Nevertheless, virilization is a common presentation for childhood adrenocortical tumors. There is a female predominance. Complete surgical excision is the treatment of choice; complete resection is required for cure. Residual or metastatic disease is associated with a poorer prognosis.[426] Histological examination may not be able to accurately differentiate benign adenomas from carcinomas. Cushing disease due to excessive pituitary ACTH secretion is extremely rare in young children, but can occur in adolescents.

Primary pigmented adrenocortical nodular disease (PPNAD) is often associated with the periodic or episodic hypercortisolism. The adrenal glands are characterized by multiple pigmented autonomously functioning nodules. PPNAD is associated with inactivating mutations of the *PRKAR1A* gene. The majority of benign lesions of the adrenal cortex appear to be associated with abnormalities of the cAMP signaling pathway, whereas adrenocortical carcinomas are linked to aberrant expression of insulin-like growth factor II, tumor protein p53 and related molecules.[427,428] Syndromes associated with adrenocortical tumors include CNC, Li-Fraumeni syndrome, and Beckwith-Wiedemann syndrome.

Confirmation of excessive glucocorticoid secretion is essential prior to imaging studies. Loss of diurnal variation in cortisol secretion, excessive 24-hour urinary free cortisol excretion, and inadequate dexamethasone-induced suppression are valid diagnostic studies. In some instances, due to periodic hypercortisolism, these studies need to be repeated. Treatment is directed by the underlying disorder.[429]

Approach to the Child with Precocious Pubertal Development

Identification of the etiology of sexual precocity begins with a thorough medical history, with questions focusing on the physical manifestations of puberty and the age and sequence of their appearance (Figs. 18.9 and 18.10). Does the patient have neurological symptoms or gelastic seizures? Has the patient received radiation therapy? Was the patient exposed to exogenous hormones? Obtaining a complete family history is important because some disorders, such as familial male-limited precocious puberty, glucocorticoid resistance, and NF1, show autosomal-dominant inheritance.

Next, a complete physical examination, including auxological measures, should be performed; particular attention should be paid to development of secondary sexual characteristics. For girls, are breast and/or pubic hair development present? For boys, is testicular volume increased? Because increased growth velocity occurs concomitantly with breast development among girls, observation of accelerated growth velocity helps to differentiate GnRH-dependent precocious gonadarche from non-progressive gonadarche or premature thelarche. The physical examination should be directed to detect physical signs indicating onset of gonadarche or adrenarche, as well as features suggestive of specific disorders such as café-au-lait spots in McCune-Albright syndrome or NF1. Does the patient have manifestations of hypothyroidism?

Laboratory studies are used to confirm or exclude specific disorders based on the possible differential diagnoses. Bone age is usually, but not always, advanced among patients with GnRH-dependent precocious gonadarche and the CAHs. Typically, bone maturation is not significantly advanced among subjects with premature thelarche or premature adrenarche. In healthy individuals, the bone age interpretation should lie within two standard deviations of the normative data.

Because gonadotropin secretion is pulsatile, random concentrations usually provide only limited information. GnRH-stimulation tests help to differentiate GnRH-dependent precocious gonadarche from premature thelarche, because an LH predominant response is typical of GnRH-dependent precocious gonadarche whereas an FSH predominant response is typical of premature thelarche.[430] The limited availability of GnRH (Factrel) has led to development of stimulation tests utilizing Lupron as the provocative agent.[431] Comparison of immunochemiluminometric (ICMA) and immunofluorometric (IFMA) assays indicated that for prepubertal children, the upper limit of normal for LH after GnRH stimulation test by ICMA was 4.1 IU/L for boys and 3.3I IU/liter for girls, and that measured by IFMA was 3.3 IU/liter for boys and 4.2 IU/liter for girls.[432] It is important to be aware of the normal values for the specific methodology used. Most current assays for gonadotropins are sandwich assays specific to the β-subunit. Using the newer third generation gonadotropin assays (e.g., Architect and Delfia) random LH concentrations greater than 1.0 U/L in girls with features consistent with precocious puberty may be sufficient to diagnosis central precocious puberty.[433] MRI of the pituitary and brain is helpful for patients with GnRH-dependent precocious gonadarche. Boys with elevated hCG concentrations may require imaging of the head, chest, and abdomen to detect an extra-testicular hCG-secreting tumor. Among girls, pelvic ultrasound can

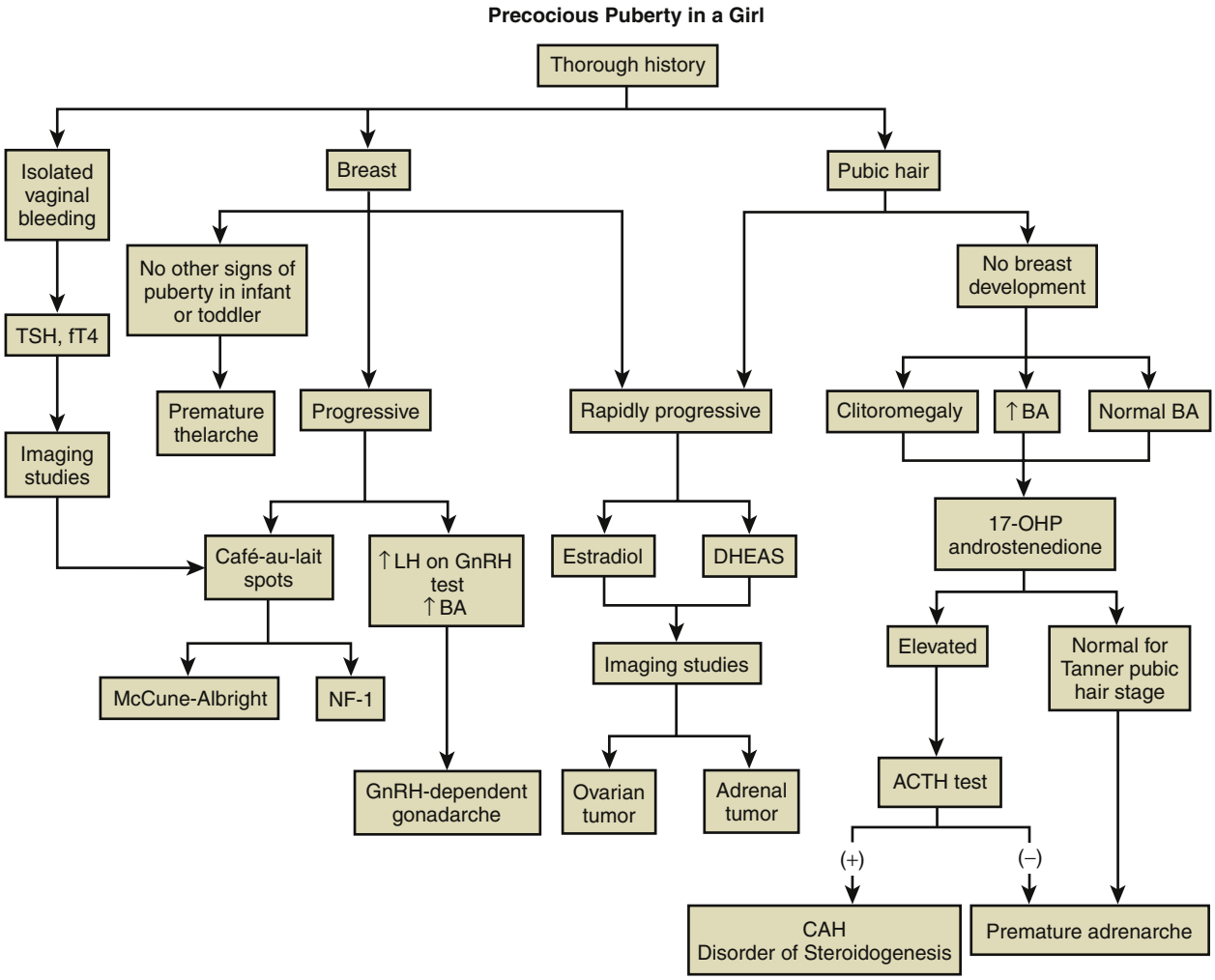


FIGURE 18.9 Algorithm for initial approach to precocious puberty in a girl. Beginning with a thorough medical history, this flowchart provides guidelines to the differential diagnosis of the most common causes of precocious puberty in girls. 17-OHP, 17-hydroxyprogesterone; ACTH, adrenocorticotropin; BA, bone age; CAH, congenital adrenal hyperplasia; DHEAS, dehydroepiandrosterone sulfate; fT4, free thyroxine; LH, luteinizing hormone; NF1, neurofibromatosis type 1; TSH, thyroid stimulating hormone.

assess for pubertal development of the ovaries and uterus; it can also detect tumors and ovarian cysts.[434]

Pelvic ultrasound is helpful for the evaluation of girls with precocious puberty because the size and morphology of the uterus and ovaries reflect function and hormone exposure. The prepubertal uterus is tubular in configuration; the cervix is similar or slightly larger than the fundus in size. Estrogen exposure causes a disproportionate enlargement of the fundus such that the uterus acquires a pear-like shape. The mean ovarian volume in prepubertal girls between 2 to 7 years of age is 1 cm^3. Small follicles (less than 9 mm) may be present.[435]

For patients with suspected disorders of steroidogenesis, liquid-chromatography—tandem mass spectrometry (LC-MS/MS) is rapidly becoming the method of choice for clinical steroid analysis because of the higher sensitivity, better reproducibility, greater specificity, and low sample volume compared to immunoassay techniques. This technique offers the ability to analyze multiple steroids simultaneously. Prior to clinical utilization, reliable reference ranges need to be established for specific analytes according to gender and pubertal stage.[436] Testicular ultrasound to assess for Leydig cell tumors or microlithiasis may be indicated especially when the testicular volumes are discordant. Adrenal ultrasound to assess for adrenal tumors or adrenal hyperplasia is appropriate for children with premature pubarche and extremely elevated DHEAS concentrations.

Disorders of Delayed Puberty

In most instances, delayed puberty is the result of delayed gonadarche and is defined as secondary sexual characteristic development at an age greater than two standard deviations more than for the normal population. Thus, for girls, lack of breast development by age 13 years or menarche by 16 years may be viewed as delayed. For boys, prepubertal testicular volume at age 14 years is considered to be delayed. Pubertal delay can be broadly sub-classified as constitutional, hypogonadotropic, or hypergonadotropic. Patients with hypogonadotropic pubertal delay can be further classified as GnRH-dependent, pituitary dependent, and hypothalamic-pituitary dependent. HH can be due to gonadal failure, defects of steroidogenesis, or defects of steroid action.

Atypical pubertal development during the adolescent years may also reveal disorders of sexual differentiation that escape diagnosis during infancy. Failure to complete puberty within 5 years also warrants evaluation.[437] In general,

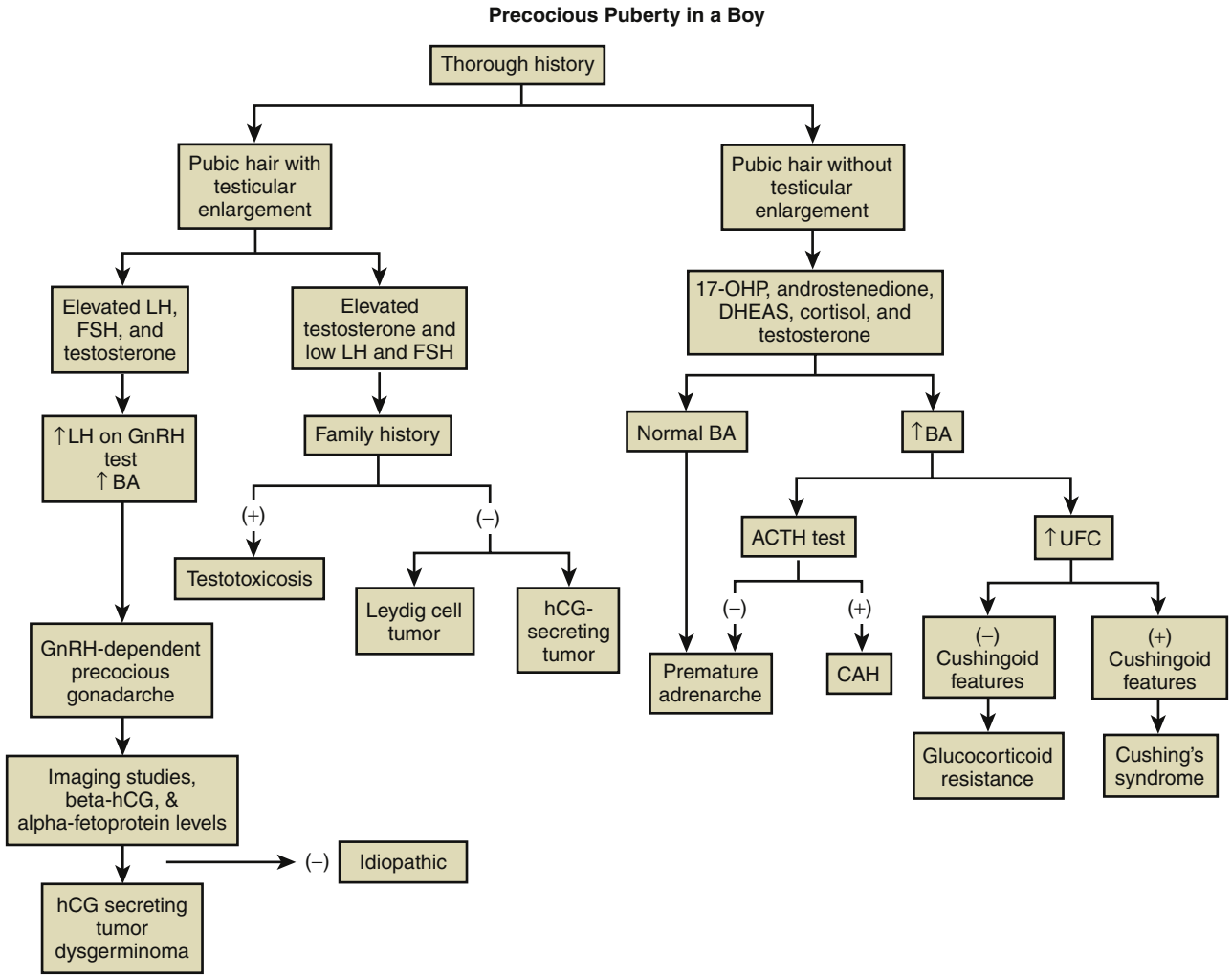


FIGURE 18.10 Algorithm for initial approach to precocious puberty in a boy. Beginning with a thorough medical history, this flowchart provides guidelines to the differential diagnosis of the most common causes of precocious puberty in boys. 17-OHP, 17-hydroxyprogesterone; ACTH, adrenocorticotropin; BA, bone age; CAH, congenital adrenal hyperplasia; DHEAS, dehydroepiandrosterone sulfate; FSH, follicle stimulating hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; UFC, urinary free cortisol.

treatment of delayed puberty involves steroid hormone replacement, which is discussed in detail at the end of the section (Box18.2; Table 18.3).

Constitutional

Rather than being a truly pathological condition, constitutional delay of puberty is considered an extreme of the normal variation of pubertal timing. Typically, both gonadarche and adrenarche are delayed. There is no definitive diagnostic test and, therefore, constitutional delay of puberty is a diagnosis of exclusion. Often, linear growth velocity during the first few years of life is decreased. Subsequently, growth velocity normalizes and tracks close to the fifth percentile during childhood. Growth velocity again decreases during the early adolescent years, associated with an apparent transient decrease in GH secretion. Typically, the family history is positive for delayed puberty. Skeletal maturation is retarded, and the predicted height is often in the lower range of genetic height potential, based on midparental height calculations.[438]

A recent report of SNPs predicted to generate missense mutations in the ghrelin receptor gene in two females with constitutional delay hints at a possible association.[439] A novel missense mutation in the leptin gene speculated to have increased bioactivity was associated with delayed puberty, poor appetite, and low BMI in a mother and son.[440] However, variants in genes associated with idiopathic HH (IHH) appear to be rare.[441,442]

GnRH-Deficiency (Hypothalamic Hypogonadism)

This category of delayed puberty is the consequence of absent or impaired GnRH secretion that may result from either a primary developmental anomaly of the hypothalamus or secondary pathophysiological conditions. GnRH deficiency or HH, reported to affect one in 7500 male patients and one in 70,000 female patients, and comprises a diverse group of reproductive disorders.[443] The term "IHH" has been used in the past for this group of disorders. However, with greater knowledge regarding the molecular causes of GnRH deficiency, the term HH is preferable.

Sporadic cases are more common than inherited forms. Inheritance patterns include X-linked, autosomal dominant,

Box 18.2 Etiologies of Delayed Puberty

CONSTITUTIONAL DELAY

GnRH-DEPENDENT (HYPOTHALAMIC-HYPOGONADOTROPIC HYPOGONADISM)
- *KAL1* mutation
- *PROK2* and *PROKR2* mutations
- *FGFR1* and *FGF8* mutations
- *HS6ST1* mutation
- *NELF* mutation
- CHARGE syndrome (*CHD7* mutation)
- *WDR11* mutation
- *SEMA3A* mutation
- *GNRH1* mutation
- *KISS1* and *KISS1R* mutations
- *TAC3* and *TACR3* mutations
- Anorexia nervosa
- Undernutrition/chronic disease/intensive exercise
- Leptin-dependent

PITUITARY-DEPENDENT (PITUITARY HYPOGONADOTROPIC HYPOGONADISM)
- *GNRHR* gene mutation
- *FSHB* gene mutation
- *LHB* gene mutation
- Developmental anomalies: *SOX2* mutation, *HESX1* gene mutation (septo-optic dysplasia), *PITX2* gene mutation (Rieger syndrome), *LHX3* mutation, *PROP-1* gene mutation, maternal cocaine abuse, valproate toxicity, intrauterine CMS vascular events

HYPOTHALAMIC AND PITUITARY-DEPENDENT HYPOGONADOTROPIC HYPOGONADISM
- Adrenal hypoplasia congenital (*DAX1/NROB1* mutation)
- Intracranial tumors
- Histiocytosis X
- Hyperprolactinemia
- Steroidogenic factor-1 (*NR5A1* mutation)
- Prader-Willi syndrome
- Bardet-Biedl syndrome
- Bloom syndrome
- Hereditary hemochromatosis

GONAD-DEPENDENT
- Primary gonadal failure:
- Gonadal dysgenesis (Turner syndrome, mixed 45,X/46,XY, pure 46,XY gonadal dysgenesis like SRY gene mutations, pure 46,XX gonadal dysgenesis, Denys-Drash syndrome, Frasier syndrome, 46,XX male, *SOX9* gene mutation),
- Klinefelter syndrome
- Testicular regression syndrome (vanishing testis)
- Auto-immune ovarian failure
- Nonautoimmune premature ovarian failure
- Galactosemia
- Noonan syndrome
- Down syndrome
- Other: trauma, torsion, post-infectious (e.g., mumps)
- Defects in steroidogenesis: *LHCGR* gene mutation, *FSHR* gene mutation, congenital lipoid adrenal hyperplasia, 17α-hydroxylase/17–20-lyase deficiency, AKR1C2/AKR1C4 gene mutations, aromatase deficiency, 17β-hydroxysteroid dehydrogenase deficiency, 5α-reductase deficiency
- Steroid hormone receptor-dependent: androgen insensitivity, estrogen receptor mutation
- Anatomical abnormalities: Mayer-Rokitansky-Kuster-Hauser syndrome
- Chemotherapy
- Radiation therapy

GnRH, gonadotropin-releasing hormone.

Table 18.3 Monogenic Disorders Associated with Hypogonadotropic Hypogonadism

Gene	***Locus***	***Associated Phenotypes***
KAL-1	Xp22.3	Anosmia, bimanual synkinesis, renal anomalies
FGFR1	8p11.2-p11.l	Anosmia, cleft lip/palate, dental anomalies
FGF8	10q24	Anosmia, cleft lip/palate, dental anomalies
HS6ST1	2q14.2	None
NELF	9q34.3	Anosmia
PROK2	3p21.1	None
PROKR2	20p13	None
CHD7	8q12.1	CHARGE syndrome
SEMA3	7q21.11	Anosmia or normosmia
KISS1R	19p13.3	None
KISS1	1q32	Normosmic
WDR11	10q26.12	Anosmia
TAC3	12q13-q21	Microphallus, cryptorchidism
TACR3	4q25	Microphallus
GNRH1	8p21-p11.2	None
GnRHR	4q21.2	None
FSHB	11p13	Hypogonadism
LHB	19q13.32	Hypogonadism
HESX1	3p21.2-p21.1	Hypopituitarism (septo-optic dysplasia)
PITX2	4q25-q26	Hypopituitarism (Rieger syndrome)
PROP-1	5q	Hypopituitarism
LHX3	9q34.4	Hypopituitarism
SOX2	3q26.33	Optic nerve hypoplasia, microphthalmia
LEP	7q31.3	Obesity
LEPR	1p31	Obesity
DAX-1	Xp21.3-p21.2	Adrenal hypoplasia congenita
NR5A1 (SF-1)	9q33	Adrenal insufficiency, hypogonadism
PTPN11	12q24.1	Noonan syndrome

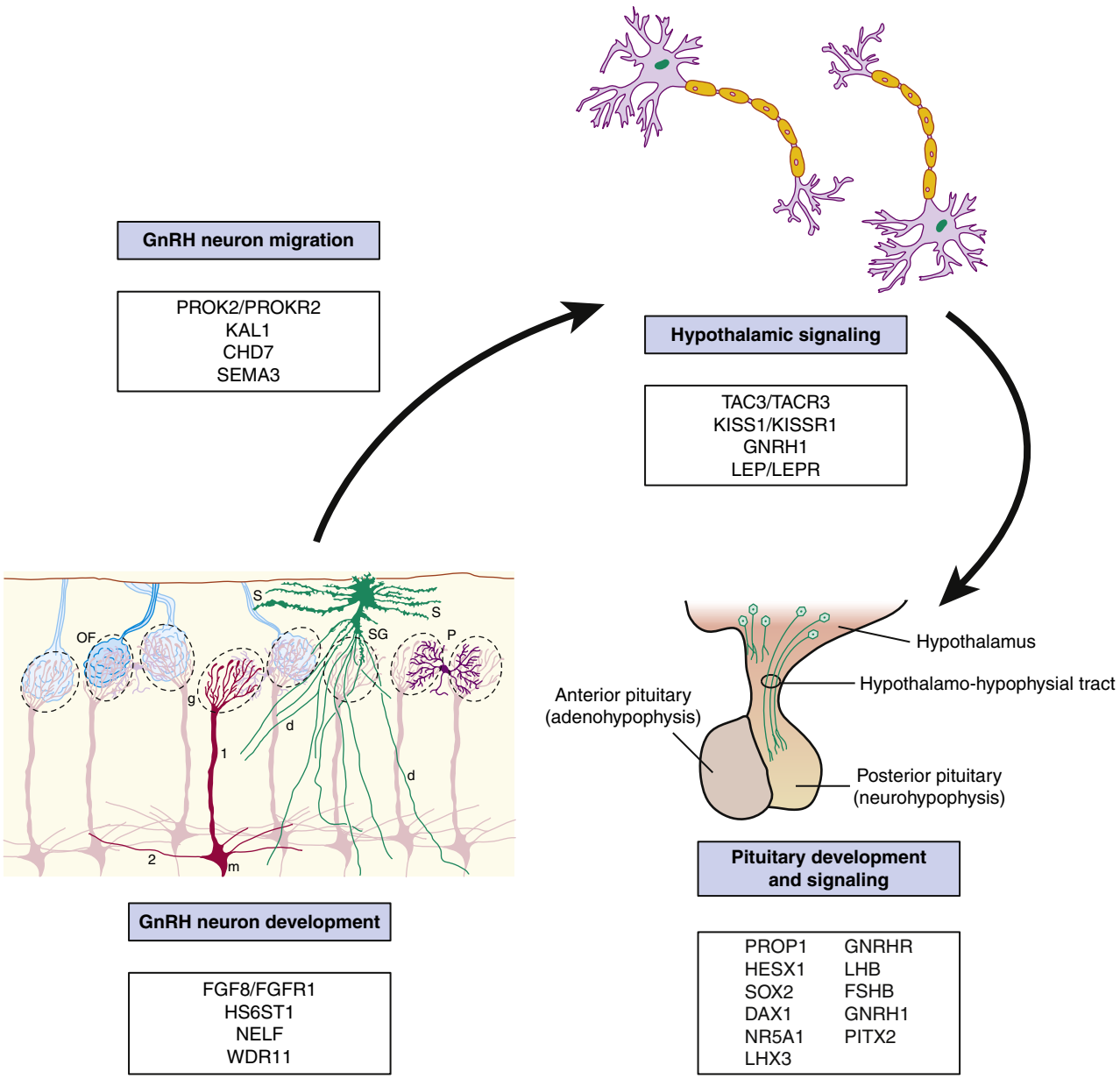


FIGURE 18.11 Genes implicated in gonadotropin-releasing hormone (GnRH) neuron development, GnRH neuron migration, and GnRH neuron activity and signaling. It also shows genes implicated in pituitary development and signaling. Genes have been classified by presumed function in these processes.

and autosomal recessive. However, it has become apparent that GnRH deficiency can be due to mutations in several genes (an oligogenic disorder).[444] In addition, there can be a spectrum of reproductive, olfactory, and nonreproductive clinical features.[445]

Traditionally, these heterogeneous disorders have been sub-classified into three major categories: (1) Kallmann syndrome with anosmia; (2) HH without anosmia; and (3) acquired HH. Correlation of olfactory function by formal smell test with genetic analysis among patients with HH revealed a spectrum ranging from normosmia to hyposmia to anosmia. Mutations associated with the hyposmia or anosmia phenotype include genes discussed above that influence GnRH neuron development and migration.[445] Mutations associated with normosmic HH include genes that influence function (Fig. 18.11).

Mutations in Genes Influencing GnRH Neuron Development and Migration

KAL1

The classic form of Kallmann syndrome is characterized by isolated gonadotropin deficiency, anosmia, X-linked inheritance, and the failure of GnRH neurons to migrate from the olfactory bulb into the hypothalamus during embryonic development.[446] The defective olfactory bulb development can be confirmed by MRI. Investigation of patients with classic Kallman syndrome led to identification of the first gene associated with anosmic HH.

Patients with *KAL1* mutations show complete loss of LH pulsatility, suggesting greater deficits in the GnRH system than patients with autosomal forms in whom low amplitude LH pulses can often be detected.[447] Men with X-linked

Kallmann syndrome have been found to have a higher incidence of cryptorchidism and microphallus, lower inhibin B concentrations, higher AMH concentrations, and increased frequency of immature testicular morphology. Nonreproductive features associated with the X-linked form include unilateral renal agenesis, synkinesia, sensorineural hearing loss, cleft palate, and cerebellar ataxia. Phenotypic heterogeneity, even with the same mutation, suggests that modifier loci or epigenetic factors modulate the clinical manifestations.[448] Rare *KAL1* missense sequence variants have been identified in women with GnRH deficiency.[449] Nevertheless, the majority of cases of Kallmann syndrome or IHH are not associated with *KAL1* mutations.

PROK2 *and* PROKR2

Mutations in *PROK2* and *PROKR2* genes have been identified in patients with IHH.[450,451] The phenotype of individuals with *PROK2* and *PROKR2* mutations includes normosmic HH as well as anosmia in the absence of a reproductive phenotype.[452] Nonreproductive features include fibrous dysplasia, obesity, synkinesia, and epilepsy.[453] Most carry heterozygous mutations, but *PROK2* mutations were detected in siblings with autosomal recessive HH; two siblings had anosmia and an affected female sibling was normosmic.[450] Patients with HH and *PROKR2* mutations are often found to carry mutations in other genes associated with HH such as *KAL1*, *PROK2*, and *FGFR1*. Heterozygous *PROKR2* gene mutations have been identified in patients with congenital hypopituitarism and pituitary stalk interruption.[454] A specific incompletely penetrant *PROKR2* mutation, Leu173Arg, has been identified in individuals with HH from diverse ethnic and geographic origins suggesting that this mutation demonstrates an ancient founder effect.[455]

FGFR1 *and* FGF8

FGFs and their receptors induce proliferation and differentiation of GnRH neurons. Investigation of families with autosomal dominant IHH led to identification of loss of function mutations in the *FGFR1* and the *FGF8* genes. Numerous point mutations and deletions in *FGFR1* have been identified in affected individuals and families. Additional clinical features include impaired development of the olfactory bulbs, skeletal anomalies, and bimanual synkinesis.[456] Cerebellar ataxia (Gordon-Holmes syndrome) is an associated clinical feature in some families in which autosomal-recessive inheritance occurs.[457] Phenotypic heterogeneity occurs associated with a broad spectrum of reproductive abnormalities. Both anosmia and normosmia have been described.[458] Mutations in *FGFR1* have been described in women with hypothalamic amenorrhea.[459] Variability in the extent of the GnRH deficiency and the olfactory phenotype has been reported for *FGF8* mutations.[460] Clinical features associated with *FGF8* mutations also includes holoprosencephaly, septo-optic dysplasia, and Moebius syndrome.[461]

HS6ST1

Loss of function mutations in the gene encoding HS6ST1 are associated with HH. Phenotypic heterogeneity occurs for both reproductive manifestations and anosmia. Among families with *HS6ST1* mutations, HH segregates as a complex trait and mutations in other genes associated with HH have been identified in some families. It remains to be determined if *HS6ST1* mutations can be solely responsible for GnRH deficiency or whether other genetic or epigenetic variations are necessary for development of clinical symptoms.[462]

NELF

Mutations in the *NELF* gene have been reported in a few patients in HH in association with anosmia.[463] In most instances, digenic inheritance is present with mutations detected in additional genes such as *KAL1* and *TACR3*.[464]

CHD7

Mutations in the C*HD7* gene are associated with the coloboma, heart anomaly, choanal atresia, mental retardation, genital and ear anomaly (CHARGE) syndrome. This syndrome is characterized by eye coloboma, heart malformations, atresia of the choanae, retardation of growth or development, genital anomalies, and ear abnormalities. Although usually sporadic, autosomal dominant cases have been described. Nonreproductive features include hearing loss, balance disturbances, and dysmorphisms affecting the ear.[465] The evaluation of patients with HH should include evaluation for features of CHARGE syndrome.[466]

WDR11

Finding a balanced translocation at chromosome 10q in a patient with HH led to investigation of the *WDR11* gene. Heterozygous mutations in this gene were identified in additional patients with HH. The protein contains multiple WD domains likely to mediate interaction with protein-binding partners; one potential binding partner is EMX1.[467] EMX1, a homeodomain transcription factor, is related to the Drosophila developmental genes empty spiracles and involved in the development of the olfactory system: no human mutations in the *EMX1* gene have yet been identified.

SEMA3A

Heterozygous mutations in the *SEMA3A* gene were identified in patients with anosmic HH.[468] However, available data suggest that *SEMA3A* mutations are unlikely to be the sole molecular basis of HH in affected individuals.[469]

Mutations in Genes Influencing GnRH Function

GNRH1

Interestingly, loss of function mutations in the *GnRH1* gene itself were not identified until 3 to 4 years ago.[470,471] Individuals with homozygous loss of function mutations of this gene have hypogonadism; affected males may have microphallus. Heterozygous missense mutations have also been described. Aberrant processing of the GnRH prohormone due to mutations in the prohormone convertase 1/3 *(PCSK1)* gene are also associated with HH.[472]

KISS1 *and* KISS1R

As noted above, investigation of familial autosomal recessive HH led to identification of mutations in the *KISS1R* gene[79,80] and to the subsequent elucidation of the *KISS1-KISS1R* (kisspeptin-GPR54) system. To date, several loss of function mutations have been reported in the *KISS1R* gene. Cryptorchidism and a small phallus have been reported in some affected males.[473] As would be anticipated from our understanding of the role of kisspeptin in GnRH pulse generation (discussed earlier in the chapter), LH and FSH concentrations are low in patients with *KISS1R* mutations.[79]

A homozygous missense *KISS1* mutation affecting a conserved amino acid was reported in a consanguineous family.[81] Autosomal recessive inheritance patterns have been described for both *KISS1* and *KISSR1* mutations.

TAC3 *and* TACR3

As described previously, loss of function mutations in the NKB gene *(TAC3)* and its cognate receptor gene *(TACR3)* were found to be associated with profound normosmic HH in consanguineous families.[82,474] Affected individuals have been found to carry mutations on both alleles, homozygous or compound heterozygous. Heterozygous mutations have also been described. Microphallus, but not cryptorchidism, is a common feature of *TAC3* mutations indicating the importance of NKB in GnRH pulse generation during fetal development.[475] Patients with *TAC3* or *TACR3* mutations tend to have low LH with relatively normal FSH concentrations consistent with diminished GnRH pulse frequency.[476]

Reversibility

Curiously, a few individuals with HH who carry loss of function mutations have demonstrated reversible HH.[477,478] Genes associated with reversal of HH in patients include *FGFR1*, *CHD7*, *TAC3*, and *TACR3*.[479,480] Regarding the NKB pathway, although reversibility has been documented in biallelic (homozygous) loss of function mutations in both the ligand *(TAC3)* and receptor *(TACR3)*[479] (Topaloglu personnal communication) there is controversy surrounding the view that frequency of reversal is typically high in these subjects.[479,481] Resolution of this issue is relevant and important not only from a clinical perspective but also because NKB signalling is a pivotal component in the contemporary model of GnRH pulse generation (discussed earlier in the chapter).

Coexistence of IHH and constitutional delay in the same pedigree raises the question as to whether, in some instances, constitutional delay and IHH reflect a phenotypic spectrum characterized by delayed reactivation of the GnRH pulse generator.[482] Nevertheless, the biological mechanism responsible for recovery of endogenous gonadotropin secretion and duration of this recovery remain to be elucidated.[483] Androgen exposure is one proposed influence.

Other Factors

Investigation of GnRH neuron development and migration in animals has identified additional factors involved in these processes,[484-486] but their role in HH has not yet been established.

Anorexia Nervosa

Anorexia nervosa is a chronic psychiatric disorder characterized by decreased caloric intake, weight loss, distorted body image, and excessive physical activity (also see Chapter 21). Approximately 90% of individuals with this disorder are females. Delayed puberty and associated oligomenorrhea or amenorrhea are secondary to deficient GnRH secretion. Primary or secondary amenorrhea can precede weight loss and can persist despite weight gain. This condition would suggest that factors other than metabolical and nutritional determinants are involved in the suppression of the GnRH pulse generator. Other hormone findings include elevated cortisol and GH concentrations and decreased IGF-I concentrations. The "low T_3 (triiodothyronine) syndrome" is also common, in which T_3 is decreased, T_4 is normal or subnormal, and TSH is normal.[487] Bulimia nervosa, characterized by binging and purging induced by vomiting or laxative abuse, is a variant of anorexia nervosa. Osteopenia and osteoporosis are serious and persistent consequences of anorexia nervosa in both sexes. The current therapies, for decreased bone mineral density include transdermal estradiol, vitamin D, and recombinant IGF1. Although these treatments are helpful, they do not fully restore bone accrual.[488]

Undernutrition, Chronic Disease, and Intensive Exercise

Undernutrition and chronic diseases, such as inflammatory bowel disease, cystic fibrosis, hypothyroidism, and poorly controlled type 1 diabetes mellitus, are associated with delayed or arrested puberty (see also Chapter 21). Primary or secondary functional amenorrhea can occur. In many chronic illnesses, inadequate nutrition leads to impaired GnRH secretion resulting in hypothalamic hypogonadism. Effective treatment of the underlying disorder is associated with pubertal progression and reversibility of the functional hypothalamic amenorrhea. For example, after successful renal transplantation, children with chronic renal disease manifest resumption of gonadotropin secretion and progressive pubertal development. Anemias, such as sickle cell anemia and thalassemia, are also associated with compromised GnRH release. It should be noted that treatment of these anemias with frequent transfusions may lead to iron overload, iron deposition in the pituitary, and exacerbation of the hypogonadotropism. Curiously, heterozygosity for mutations in genes associated with HH may increase susceptibility for hypothalamic amenorrhea.[488a]

Intensive exercise and other athletic training schedules including caloric restriction to maintain a weight class, may delay or interrupt pubertal development due to decreased GnRH secretion. Long-distance runners, gymnasts, ballerinas, and wrestlers are among the most commonly affected athletes. During the competitive season, male wrestlers lose body weight, fat mass, and lean body mass, such that disruption of the neuroendocrine axes governing testicular function and growth may ensue. These changes, however, reverse within months after conclusion of the wrestling season.[489] Administration of leptin to eight women with hypothalamic amenorrhea was associated with improvement in reproductive, GH, and thyroid function providing additional evidence for leptin's important role as a signal indicating energy status.[490]

Leptin-Dependent Obesity

Retarded pubertal development has been associated with early onset morbid obesity due to autosomal homozygous mutations of *LEP* or *LEPR* genes.[151,491,492] The consequences of leptin replacement administered to patients with mutations of *LEP* have been described in detail above.

Pituitary-Dependent (Pituitary Hypogonadotropism)

Pituitary hypogonadotropism in subjects with delayed puberty may be due to a mutation in the gene for the GnRH-R, a mutation in a gene coding for one of the gonadotropin subunits, or a more generalized developmental anomalies of the pituitary (Fig. 18.11).

GnRH-R Gene Mutations

The inheritance pattern of HH secondary to mutations in the *GNRHR* gene is autosomal-recessive, but sporadic cases are

observed.[493] Reported phenotypes range from microphallus and undescended testes at birth to presentation with delayed puberty in adolescence.[494] In general, the clinical phenotype correlates with biochemical LH pulsatility profiles and responses to exogenous GnRH. Complete HH is usually associated with low levels of apulsatile LH, whereas some spontaneous LH pulses, albeit reduced in amplitude, may be seen in patients with partial forms. The clinical phenotype correlates with the magnitude of the functional consequence of the mutation upon receptor function. Nevertheless, phenotypic heterogeneity for the same mutation has been observed.[495,496] Thus, the phenotype of individuals with *GNRHR* mutations ranges from partial to complete hypogonadism. The mutations in *GNRHR* reported to date appear to impair receptor folding and ligand binding, and decrease activation of the inositol phosphate or phospholipase C signaling pathways.[497-499]

FSH-β Gene Mutations

Women found to have mutations in the *FSHB g*ene have presented with absent breast development and primary amenorrhea.[500] Circulating FSH and estrogen concentrations were low, while LH secretion was elevated.[501,502] Three affected males have been reported with phenotypes ranging from absent to normal secondary sexual development. All three had azoospermia,[503-505] and, as in females, FSH concentrations were low whereas LH tended to be elevated.[506]

LHβ Gene Mutations

One male homozygous for a missense mutation, Gln55Arg, in the *LHB* gene had delayed puberty, elevated immunoreactive LH concentrations, few Leydig cells, and arrested spermatogenesis; treatment with hCG stimulated testosterone synthesis and spermatogenesis (also see Chapter 2).[507] Another variant, Gly102Ser, has been associated with male and female infertility.[508] Functional characterization of the Gly102Ser variant at high LH concentrations showed impaired receptor binding and decreased ability to stimulate progesterone biosynthesis compared with wild-type LH.[509] Homozygosity for a splicing mutation in the *LHB* gene was associated with hypogonadism in the affected male siblings and spontaneous pubertal development with secondary amenorrhea and infertility in the female sibling.[510]

One *LHB* variant appears to be relatively common; the frequency varies widely between ethnic groups ranging from near 0% in Kotas of Southern India up to 50% in Australian aborigines.[511] This variant has two single nucleotide polymorphisms (SNPs) in the coding region, which are predicted to generate the missense mutations, Tyr8Arg and Ile15Thr, and eight SNPs in the promoter region. Differences in transcriptional activity between the variant and wild-type have been attributed to the SNPs in the promoter of the variant.[512] Comparisons of the recombinant variant peptide subunit with the native subunit revealed differences in carbohydrate side chains and in intracellular trafficking.[513] More specifically, the Arg8/Thr15 variant contains an additional sialic acid residue per molecule and a longer half life.[514] Among healthy boys, heterozygosity for the Arg8/Thr15 variant was associated with smaller testicular volumes, slower growth rates, and lower IGFBP3 concentrations, suggesting that this variant affects the tempo of puberty.[513] Existing data suggest that, in girls, this variant may be associated with subfertility, lower frequency of PCOS, and delayed appearance of breast cancer.

Developmental Anomalies of the Pituitary

The pituitary consists of the anterior and posterior portions. The anterior portion is derived from an invagination of the oral ectoderm. The posterior pituitary originates from the neural ectoderm. Transcriptional activators, repressors, and other signalling molecules direct the development of the pituitary. Genetic variants in these factors have led to identification of genes associated with human disease. These developmental anomalies of the CNS, such as midline defects or septo-optic dysplasia, may be associated with gonadotropin and other pituitary hormone deficiencies, and, therefore, with delayed pubertal development. Septo-optic dysplasia, also known as de Morsier syndrome, is characterized by abnormalities of the septum pellucidum, thinning of the corpus callosum, and hypoplasia of the optic nerve. With optic nerve hypoplasia, physical examination reveals pendular nystagmus and pale hypoplastic optic discs.

The homeobox gene expressed in ES cells *(HESX1)* gene is an early marker of pituitary differentiation with limited spatiotemporal expression early in gestation. The protein functions as a transcriptional repressor and is important for midline development. Mutations in *HESX1* have been reported in familial cases of septo-optic dysplasia.

Mutations in the sex-determining region Y (SRY) box 2 *(SOX2)* gene are associated with hypopituitarism, eye anomalies, and HH.[515,516] Nonreproductive features include hypoplasia of the corpus callosum, sensorineural hearing loss, and esophageal atresia. SOX2 plays a role in regulation of cell proliferation in the anterior pituitary during early development. *SOX2* mutations appear to decrease the number of anterior pituitary progenitor cells.[517] SOX2 interacts with CHD7 to regulate common target genes.[518]

Mutations in other genes involved in pituitary differentiation have been identified among patients with multiple anterior pituitary hormone deficiencies. Rieger syndrome is an autosomal-dominant disorder characterized by eye anomalies, dental hypoplasia, and anterior pituitary hormone deficiencies associated with mutations in the paired-like homeodomain transcription factor 2 *(PITX2)* gene, which encodes a transcription factor involved in pituitary cell differentiation and maintenance.

Delayed puberty associated with impaired gonadotropin secretion has been reported in patients with mutations in the *LHX3* gene, which codes for a homeo-domain-containing transcription factor expressed early in the differentiation of the anterior pituitary.[519] Mutations in the *PROP-1* (paired like homeodomain factor 1, Prophet of PIT1) gene are associated with combined pituitary hormone deficiencies. The *PROP-1* gene codes for a transcription factor involved in development of somatotrophs, thyrotrophs, and lactotrophs.[520] Most patients with *PROP-1* gene mutations reported to date have presented in childhood with TSH and GH deficiencies. LH and FSH deficiencies develop later with a variable age of onset, such that the clinical presentation ranges from delayed puberty to secondary amenorrhea. Corticotroph dysfunction associated with ACTH deficiency and acquired adrenal insufficiency can also develop over time.[521,522] Thus, phenotypic heterogeneity occurs in regard to age at presentation and the spectrum of anterior pituitary hormone deficiencies.[523]

Nongenetic factors may also lead to CNS developmental abnormalities with hypogonadotropism and should therefore be considered in the etiology of pituitary-dependent delayed

puberty. Three such entities, maternal cocaine abuse, valproate toxicity, and intrauterine vascular disruptive events, have been implicated as possible etiologies of septo-optic dysplasia.[524-526]

Hypothalamic and Pituitary-Dependent Hypogonadism

This section discusses disorders that involve both the hypothalamus and pituitary. Some disorders, such as adrenal hypoplasia congenita (AHC), also affect the development of the adrenal cortex. For some disorders, inconsistency of investigative reports precludes precise definition of the anatomical localization.

Adrenal Hypoplasia Congenita

Adrenal hypoplasia congenital (AHC), which is characterized by primary adrenal insufficiency and HH, is an X-linked recessive disorder due to mutations in the *DAX-1* (dosage-sensitive sex reversal, AHC-critical region of the X chromosome, gene 1 which is also known as *NROB1*) gene located on the short arm of the X chromosome.[527] In AHC, the development of the fetal zone of the adrenal cortex during fetal development and its regression during the first year of postnatal life occur normally. The adult zone of the adrenal cortex, on the other hand, fails to develop. Thus, symptoms of adrenal insufficiency in AHC generally do not manifest until after 6 to 8 weeks of age, and affected males can present in infancy or childhood with primary adrenal insufficiency. Delayed adrenarche may occur. In addition, complete or partial HH can occur. The HH reflects a combination of inconsistent hypothalamic GnRH secretion and/or abnormal pituitary responsiveness to GnRH.[527]Isolated HH due to *NROB1* mutations may occur rarely in the absence of adrenal insufficiency or a family history of hypogonadism.[528] Heterozygotic females may have delayed puberty but show normal fertility.[529]

The *NROB1* gene codes for a receptor protein expressed in the hypothalamus, pituitary, adrenal, and gonads. Because no ligand has been identified for the receptor as of yet, it is classified as an orphan nuclear receptor. Through its C-terminus transcriptional repression domain, the protein silences transcription of many genes involved in steroid hormone metabolism. The majority of mutations detected in patients with AHC cluster in the region encoding the C terminal of the protein.[530] Structure—function analysis suggests the mechanism through which *NROB1* mutations cause AHC involves abnormal folding of the receptor leading to impaired nuclear translocation and transcriptional silencing.[531] Mutations in this region of the X chromosome may be part of a contiguous gene deletion syndrome, which includes glycerol kinase deficiency and Duchenne muscular dystrophy. Phenotypic heterogeneity can occur even within a family.[532]

Intracranial Tumors

Depending on their precise location and size, intracranial tumors can suppress gonadotropin secretion either directly or indirectly, via interruption of GnRH release. Those that present before or during puberty, therefore, lead to delayed or arrested gonadarche. Deficiencies of other pituitary hormones may be present or develop at a later time. Thus, concurrent GH, TSH, ACTH, and ADH deficiencies may be present. Acquired pituitary hormone deficiencies, especially if accompanied by diabetes insipidus, suggest the presence of a CNS tumor.

The most common neoplasm is a craniopharyngioma, which is believed to arise from remnants of Rathke's pouch, the embryonic anlagen of the anterior pituitary. These tumors account for approximately 10% of childhood CNS tumors with the peak chronological age at presentation occurring between 5 and 20 years.[533] In addition to delayed pubertal development, signs and symptoms suggestive of a craniopharyngioma include decreasing growth velocity, headache, polyuria, and visual disturbance (bilateral temporal field deficits, optic atrophy, or papilledema). Suprasellar or intrasellar calcifications may be seen on skull radiographs. MRI and CT scans confirm the presence of the tumor and reveal whether it is cystic or solid. Treatment usually involves surgical resection, with outcome dependent on the size and precise location of the tumor. Radiation therapy may be used as adjunctive therapy. Recurrences are common.[534]

Other types of tumors include germ cell tumors, epidermoid and dermoid cysts, chromophobe adenomas, prolactinomas, and optic gliomas. Germ cell tumors include germinomas, dysgerminomas, embryonal cell carcinomas, and teratomas. Such tumors account for approximately 6.5% of intracranial neoplasms in children. Diabetes insipidus is a common manifestation, however, other anterior pituitary hormone deficiencies may occur. Germ cell tumors often originate in the suprasellar hypothalamus or pineal, and they may show subependymal spread along the lining of the third ventricle such that seeding of the cerebrospinal fluid leads to involvement of the lower spinal cord and cauda equina. Elevated β-hCG or α-fetoprotein concentrations may be detected secondary to secretion by the tumor cells. Usually, the response to radiation therapy is good which averts the need for surgery unless a tissue diagnosis is necessary.

Epidermoid and dermoid cysts are epithelial-lined intracranial cysts that are often located in the suprasellar region or at the cerebellopontine angle. Chromophobe adenomas are rare in children but may occur in older adolescents, leading to delayed puberty, secondary amenorrhea, or acquired testosterone deficiency. Optic nerve and chiasmatic gliomas (astrocytomas) account for approximately 5% of pediatric brain tumors and tend to present during the first 2 decades of life.[535] Although optic nerve gliomas are often associated with NF1 and precocious puberty, delayed puberty due to hypogonadotropism (with other anterior pituitary hormone deficiencies) may also occur.

Histiocytosis X

Histiocytosis X, also known as Langerhans cell histiocytosis or Hand-Schuller-Christian disease, is characterized by infiltration of lipid-laden histiocytes into skin, bone, or visceral organs. These specific dendritic cells can infiltrate almost any organ. The clinical course can be unpredictable such that spontaneous resolution or progression to a disseminated form may occur. Infiltration of the orbit can cause exophthalmos. The typical radiographic findings include cyst-like areas involving flat bones of the skull, ribs, pelvis, scapula, lower spine, and long bones of the limbs. Pituitary MRI reveals loss of the posterior pituitary bright spot and thickening of the infundibulum. Although not malignant, the histiocytes can invade adjacent tissues.

Diabetes insipidus is the most common endocrine manifestation occurring in approximately 25% of affected children, but other pituitary hormone deficiencies can occur.[536] Once established, anterior pituitary hormone deficiencies

are generally permanent. Gonadotropin deficiency may develop several years following the initial diagnosis. Thus, delayed puberty may be seen. Hypothalamic infiltration can manifest as neuropsychiatric and behavioral disorders as well as autonomic dysregulation.[537]

Hyperprolactinemia

Hyperprolactinemia can lead to hypogonadotropism as a result of the action of prolactin to directly suppress pulsatile GnRH secretion (see also Chapter 3).[538] Thus, if hyperprolactinemia occurs prior to puberty, delayed or interrupted gonadarche with galactorrhea may be observed.[539] Even if galactorrhea is not readily apparent, nipple manipulation may induce fluid release. Because prolactin release may be stimulated by nipple manipulation, evaluation of serum prolactin levels should be conducted on blood samples collected in the morning from fasting subjects prior to breast examination. Hyperprolactinemia can be due to microadenomas (adenomas measuring less than 10 mm) and macroadenomas (larger than 10 mm).[540] Although CT scans can be used to identify pituitary adenomas, MRI provides more anatomical detail. Individuals taking antipsychotic medications and patients with primary hypothyroidism may also have hyperprolactinemia.[540,541] Prolactin secretion is tonically inhibited by hypothalamic dopamine release, and dopamine receptor agonists (bromocriptine or cabergoline) are effective to decrease tumor size and prolactin concentrations.[542,543]

Steroidogenic Factor-1/NR5A1 Mutations

This gene encodes Steroidogenic factor-1 *(NR5A1/SF1)*, which plays a major role in male sexual differentiation, steroidogenesis, and development of the hypothalamus, pituitary, adrenal glands, and testes. Because inactivating mutations impair testicular differentiation and testosterone secretion, affected 46,XY individuals would be predicted to have undervirilization of the external genitalia. The phenotype reported for one 46,XY patient who was heterozygous for an inactivating mutation included undervirilization of the external genitalia (46,XY male to female sex-reversal), adrenal insufficiency in the neonatal period, and delayed puberty.[544] A 46,XX patient presented with adrenal insufficiency and apparently normal ovarian differentiation, but it is unclear whether or not she will undergo spontaneous pubertal development.[545] Haploinsufficiency of *NR5A1* can also manifest as a predominantly gonadal phenotype characterized by hypergonadotropic hypogonadism. Heterozygous mutations in *NR5A1* have been reported among patients with 46,XY partial gonadal dysgenesis and underandrogenization but normal adrenal function.[546,547] Müllerian structures are generally absent. Penoscrotal hypospadias, bilateral anorchia, and testicular regression syndrome have been described. Haploinsufficiency of *NR5A1* can also manifest associated with delayed puberty or premature ovarian failure in 46,XX individuals. More recently, the phenotypic spectrum associated with *NR5A1* mutations has greatly expanded. The phenotypic spectrum extends to include 46,XY girls who present with delayed puberty, primary amenorrhea, and low testosterone concentrations in the absence of adrenal insufficiency.[548]

Other Syndromes Associated with Delayed Puberty

Delayed puberty due to HH is observed in several unrelated syndromes such as Prader-Willi, Bardet-Biedel, Alström, and Bloom syndromes. While both premature pubarche and GnRH-dependent premature gonadarche have been reported to occur in Prader-Willi syndrome, the majority of affected boys have cryptorchidism. When spontaneous pubertal development occurs, it generally progresses slowly and is often incomplete.[549] Hormone replacement therapy may not be necessary for all patients with Prader-Willi syndrome. Prader-Willi syndrome is also characterized by short stature, hypotonia, hyperphagia, obesity, mental retardation, and decreased fetal movements. It is a contiguous gene deletion syndrome involving loss of the paternal allele or uniparental disomy of the maternal allele for several genes, including the necdin and small nuclear ribonucleoprotein polypeptide N *(SNRPN)* genes located in the region of chromosome 15q11-13.[550]

The Bardet-Biedl syndrome is a rare autosomal recessive ciliopathy. Typical clinical features include include retinal dystrophy, dystrophic extremities (polydactyly, syndactyly, brachdactyly), mental retardation, learning difficulties, hypogonadism, and obesity.[551] Affected females may have structural abnormalities involving the internal genitalia, and they may experience hydrometrocolpos.[552] Inheritance is autosomal recessive with linkage to least 14 distinct loci.[553]

Alström syndrome is a progressive autosomal-recessive disorder characterized by cone–rod retinal dystrophy, cardiomyopathy, hypertriglyceridemia, and type 2 diabetes mellitus. Hypogonadism is an inconsistent feature. Weight gain leading to obesity begins during early childhood. Affected males often have small external genitalia and testicular atrophy with obliterating fibrosis of seminiferous tubules.[554] The reproductive phenotype in females ranges from polycystic ovary-like features presumably related to the insulin resistance to abnormal breast development, precocious puberty, endometriosis, irregular menses, or amenorrhea. The syndrome is due to mutations in the *ALMS1* gene mapped to chromosome 2p13. The gene is widely expressed; the protein appears to be involved in ciliary function, cell cycle control, and intracellular transport.[555]

Bloom syndrome is an autosomal-recessive disorder characterized by impaired prenatal and postnatal growth, predisposition to neoplasia, chromosomal instability, and hypogonadism due to inactivating mutations in the gene coding for DNA helicase RecQ protein-like-3 *(RECQL3)* which is located at chromosome 15q26.1. Men have azoospermia and are infertile and women are subfertile due to premature ovarian failure.

Hereditary hemochromatosis is associated with iron overload and iron deposition in endocrine organs. The typical endocrine manifestations include HH, impaired glucose tolerance, and diabetes mellitus. Mutations in several different genes are associated with the iron overload phenotype.[556] Iron accumulation in endocrine disorders is responsible for the clinical endocrine manifestations which include hypogonadism, diabetes, and hypothyroidism.

Gonadal Causes of Delayed Puberty (Primary Gonadal Failure)

Primary gonadal failure is associated with hypergonadotropic hypogonadism (see also Chapter 17). Etiologies include sex chromosome abnormalities leading to aberrant differentiation of the gonads (gonadal dysgenesis), absence of the

Table 18.4 Monogenic Disorders Associated with Hypergonadotropic Hypogonadism

Gene	*Locus*	*Associated Phenotypes*
WT1	11p13	Deny-Drash and Fraser syndromes
SRY	Yp11.3	Gonadal dysgenesis
SOX9	17q24.3	Campomelic dysplasia (loss of function)
SOX9	17q24.3	Sex reversal (duplication)
SOX10	22q13	Sex reversal
SOX3	Xq27.1	Sex reversal (over-expression)
RSPO1	1p34	Sex reversal
AIRE	21p22.3	Auto-immune polyendocrinopathy candidiasis ectodermal dystrophy
FOXL2	3q23	Blepharophimosis ptosis epicanthus inversus syndrome
NOBOX	7q35	Premature ovarian failure
FIGLA	2p13.3	Premature ovarian failure
BMP15	Xp11.22	Premature ovarian failure
FMR1	Xq27.3	Premature ovarian failure
LHCGR	2p16.3	Leydig cell hypoplasia
FSHR	2p16.3	Premature ovarian failure, oligospermia
StAR	8p11.23	Congenital lipoid adrenal hyperplasia
CYP17A1	10q24-q25	Hypertension, glucocorticoid deficiency, delayed puberty
AKR1C2/AKR1C4	10p15.1	Under-virilized males
CYP19A1	15q21.2	Estrogen deficiency
HSD17B3	9q22.32	46,XY Sex reversal with virilization at puberty
SRD5A2	2p23.1	46,XY Sex reversal with virilization at puberty
AR/NR3C4	Xq11-12	Androgen insensitivity

gonads, and injury to the gonads (Table 18.4). The hypergonadotropism associated with primary gonadal failure and with disorders of steroidogenesis or steroid hormone action is a result of loss of negative feedback inhibition by gonadal steroids at both the hypothalamic and pituitary level.

Gonadal Dysgenesis

Gonadal dysgenesis is used to describe disorders in which gonadal differentiation is aberrant (see also Chapter 17). Gonadal dysgenesis can be due to haploinsufficiency for the X chromosome (e.g., Turner syndrome). Other forms may result from mutations in genes involved in the process of sexual differentiation.

Turner Syndrome

Turner syndrome (TS) is due to deletions or structural rearrangements of the X chromosome. The reported incidence of liveborn females with this form of gonadal dysgenesis is 1:2000 to 1:5000.[557] Initially, the process of ovarian differentiation proceeds normally in fetuses with TS. During the 4th week of gestation, the primitive germ cells begin to migrate from their extra-gonadal site of origin to the genital ridge. Premature degeneration of ovarian follicles begins by 18 weeks of gestation. Ovarian follicles are replaced by abundant connective tissue (streak gonad). This accelerated prenatal and postnatal follicular atresia is generally associated with ovarian failure; the majority of girls with TS do not spontaneously enter puberty. Nevertheless, 25% to 30% of girls with TS show partial pubertal development at the appropriate time, and 2% to 5% may have spontaneous menses. As anticipated with primary ovarian failure, LH and FSH concentrations show a biphasic pattern (Fig. 18.6) with elevated concentrations during the first year of life and again at the expected age of puberty. For this reason, gonadotropin concentrations obtained between ages 5 to 9 years old cannot be reliably used to assess ovarian function.

Spontaneous pubertal development is more common among girls with mosaic karyotypes. AMH concentrations have been correlated with ovarian function. In general, AMH values less than 8 pmol/L have a high sensitivity for predicting ovarian failure. AMH concentrations appear to be a marker of ovarian function in girls, adolescents, and adult women with TS.[558] Low inhibin B concentrations may be another indication of ovarian failure.[559]

Short stature is a characteristic feature of TS.[560] Approximately 95% of individuals with TS have short stature. Common skeletal features include cubitus valgus, shortened fourth metacarpals, short neck, high arched palate, and Madelung deformity. Scoliosis may develop during the adolescent years.[561] Haploinsufficiency for the short stature homeobox-containing *(SHOX)* gene, located in the pseudoautosomal region of the X chromosome, contributes to the short stature and skeletal abnormalities associated with TS.[562] Because the majority of patients with TS present with short stature, exclusion of this form of gonadal dysgenesis is essential in the evaluation of all girls with short stature.[563] Bone mineral density is decreased with bone markers, indicating both increased resorption and normal to decreased bone formation.[564]

Aberrant development of the lymphatic system, especially in the nuchal region, is associated with a short webbed neck, peripheral lymphedema, and low posterior hairline. Additional features include bilateral otitis media, sensorineural hearing loss, cardiac malformations (especially of the left side of the heart), renal abnormalities, hypoplastic and hyperconvex nails, and multiple pigmented nevi. Phenotypic heterogeneity is common among girls with TS. Only about 25% of affected girls are diagnosed with TS as infants or toddlers.

Mosaicism with coexistence of multiple cell lines is not uncommon, although detection may require evaluation of multiple tissues. It has been speculated that mosaicism in critical cell lines is essential for fetal viability.[565,566] If the clinical suspicion for TS is high despite a normal peripheral blood karyotype, it may be worthwhile to examine a second tissue, such as skin. When a cell line containing a Y chromosome is present, the external genital phenotype may vary from female to ambiguous to male and the gonadal structure can range from a streak to a functional testis. In some instances, Y chromosome-specific sequences can be detected using sensitive molecular techniques.[567] The requirement to assess for the SRY gene or other Y chromosomal material remains controversial for girls with TS without any signs of virilization.[568] Girls with TS and evidence of virilization or presence of a marker chromosome should be thoroughly evaluated for presence of Y chromosomal material. If Y chromosomal sequences are present, the risk for development of gonadoblastoma is estimated to be 7% to 10%.[569] Such gonadoblastomas may secrete testosterone or estrogens and become calcified, but rarely become malignant.

Girls with Turner syndrome tend to have normal verbal abilities. As a group, however, they demonstrate specific deficits in visuospatial processing, visuoperceptual skills, motor function, and nonverbal memory compared with normal girls. These deficits may be associated with one or more deletions of a critical region in the pseudoautosomal region of the X chromosome.[570] For example, some girls with Turner syndrome may experience difficulty with interpretation of body language and facial expressions, trouble in maintaining relationships with peers, reading maps, and geometry. Although most deficits persist into adulthood, some cognitive deficiencies improve either due to a maturational or developmental lag, or due to effects of estrogen therapy. Nevertheless, the neurocognitive deficits, psychosocial issues, and school difficulties can lead to frustration and poor self-esteem.[571] Mental retardation is typical for girls with ring X chromosomes lacking the *XIST* gene (the gene responsible for X inactivation) because one copy of certain genes is not activated.[572]

Prior to the use of GH treatment, girls with TS who did not receive GH therapy achieved an average adult stature approximately 20 cm shorter than normal. The growth deficit of girls with Turner syndrome can be partially ameliorated by GH treatment. Earlier treatment and use of GH doses of 0.36 mg/kg per week are associated with improved final height outcome.[573] One study showed a modest growth benefit and improved final height outcome with the combination of ultra-low-dose childhood estrogen replacement and growth hormone.[574] Estrogen replacement therapy is generally necessary to induce pubertal development.[575] Yet, despite adequate estrogen replacement therapy, uterine maturation may be incomplete and may contribute to a high rate of miscarriage in both spontaneous and assisted pregnancies among women with TS.[576]

Girls with TS have an increased risk of chronic lymphocytic thyroiditis; thyroid function studies should be obtained regularly. Screening for renal structural abnormalities, skeletal malformations (congenital dislocated hips, Madelung deformity, scoliosis), hypertension, diabetes mellitus, and celiac disease is also necessary.[577] At diagnosis, a thorough cardiac evaluation is essential to ascertain for specific cardiovascular abnormalities such as coarctation of the aorta, bicuspid aortic valve, or mitral valve prolapse. Prophylactic antibiotic therapy is generally indicated if bicuspid aortic valve or mitral valve prolapse are detected. Repeat echocardiograms for adolescent and young adult women are necessary to evaluate for changes (dilatation) of the aortic root because of the increased risk of aortic dissection.[578] Although echocardiology may be sufficient for some, MRI with gadolinium provides better visualization of the entire aortic arch.[561]

Available data have led to reconsideration of oocyte donation and pregnancy in women with TS. In the past, adolescent girls were informed that reproductive options included adoption and oocyte donation. At the present time, counseling mandates frank and brutally honest discussions of the risk for aortic dissection during, or years after, pregnancy. As noted by the Practice Committee of ASRM, "Turner syndrome is a relative contraindication for pregnancy, and patients should be encouraged to consider alternatives, such as gestational surrogacy or adoption."[579] With the vast diversity of clinical features, a multidisciplinary approach is essential for the long-term clinical management of girls with TS.[580]

46,XY Gonadal Dysgenesis

Aberrant testicular differentiation in 46,XY fetuses is one cause of gonadal dysgenesis (see also Chapter 17). Because the degree of gonadal dysgenesis and testosterone deficiency vary, the external genital development can range from normal female external genitalia to undervirilization of male external genitalia. In "complete" or "pure" 46,XY gonadal dysgenesis, affected individuals appear to be normal females at birth. This disorder of gonadal differentiation is also known as Swyer syndrome or 46,XY sex reversal disorder of sexual differentiation. Affected individuals usually present with delayed puberty and primary amenorrhea; they are usually found to have streak gonads. The internal genital structures are usually female due to prenatal testosterone and AMH deficiencies. "Partial" or "incomplete" 46,XY gonadal dysgenesis is characterized by defective testicular differentiation, ambiguous external genitalia, and a combination of wolffian and müllerian duct structures. Compared to healthy controls, women with complete gonadal dysgenesis were taller and had smaller uterine sizes.[581]

Patients with 46,XY sex reversal have an increased risk of gonadoblastoma because the dysgenetic gonad carrying a Y chromosome has an increased risk for neoplastic changes. Although gonadal tumors typically do not develop until the second decade of life, they can occur earlier. Indeed, some patients present with the gonadal tumors.[582] In one series of 22 patients, 32% were found to have dysgerminoma and 14% had gonadoblastoma.[581] Spontaneous breast development in patients with gonadal dysgenesis may be secondary to estrogen secretion by these gonadal tumors.[583,584] Early diagnosis is beneficial to detect gonadal neoplasia and to initiate hormone replacement therapy.

Laboratory evaluation shows elevated gonadotropin concentrations, low testosterone concentrations, and 46,XY karyotype. Approximately 15% of patients with 46,XY gonadal dysgenesis have mutations in the testis-determining *SRY* gene. Most *SRY* mutations are *de novo* events. Curiously, some mutations are familial and present in unaffected fathers.[585,586] Because the majority of patients with 46,XY sex reversal do not have mutations in the *SRY* gene, it is presumed that they carry mutations involving other genes either upstream or downstream of *SRY* in the process of sexual differentiation.

Other Forms of Gonadal Dysgenesis

Denys-Drash and Frasier syndrome are associated with gonadal dysgenesis. Both disorders are due to heterozygous mutations in the Wilms' tumor-1 *(WT1)* gene.[587,588] Denys-Drash syndrome is characterized by 46,XY male to female sex reversal, Wilms' tumor, and chronic renal failure due to diffuse mesangial sclerosis. In addition to 46,XY male-to-female sex reversal and renal disease, patients with Frasier syndrome have a higher incidence of gonadoblastomas than patients with Denys-Drash syndrome. Patients with either of these syndromes and 46,XX karyotypes tend to have normal female external genitalia.

Ovotesticular disorder of sexual differentiation (previously known as true hermaphroditism) is characterized by

the concurrent presence of testicular tissue with seminiferous tubules and ovarian tissue with follicles. Although an ovotestis is the most commonly identified gonad, an ovary may be present on one side with a testis on the other side. The development of müllerian and wolffian duct derivatives is variable and depends on the amount of functional testicular tissue. Patients with true hermaphroditism are usually recognized in infancy due to genital ambiguity. The most frequent karyotypes are 46,XX (60%), mosaic 46,XX/46,XY (33%), and 46,XY (7%).[589]

Heterozygous inactivating mutations in the *SOX9* gene are associated with campomelic dwarfism and 46,XY male-to-female sex reversal.[590] Because some of the related skeletal abnormalities (such as small thoracic cage, undermineralized ribs, and tracheobronchomalacia) can cause respiratory distress, affected individuals often die early. However, milder forms can present with delayed puberty due to primary gonadal failure.[591] Another form of gonadal dysgenesis is 46,XX gonadal dysgenesis, which is characterized by female internal and external genital structures, delayed puberty, and streak gonads.

46,XX Males

The frequency of the XX male syndrome is approximately 1 in 25,000 males. Since 46,XX males often have normal male external genital development, they typically seek medical attention for delayed puberty, infertility, or gynecomastia. On physical examination, the testes are usually small. Some present in the newborn period with genital ambiguity or hypospadias.

This form of a disorder of sex development (DSD) can be subclassified into *SRY*-positive and *SRY*-negative groups.[592] In approximately 80% of 46,XX males, the *SRY* gene is detected by molecular genotype analysis. When the *SRY* gene is present, it is usually located on an X chromosome due to recombination between the X and Y chromosomes. However, translocation to an autosome can occur. The *SRY*-positive 46,XX males generally have normal male external genitalia, small azoospermic testes, hypergonadotropic hypogonadism, and often present with infertility.

Molecular etiologies are more diverse for the *SRY*-negative 46,XX patient. Overexpression of the *SOX10* gene at 22q13 was found in one patient with 46,XX sex reversal in association with multiple congenital anomalies.[593] Duplication of *SOX9* was described in one family; all affected family members had normal male secondary sexual characteristics and azoospermia.[594] Overexpression of the *SOX3* gene has also been associated with XX male sex reversal.[595] The R-spondin 1 *(RSPO1)* gene codes for a secreted furin-like domain protein that stabilizes β-catenin in the wingless-type mouse mammary tumor virus integration site-4 (WNT4) signaling pathway. Mutations in this gene are associated with 46, XX sex reversal.[596]

Klinefelter Syndome (KS)

KS is a chromosomal abnormality characterized by 47,XXY karyotype and primary testicular failure that may manifest before or after puberty (see also Chapter 17). Parental origin of the supernumerary X is approximately equal regarding maternal and paternal origin.[597] Gonadarche may be variable ranging from normal to absent. In one study, the incidence of KS was 1 in 667 males when diagnosed by prenatal cytogenetic analysis, whereas the incidence was much lower when patients were detected by clinical features alone. Thus, incomplete clinical ascertainment of patients with KS likely occurs.[598] Clinical features include gynecomastia, small firm testes, clinodactyly, and tall stature with disproportionate increase in limb lengths. Affected boys may have reduced penile length and small testes in childhood. The phenotype in boys with KS does not differ according to presenting clinical features or parental origin of the extra X chromosome.[599] It has been suggested that the extra copy of the short stature homeobox-containing *(SHOX)* gene located in the pseudoautosomal region on the short arm of the X chromosome contributes to the increased height in boys with KS.[600]

The initial development of the testis during gestation appears to be comparable to normal boys. Although the incidence of cryptorchidism and micropenis are slightly increased, anomalies of external genital development are uncommon.[601] KS affects both androgen secretion (Leydig cells) and spermatogenesis (Sertoli cells and germ cells). As would be anticipated, gonadotropin concentrations are elevated and testosterone concentrations tend to be low. Inhibin B concentrations tend to be normal during the prepubertal years but decline during puberty consistent with progressive testicular failure at the time of gonadarche.[602] AMH concentrations follow a similar pattern, but show a slower decline during puberty.

Affected boys may experience academic difficulties. The cognitive phenotype is characterized by deficits in language and executive function. Although IQ scores are generally in the normal range, boys with KS tend to have learning disabilities in arithmetic, reading, and spelling, which become more apparent and limiting over time.[603] Indeed, the learning disabilities may be the presenting clinical feature for some boys. Additional nonreproductive features include an increased incidence of mediastinal germ cell tumors, breast cancer, and vascular disease.[603] Patients with KS tend to have altered body composition with increased total body and truncal fat and risks to develop type 2 diabetes mellitus appear to be increased.[604,605]

The magnitude of primary testicular failure varies between patients. Goals of treatment include preservation of linear growth velocity, development of secondary sexual characteristics, increased muscle mass, and optimal bone mineral density. Hence, dosage and age at initiation of hormone replacement therapy need to be individualized.

Fertility preservation is an important topic to discuss with patients and their families. Paternity has been achieved for some using testicular sperm extraction combined with intracytoplasmic sperm injection (TESE-ICSI).[606] One reason for the success of this procedure is that foci of spermatogonia seem to exist within the testes. It has been speculated that these foci arise from clones of spermatogonia that have lost one of the X chromosomes.[607] Among men with KS undergoing TESE, testicular volume, testosterone concentrations, and hCG-stimulated testosterone responses correlated with sperm production.[608] It has been suggested that spermatogenesis decreases with age in men with KS. This hypothesis has been used to justify collection of testicular tissue from younger boys with hopes for *in vitro* sperm maturation. However, this approach is currently considered to be experimental and may prove to be

deleterious by removing the few functional spermatogonia resident in the testis.[609]

Testicular Regression Syndrome

This disorder, also known as "vanishing testis syndrome," affects at least 5% of boys who present with cryptorchidism. Typically, the external genitalia are male, but one or both testes cannot be identified. Rather, a nubbin of discrete vascularized fibrosis usually located in close proximity to a blind-ending spermatic cord structure is found at the time of orchidopexy. Histological examination often reveals calcification or hemosiderin deposits.[610] Often attributed to testicular torsion, the precise etiology of this condition responsible for primary testicular failure remains to be established. A novel heterozygous missense mutation (Val-355Met) in the steroidogenic factor-1 *(NR5A1)* gene was identified in a single boy with a micropenis and testicular regression syndrome.[611]

Auto-Immune Ovarian Failure

Autosomal-recessive type I autoimmune polyglandular syndrome (APS-1), also known as auto-immune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), is characterized by mucocutaneous candidiasis, hyperparathyroidism, and adrenal insufficiency.[612] APS-1 is due to mutations in the *AIRE* gene.[613] Ovarian failure is common in APS-1 and may be associated with autoantibodies to the P450 steroidogenic enzymes expressed in the gonads and adrenal glands such as 3β-hydroxysteroid dehydrogenase.[614] Auto-immune testicular failure may also occur. Women with autoimmune Addison disease, particularly in association with APS-1, manifest an increased risk for premature ovarian failure.[615] The incidence of auto-immune primary gonadal failure is low in APS-2, which is characterized by immune-mediated type 1 diabetes mellitus, chronic lymphocytic thyroiditis, vitiligo, and pernicious anemia.[613] Some researchers have suggested that, in unexplained infertility, ovarian antibodies are an independent marker of ovarian failure that may precede changes in FSH and inhibin B concentrations.[616]

Nonimmune Premature Ovarian Failure

With increasing awareness that *in utero* ovarian development is an active process, disorders due to mutations in genes involved in this process may manifest as delayed puberty, primary amenorrhea, or premature ovarian failure (POF).[617] Genes associated with a POF phenotype include Forkhead box L2 *(FOXL2)*, Newborn Ovary Homeobox *(NOBOX)*, Factor in germline alpha *(FIGLA)*, and bone morphogenetic protein 15 *(BMP15)*.[618] The autosomal dominant blepharophimosis ptosis epicanthus inversus syndromes (BPES) is associated with mutations in *FOXL2*,[619] BPES is characterized by eyelid dysplasia consisting of small palpebral fissures (blepharophimosis), ptosis, epicanthus inversus, and a broad nasal bridge. Two BPES phenotypes have been described. The type 1 consists of eye findings and POF. Patients with BPES type 2 manifest only the eye features. Patients heterozygous for *FOXL2* mutations generally experience spontaneous pubertal development culminating in menarche, but undergo premature follicle depletion leading to POF. An increased number of CGG trinucleotide repeats ranging from 55 to 200 repeats in the fragile X gene *(FMR1)* is associated with POF.

Noonan Syndrome

Clinical features of Noonan syndrome include short stature, hypertelorism, short webbed neck, mental retardation, bleeding disorders, and right-sided cardiac anomalies. The estimated incidence ranges from 1:1000 to 1:2500 live births. Though inherited in an autosomal-dominant manner, 60% of cases are sporadic.[620] Cryptorchidism is common in affected males. Noonan syndrome is not a homogenous entity; patients with Noonan syndrome are found to carry mutations in genes within the RAS/MAPK pathway. At least 50% of cases have missense mutations in the *PTPN11* gene, which encodes the nonreceptor protein tyrosine phosphatase SHP2.[621] Other genes in the RAS-MAP kinase pathway found to be associated with Noonan syndrome include *KRAS*, *SOS1*, NRAS, *MEK1*, *BRAF*, *SHOC2*, and *NF1*.[622] Reproductive function tends to be normal in women. Postpubertal men tend to have elevated LH and FSH concentrations and declining AMH and inhibin B concentrations suggesting that Sertoli and/or germ cell function progressively deteriorates, resulting in impaired spermatogenesis and subfertility.[623]

Galactosemia

Galactosemia is an autosomal-recessive metabolical disorder characterized by abnormal galactose metabolism. Three different genes encode proteins involved in galactose metabolism: galactokinase *(GALK)*, galactose-1-phosphate uridyl transferase *(GALT)*, and UDP-galactose 4′epimerase *(GALE)*. Mutations in any of these genes can result in human disease. Affected women have elevated FSH concentrations and ovarian failure.[624] AMH concentrations may be useful to assess ovarian reserve in women with galactosemia.[625] Affected males appear to have normal testicular function.[626]

Down Syndrome

The occasional child with Down syndrome presents with abnormal pubertal development. Normal uterine size and the presence of follicles in ovaries have been documented.[627] However, for both sexes, gonadal failure with elevated gonadotropins may develop in adulthood.[628] For counseling purposes, women with Down syndrome can conceive, but affected men tend to be infertile. Children with Down syndrome have an increased risk for other chronic disorders, which may affect onset and progression of puberty. These disorders include hypothyroidism, celiac disease, and diabetes mellitus.[629]

Defects in Steroidogenesis or Steroid Hormone Action

LH-R Gene Mutations

In males, inactivating *LHCGR* mutations impair testosterone biosynthesis; such patients may present with delayed puberty (see Chapter 2). Inherited as an autosomal-recessive disorder, the most severe form is characterized by Leydig cell hypoplasia, testosterone deficiency, and 46,XY male-to-female sex reversal. Müllerian duct derivatives are absent. Milder forms may present with hypogonadism in 46,XY

individuals. The typical presentation for 46,XX individuals who carry severe inactivating mutations of this gene is the absence of puberty with primary amenorrhea, which may be associated with LH hypersecretion, cystic ovaries, and infertility.[630,631] A 46,XY patient who presented with delayed puberty was found to have a homozygous deletion of exon 10. Functional analysis to determine the consequences of the mutation showed that despite being able to bind LH, the mutant receptor was less effective at stimulating increased cAMP production than the normal receptor.[632]

FSH-R Gene Mutations

Although inactivating mutations of the *FSHR* gene are associated with primary ovarian failure and infertility, the development of secondary sex characteristics is variable in this autosomal-recessive disorder (also see Chapter 2).[633-635] While pubarche appears normal, breast development varies from absent to normal. Menarche is typically absent, although early secondary amenorrhea is occasionally observed. The variation in phenotype would suggest that the FSHR is not completely inactivated and that low levels of estradiol may be secreted. The phenotype is less severe in males, and pubertal development has occurred in those cases reported to date.[636] Testicular volume and sperm count, however, were decreased.

Congenital Lipoid Adrenal Hyperplasia

Mutations in the *StAR* gene are the molecular basis of congenital lipoid adrenal hyperplasia (see also Chapter 4). Consequently, sustained trophic hormone stimulation of the adrenal and the testis or ovary leads to the accumulation of cholesterol esters and cholesterol auto-oxidation in the cytoplasm of steroidogenically active cells. Cell destruction ensues because the accumulated lipid droplets lead to both physical engorgement and chemical damage from the cholesterol auto-oxidation products. Thus, the phenotype of congenital lipoid adrenal hyperplasia reflects the consequences of two separate events—the inactivating mutation and subsequent cellular damage.[637] These two hits interfere with fetal testicular testosterone biosynthesis, leading to androgen deficiency and undervirilization of affected 46,XY fetuses. Yet, despite the genetic mutation in the *StAR* gene, StAR-independent steroidogenesis allows for normal placental steroidogenesis and low, but measurable, adrenal steroid concentrations in the immediate newborn period. Subsequent cellular damage, however, leads to complete loss of adrenal steroidogenesis. Because the fetal, neonatal, and prepubertal ovary are relatively quiescent, ovarian lipid accumulation is minimal at these stages of development, and gonadarche may be initiated in affected 46,XX individuals.[638] Milder non-classical forms of congenital lipoid adrenal hyperplasia have been reported.[639]

17α-Hydroxylase or 17,20-Lyase Deficiency

The clinical features of decreased 17α-hydroxylase or 17,20-lyase activity, resulting from mutations in the *CYP17A1* gene can include glucocorticoid and sex steroid hormone deficiencies (see also Chapters 4 and 17). Because mineralocorticoid biosynthesis is unaffected, patients may have hypertension and/or hypokalemia due to increased aldosterone production. Affected males may present with complete or partial male-to-female sex reversal due to impaired testosterone secretion. Affected females have normal female internal and external genitalia; they typically present with delayed puberty secondary to inadequate estrogen production.[640]

In some patients, the lyase activity of the enzyme is more impaired than its hydroxylase activity. The molecular basis for this differential effect appears to be related to the consequences of mutations in specific domains of the mature protein such that mutations in the steroid-binding domain are associated with deleterious effects on both activities. The 17,20 lyase activity is particularly sensitive to interactions with the accessory proteins P450 oxidoreductase and cytochrome b_5.[641] Documented isolated 17,20-lyase deficiency is extremely rare.[642]

AKR1C2 and AKR1C4 Deficiencies

Genetic investigation of the original Swiss families purported to have isolated 17,20-lyase deficiency uncovered the importance of the alternative "backdoor pathway" for normal male sexual development. Through this pathway, DHT can be synthesized without testosterone as an intermediary hormone. Dihydrodiol dehydrogenase type 2 (AKR1C2) and dihydrodiol dehydrogenase type 4 (AKR1C4) are members of the aldo-oxo reductase (AKR) superfamily. Loss of function mutations in these genes were identified in these Swiss families.[643] Adrenal insufficiency and virilisation at puberty do not occur.

Aromatase Deficiency

Aromatase deficiency is an autosomal recessive disorder due to inactivating mutations in the aromatase *(CYP19A1)* gene (see Chapter 4).[644] The major consequence of decreased aromatase activity is impaired conversion of androgens to estrogens. Affected female infants may present with genital ambiguity due to excessive androgen exposure *in utero*. Subsequently, affected females show absence of breast development, primary amenorrhea, estrogen deficiency, progressive virilization, and hypergonadotropic hypogonadism.[645] From the small number of male patients who have been reported with this mutation, gonadarche appears to be initiated and male secondary sexual characteristics are normal.[646] When semen analysis has been performed, oligozoospermia or azoospermia has been noted. The cardinal feature of the mutation in the male is delayed bone maturation and decreased bone mineralization. Interestingly, women pregnant with affected fetuses can virilize due to impaired placental aromatase activity leading to hyperandrogenism.

17β-Hydroxysteroid Dehydrogenase Deficiency

In this autosomal-recessive disorder, testicular conversion of androstenedione to testosterone is impaired secondary to mutations in the *HSD17B3* gene (also see Chapters 4 and 17). Affected 46,XY individuals show varying degrees of external genital ambiguity at birth ranging from microphallus to perineoscrotal hypospadias with bilateral labial gonads. Affected 46,XY individuals have hypoplastic Wolffian duct development. Progressive virilization occurs at puberty, which is presumed to be due to increased peripheral conversion of sex steroids to testosterone. Affected individuals, raised as females, may convert to male gender

roles as puberty progresses. Appropriate gender assignment can be made when the diagnosis is made in the neonatal period. Phenotypic heterogeneity even within a single family has been reported.[647] Although increased basal androstenedione: testosterone ratios are anticipated, hCG stimulation and molecular genetic analysis may be necessary to confirm the diagnosis.

5α-Reductase Deficiency

In specific androgen target cells, such as the developing external genitalia and prostate, testosterone is converted to DHT by 5α-reductase type 2 (also see Chapters 4 and 17). In this autosomal-recessive disorder, the conversion of testosterone to DHT is impaired due to mutations in the 5α-reductase type 2 *(SRD5A2)* gene. This disorder is prevalent in the Dominican Republic; southern Lebanon; eastern highlands of Papua, New Guinea; and southern Turkey.[648] Affected 46,XY individuals show phenotypic heterogeneity with varying degrees of male-to-female sex reversal at birth.[649] Typically, at birth, they have perineoscrotal hypospadias, bilateral labial gonads, and absence of müllerian structures. At puberty, affected individuals virilize with phallic enlargement and development of pubic hair. When initially raised as girls, some affected individuals change to a male gender when virilization occurs. Spermatogenesis is usually impaired. Females homozygous for *SRD5A2* mutations generally have no reproductive symptoms.

Although random testosterone to DHT ratios may be elevated, hCG-stimulation testing may be necessary to confirm the diagnosis. Urinary steroid profiles with measurement of specific metabolites (androsterone/aetiocholanolone, 5α-tetrahydrocortisol/tetrahydrocortisol, and 5β-tetrahydrocorticosterone/tetrahydrocorticosterone) may be helpful to screen for 5α-reductase deficiency for females who present with primary amenorrhea, 46,XY karyotype, mild virilisation, and absent uterus.[650]

Androgen Receptor (AR) Gene Mutations

The phenotype of 46,XY individuals with mutations in the *AR* gene varies from male-to-female sex reversal to male infertility (also see Chapter 17). Inheritance is X-linked recessive; the *AR* gene is located on the X chromosome. This gene is also known as *NR3C4*. Similar to other members of the steroid and thyroid nuclear hormone receptor family, AR consists of a modular structure with ligand binding, DNA binding, N-terminal regulatory, and hinge region domains. Over 500 mutations have been reported in the *NR3C4* gene.[651] Mutations in the ligand-binding domain interfere with androgen binding. Although ligand binding is normal if the mutation is located in the DNA binding domain, the AR activity is impaired.

Individuals with complete androgen insensitivity have female external genitalia with labial masses or undescended testes. Müllerian-derived structures are absent because testicular AMH secretion *in utero* is normal. They are often considered to be females at birth and can present with either inguinal hernias in childhood or delayed menarche with sparse sexual hair development in adolescence. Typically, in the adolescent girl presenting with primary amenorrhea, LH and testosterone concentrations are elevated. FSH and inhibin B concentrations are usually normal. As noted previously, infants with CAIS often lack the neonatal testosterone surge. Recommendations for interventions upon the unanticipated finding of a gonad during a hernia repair in a female child include biopsy of the gonad, leaving the gonad *in situ*, obtaining a karyotype and other appropriate hormone measurements, and detailed discussion with the parents.

If the diagnosis of CAIS is confirmed, therapeutic options in the child include gonadectomy or leaving the gonad intact to allow for spontaneous breast development. The risk for neoplastic degeneration of the gonad is low in childhood.[652] Females with CAIS have a blind vaginal pouch and, generally, vaginal dilation is the only therapy needed to create a functional vagina.[653]

Full disclosure and psychosocial support are fundamental to the management of females with CAIS. From the first discussion regarding inheritance, it is important to realize that mothers of girls with CAIS may feel guilty for transmitting this X-linked disorder. Topics for the dialogue with the patient and her parents include implications of an XY karyotype, status of the testes, infertility, and sexual function. Although this information has been discussed at diagnosis with the parents, repeated age-appropriate discussions benefit the child. The complexity of the information disclosed increases as the child matures. A reasonable goal is to complete education about AIS by midadolescence.[654]

In partial androgen insensitivity, the phenotype is often that of an undervirilized male. For partial androgen insensitivity, presenting complaints can include gynecomastia, decreased sexual hair, hypospadias, cryptorchidism, small penis, and infertility. Androgen treatment may be beneficial in the adolescent male.[655]

Even within a family, phenotypic heterogeneity can occur.[656] In some instances, phenotypic heterogeneity appears to be secondary to somatic cell mosaicism.[657] No mutation in the coding region of *NR3C4* has been identified for some patients with prototypic features of AIS. Some researchers have speculated that these cases of apparent androgen insensitivity may be due to abnormalities of receptor cofactors or due to mutations in distant regulatory regions of *NR3C4* gene.[658]

Mayer-Rokitansky-Kuster-Hauser Syndrome

This congenital disorder is characterized by anatomical abnormalities of müllerian duct derivatives with prevalence reported between 1 in 4000 to 1 in 10,000 female patients. The disorder is due to abnormal development of the müllerian duct derivatives during the first trimester. Despite much investigation, the molecular etiologies have not been elucidated apart from a few women found to have *WNT4* mutations or duplications of the *SHOX* gene.[659,660] Affected patients usually have normal ovarian function such that breast development occurs normal. These patients typically present with primary amenorrhea or cyclic abdominal pain. Müllerian duct remnants may be palpable on bimanual recto-abdominal examination as a midline band of tissue. While ultrasound and MRI are helpful, laparoscopy may be needed to confirm the diagnosis.[661-663] Renal abnormalities especially ectopic kidneys or unilateral renal agenesis and skeletal abnormalities may be present. Vaginal development is variable, and often, vaginal dilation is helpful to enlarge the vagina for intercourse. Successful outcome of vaginal dilation depends on the patient's motivation and compliance.

Gynecomastia

Gynecomastia is the presence of palpable breast tissue in males. Approximately 50% of boys experience gynecomastia during midpuberty. Pathological causes of gynecomastia in boys include alterations in steroidogenesis such as Klinefelter syndrome, disorders of androgen biosynthesis, and androgen insensitivity. Other etiologies include hormone exposures, drug exposures, hyperprolactinemia, and other chronic disorders. Increased endogenous estrogen exposure can be due to hormone secreting tumors or the familial aromatase excess syndrome. Chronic medical conditions associated with gynecomastia include hepatic cirrhosis, renal failure, and hyperthyroidism. Drugs associated with gynecomastia include marijuana, cimetidine, spironolactone, and digitoxin.[664]

Chemotherapy and Radiation Therapy

As over 75% of young cancer patients are long-term survivors, quality of life concerns have become increasingly important considerations for adolescents and young adults especially regarding fertility. Chemotherapy and radiation therapy for childhood cancers can affect multiple components of the hypothalamic-pituitary-gonadal axis. The specific consequences depend on the specific drugs and fields of irradiation. The germ cells of the gonads are particularly vulnerable to radiation. For example, spermatogenesis is more sensitive to the effects of low-dose irradiation (greater than 1 or 2 Gy) than Leydig cell steroidogenesis (approximately 12 Gy).[665,666] Chemotherapeutic agents can be classified by gonadotoxicity into high, medium, and low-risk categories. High-risk drugs include alkylating agents such as cyclophosphamide which can injure germ cells.[667] Alkylating agents can generate bonds between DNA strands disrupting cell division. The net result is destruction of oocytes and follicular depletion in females and oligo or azospermia in males.[668]

CNS irradiation may lead to gonadotropin deficiency and other pituitary hormone deficiencies. Boys with brain tumors who received cranial irradiation and adjuvant chemotherapy have an increased risk for HH. In such patients, low inhibin B concentrations suggest damage to the seminiferous tubules and impaired spermatogenesis.[669] Pituitary hormone deficiencies may develop many years after CNS irradiation necessitating long-term follow-up of childhood cancer survivors.

Because the indications for bone marrow transplant are expanding to include malignant and nonmalignant hematologic disorders, solid tumors, and inherited metabolical disorders, more children are undergoing this procedure. After total body irradiation and bone marrow transplant, boys tend to have normal pubertal development whereas girls tend to have delayed puberty. However, low serum testosterone and elevated FSH concentrations among boys suggest that the prognosis for fertility is poor.[670] Other anterior pituitary hormones, in addition to the gonadotropins, can be affected by cancer treatment in childhood.[671]

Discussions regarding fertility preservation are essential for pediatric patients with cancer and their parents. Detailed discussion of this important topic is beyond the scope of this chapter.[672,673]

Approach to the Child with Delayed Puberty

As with sexual precocity, a thorough medical history and physical examination are crucial to the identification of the etiology of delayed puberty (Figs. 18.12 and 18.13). Is the patient undergoing treatment for any other disorder? What,

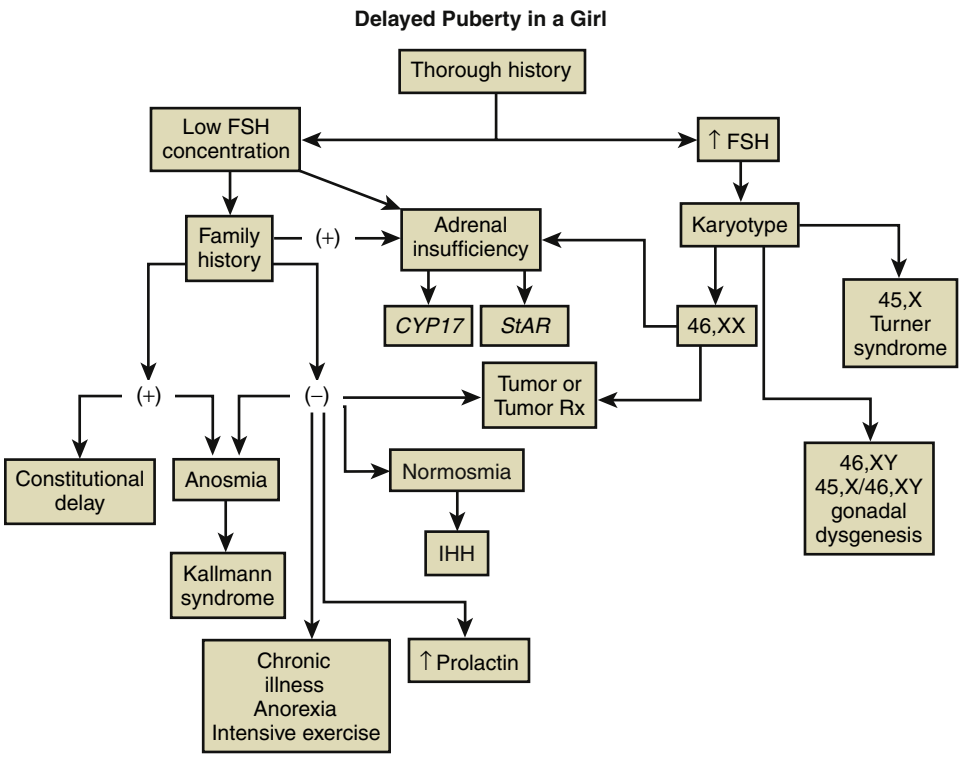


FIGURE 18.12 Algorithm for initial approach to delayed puberty in a girl. Beginning with a thorough medical history, this flowchart provides guidelines to the differential diagnosis of the most common causes of delayed puberty in girls. FSH, follicle stimulating hormone; CYP17, 17-alpha hydroxylase/17,20-lyase; StAR, steroidogenic acute regulatory protein.

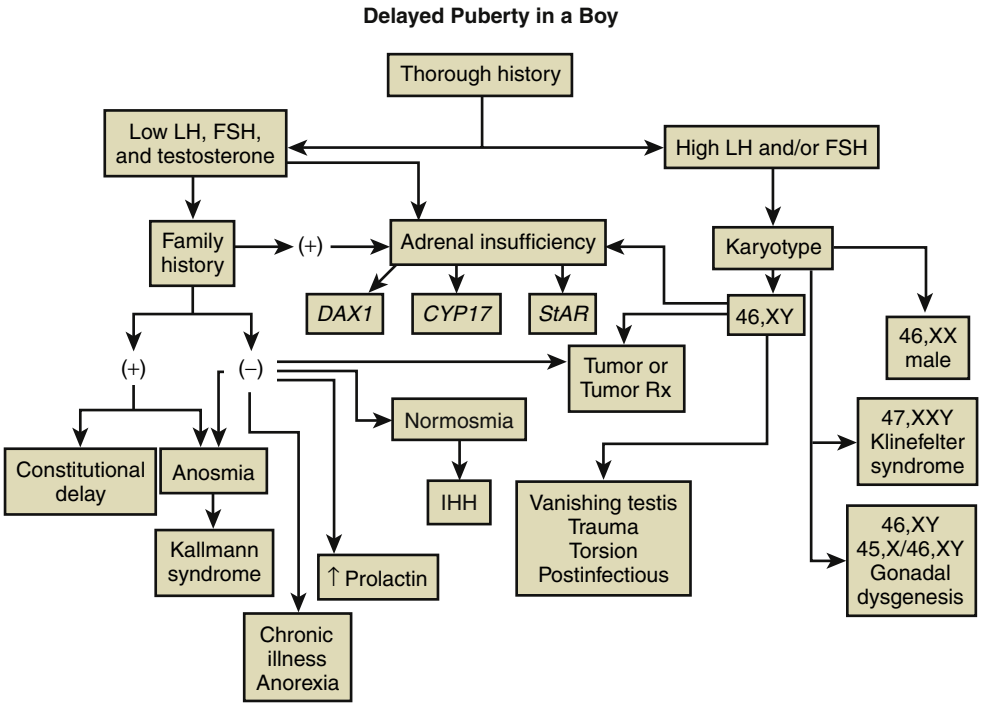


FIGURE 18.13 Algorithm for initial approach to delayed puberty in a boy. Beginning with a thorough medical history, this flowchart provides guidelines to the differential diagnosis of the most common causes of delayed puberty in boys. LH, luteinizing hormone; FSH, follicle stimulating hormone; DAX1, dosage sensitive sex reversal, AHC-critical region of the X chromosome, gene 1; CYP17, 17 alpha-hydroxylase/17,20-lyase; StAR, steroidogenic acute regulatory protein.

if any, pubertal development has occurred? Is there a family history of delayed puberty? What was the age of onset and sequence of any pubertal development? If the patient is a boy, was he treated for a micropenis or cryptorchidism? If the patient is a girl, is she experiencing hair growth in androgen-dependent regions? What is the family history regarding age at onset of puberty? Is there any history of infertility in the family? Does the patient or any family members have anosmia or neurological abnormalities? What does the child eat? Are there any concerns or clues suggesting an eating disorder? Is the child a competitive athlete or dancer?

Growth records to assess for stature, changes in growth velocity, weight loss, body mass index (BMI), and decreased weight for height should be examined. A thorough physical examination focusing on the physical features associated with estrogen and androgen actions should be performed. In boys, testicular length greater than 2.5 cm along the longitudinal axis or testicular volume greater than 4 mL suggests the onset of gonadarche. Breast development and pubic hair development should be assessed in girls. Are labial masses or clitoromegaly present in girls?

Features suggestive of specific disorders should be sought on physical examination (e.g., Turner syndrome, midline defects, and Prader-Willi syndrome). Short stature in a girl is suggestive of Turner syndrome and warrants measurement of gonadotropin concentrations and a karyotype, even in the absence of other stigmata of the condition. Small, firm testes, especially when gynecomastia is present, suggests Klinefelter syndrome. Height, upper-to-lower segment ratios, and arm span help define eunuchoid habitus. Tall stature, especially with long limbs, suggests hypogonadism, such as Kallmann syndrome or 46,XY gonadal dysgenesis. Patients should be carefully examined for features associated with *CHD7* mutations.

Useful laboratory studies include LH, FSH, DHEAS, sex steroid, inhibin B, AMH, and prolactin concentrations. Hypothyroidism should be excluded by measurement of free T_4 and TSH concentrations. Bone age determinations are used to assess the degree of delay, monitor subsequent development, and estimate final adult height. The bone age and height prediction should be assessed and related to the mid-parental height. Urinalysis, erythrocyte sedimentation rate, and renal function studies help exclude chronic disorders. Assessment for celiac disease, chronic inflammatory bowel disease, hemoglobinopathies, or hematochromatosis may be indicated based on clinical features. Even if the patient declares that he or she has a normal sense of smell, formal olfactometry may be necessary.

Imaging studies may be warranted depending upon the differential diagnosis formulated from the history and physical examination. Brain MRI studies may identify hypoplastic or absent olfactory bulbs. Renal anomalies can be associated with *KAL1* mutations. Dental or skull malformations can be associated with *FGFR1* mutations and may be identified by imaging.

Elevated basal gonadotropin concentrations confirm primary gonadal failure, but this assessment is less reliable when skeletal maturation is less than 11 years in girls and 12.5 years in boys. In most instances when the gonadotropin concentrations are elevated, cytogenetic analysis is indicated to assess for Turner syndrome in girls, Klinefelter syndrome in boys, disorders associated with gonadal dysgenesis, or defects in sex steroid synthesis or action. The current recommendation for first tier cytogenetic evaluation for patients with congenital anomalies or developmental disorders includes chromosomal microarray analyses because these analyses more readily detect subtelomeric copy number variations than G-banded karyotype studies.[674] Custom genome microarrays may also be helpful to detect genetic variations associated with hypogonadism.[675] For patients diagnosed to have HH, sequencing of specific genes can be performed through both commercial and

research laboratories. The sequence of genes to be tested should be based on the patient's clinical presentation, physical examination, laboratory data, and family history.[676]

After ensuring that pregnancy is not the cause of primary amenorrhea, progesterone withdrawal can be performed to assess estrogenization of the uterus and patency of the vaginal outflow tract. Withdrawal bleeding generally occurs within 24 to 72 hours of completing a course of progesterone (5 to 10 mg daily for 10 days). If no bleeding occurs, imaging studies to assess for a uterus should be performed. Another explanation for lack of withdrawal bleeding is estrogen deficiency.

In males, if random gonadotropin concentrations are not elevated, testing with hCG may be indicated when the testes are not palpable or if defects in testosterone biosynthesis are suspected. Multiple regimens have been described for hCG-stimulation tests, ranging from daily injections for 5 days to biweekly injections over several weeks. At least 5000 units should be administered over the treatment period, with total dosage not exceeding 3000 units per injection or 15,000 units over the treatment period. A blood sample to measure testosterone, DHT, DHEA, and androstenedione should be obtained within 24 hours of the last injection. When *CYP17A1* mutations are being considered, serum pregnenolone and 17-OHP levels should also be determined.

Discrimination of hypothalamic and pituitary gonadotropin disorders from constitutional delay may be difficult. In both situations, basal gonadotropin concentrations and GnRH-stimulated (100 μg GnRH) gonadotropin responses are low and show considerable overlap. Bone age x-rays generally show delayed skeletal maturation. A family history of delayed puberty is more common in constitutional delay. The presence of coincidental disorders, such as other anterior pituitary hormone deficiencies, decreases the chances of the delay being constitutional. In addition, constitutional delay of puberty is usually associated with delayed adrenarche; however, adrenarche may also be delayed among subjects with ACTH deficiency.

Comparison of various diagnostic studies suggests that low inhibin B concentrations may best distinguish HH from constitutional delay.[677] Two studies suggest that boys with constitutional delay tend to have inhibin B concentrations greater than 35 pg/mL.[678,679] Nevertheless, constitutional delay remains a diagnosis of exclusion. No single blood test, stimulation study, or imaging study accurately distinguishes constitutional delay from HH. Despite the importance of genetic factors underlying these disorders, detection of specific genetic variants associated with either disorder is uncommon. Thus, serial observation remains the best way to distinguish constitutional delay from HH.

Girls with 46,XY karyotypes associated with gonadal dysgenesis will likely require gonadectomy due to the risk for malignancy in dysgenetic gonads. Surgery may be deferred until later adolescence in girls with CAIS to allow for breast development.[680]

Estrogen Replacement

The goal of estrogen replacement therapy is to mimic the normal pattern of ovarian estradiol secretion to induce development of secondary sexual characteristics. Considerations such as chronological age, actual height, and predicted height are factored into the decision-making process as to when to initiate therapy. Estrogens promote both linear growth acceleration as well as stimulating bone maturation culminating in epiphyseal fusion.

It is important for girls with hypogonadism to experience menarche at the same time as their peers; for this reason estrogen therapy is generally initiated around age 12 years. Estrogen replacement therapy can be administered by oral or transdermal routes. Since oral estrogens undergo first-pass metabolism in the liver, oral estrogens can trigger an imbalance between antithrombotic mechanisms and procoagulant factors and are associated with higher estrone concentrations.[681] Currently, oral ethinyl estradiol is no longer commercially available as a single agent in the United States. Conjugated equine estrogens are available, but this formulation contains multiple estrogenic substances with varying biological potencies and is, thus, not appropriate for this population. Thus, transdermal estrogen is often preferred for the initial stages of pubertal development. Beginning doses can be as low as 6 to 7 mcg estradiol daily; transdermal estradiol patches can be cut into halves and quarters. The dose can be gradually increased over several years. The use of transdermal matrix patches only at night can mimic the spontaneous estrogen concentrations, as well as diurnal variation observed in early gonadarche.[682] Initially, estradiol is administered for 1 to 2 years to induce breast development. Two types of transdermal patches are available: reservoir and matrix. One advantage of matrix patches is the ability to cut the patches into pieces to administer lower doses.

Once breakthrough bleeding occurs, or after 1 to 2 years of unopposed estrogen, a progestin should be added. The girl can then be switched to an oral contraceptive or a combination of patches and pills can be used for cyclic estrogen-progesterone therapy. The estrogen dosage can be titrated according to the tempo and adequacy of pubertal development. Medroxyprogesterone (5 to 10 mg) or prometrium can be added to achieve cyclic estrogen and progestagen treatment. Upon completion of pubertal development, the estrogen dose should be the minimum necessary to prevent breakthrough bleeding, support regular menstrual cycles, and promote calcium deposition.

Hormone replacement therapy for women with TS and HH begins in adolescence and ends 40 to 50 years later. Women receiving estrogen-containing hormone therapy may have an increased risk to develop venous thromboembolism. In addition, certain progestagens influence thrombotic risk. Inherited forms of thrombophilia include point mutations in Factor V (Factor V Leiden) and Factor II (prothrombin G20210A). Protein C, protein S, and antithrombin deficiencies are rare inherited causes of thrombophilia. Although VTEs can occur at any time, the risk is highest in the first year of oral contraceptive use. Hence, understanding and identifying potential risk factors for thromboembolic events is essential.

For females with complete androgen insensitivity, congenital adrenal hyperplasia, or Mayer-Rokitansky-Kuster-Hauser syndrome, nonsurgical self-vaginal dilation supervised by a multi-disciplinary team is an effective alternative to vaginal surgery and often improves vaginal length.[683]

Androgen Replacement

Testosterone replacement therapy will induce and maintain secondary sexual characteristics in boys. Other goals include enhancement of libido and increased bone mineralization. Testosterone can be administered by intramuscular injection or transcutaneous absorption. To induce pubertal genital changes, intramuscular depot testosterone esters (such as testosterone enanthate or testosterone cypionate) are administered monthly, initially at low doses such as 50 mg per month with subsequent increases in dose. Medication impregnated patches or testosterone gel can be used. Some individuals complain of itchiness or inadequate adherence of the patches.

Studies in adult men showed achievement of steady-state testosterone concentration within 72 hours of therapy with the gel.[684] However, optimal dosing regimens have not been established for induction of puberty with these newer forms of testosterone. Adult testosterone replacement therapy consists of weekly depot injections of 100 mg given at one-, two-, or three-week intervals. When the interval between injections is increased beyond 3 weeks, testosterone concentrations may be supraphysiological immediately after the injection and decline to sub-therapeutic concentrations prior to the next injection. Transdermal testosterone impregnated patches and testosterone gel provide less variable serum concentrations. Testosterone gels must be allowed to dry completely to avoid transfer of the gel to clothing or bedding with the potential for accidental exposure to female family members and younger siblings.

Boys with anorchia can be offered the choice of having saline-filled testicular prostheses surgically placed into the scrotum. The scrotum should be able to accommodate adult-sized prostheses so that only a single operation is necessary to minimize the risk for infection and scarring.

Psychosocial Considerations for Precocious and Delayed Puberty

Early puberty, especially among girls, negatively influences self-esteem and is associated with increased risks for psychosocial stress.[685,686] Thus, older girls with residual behavioral issues as a consequence of precocious puberty may benefit from counseling.[687] Although premature adrenarche is usually benign, some girls meet diagnostic criteria for psychological disorders.[688] Boys with precocious puberty may demonstrate increased activity, increased aggressive behavior, and may masturbate. Parents report decreased self-esteem and self-confidence in some late maturing boys. Because early or late puberty can create stress for a child and his or her family, discussion to ascertain how the deviation from the normal timing of puberty impacts the patient is crucial. Importantly, maturation of cognitive function appears to correlate better with chronological age than biological maturation.[689]

Among children with delayed puberty, in the absence of major psychosocial issues, longitudinal re-evaluations to ensure pubertal development and progression may be sufficient. For those children suffering significant consequences, short courses of sex-appropriate steroids in small doses may be beneficial. For prepubertal boys, intramuscular depot testosterone (50 to 75 mg) can be administered monthly for three injections. After a medication-free period of at least 2 months, testosterone concentrations should be measured to assess for gonadarche. If the testosterone concentration remains low, a second course of therapy may be indicated. When used in small doses, testosterone increases growth velocity (by direct steroid action on bone and indirectly by stimulating GH secretion) and does not compromise adult height.[437] If testosterone, LH, and FSH concentrations remain low after a second course of testosterone therapy, the boy may have HH and require long-term testosterone replacement therapy.

The ability to reproduce is presumed, and is closely linked to self-esteem, gender identity, and body image. Thus, when infertility is likely to occur based on the underlying disorder, it is important to be honest and discuss the possibility of infertility. Patients and their families may need to mourn this loss of fertility, and thus involuntary childlessness, without the availability of established religious and social rituals that are extant in the case of death.[690] Subsequent discussion should include alternative ways to achieve a family such as adoption or marriage to someone with children from a prior relationship. Advancements in assisted reproductive technologies offer the opportunity for parenthood. For example, girls with premature ovarian failure can carry pregnancies. Short-term hCG treatment successfully stimulated spermatogenesis in a man with HH.[691]

Conclusion

Puberty, which normally unfolds toward the end of the first, or the beginning of the second, decade of life, results from initiation of two independent developmental processes: gonadarche and adrenarche. Gonadarche describes the pubertal activation of the gonad that results in gametogenesis and the production of adult patterns in gonadal steroid synthesis. Gonadarche results from increased gonadotropin secretion, which, in turn, is triggered by a resurgence in the activity of the hypothalamic GnRH pulse generator that has been held in check by a neurobiological brake (viewed to be conceptual in nature) from the time of late infancy.

Current models posit that the GnRH pulse generator is comprised of KNDy neurons in the arcuate nucleus of the hypothalamus, and that the output of the pulse generator is relayed to the GnRH neuronal network by the intermittent release of kisspeptin. The components of the neurobiological brake that dictates the postnatal pattern of GnRH pulse generator activity remain a mystery. The lifting of the brake at the end of juvenile development, and therefore the timing of the pubertal resurgence of pulsatile GnRH release probably involves somatic cues—although the identity of these signals remains unclear.

Adrenarche describes the pubertal increase in adrenal androgen production that is responsible for pubarche. The timing of adrenarche, in contrast to that of gonadarche, does not appear to require a signal from the brain. The timing and tempo of puberty may be modulated by genetic and environmental factors. Understanding the genomics of puberty and the interaction of environmental determinants to time the onset of human fertility is critical to improved therapeutic approaches for disorders of pubertal development.

With an appreciation and understanding of the physiology underlying gonadarche and adrenarche, a differential

diagnosis can be derived for patients presenting with disorders of pubertal development. With the tools of molecular genetics, the molecular etiology and risk for recurrence can often be determined. Evaluation and treatment include consideration of the psychosocial complications of "off-time" pubertal development or possible infertility.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 19

Nutrition and Reproduction

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In males, pulsatile gonadotropin releasing hormone (GnRH) and gonadotropin secretion occurs at a mean interval of every 2 hours,[1] a frequency sufficient to maintain testosterone secretion, normal virilization, and spermatogenesis. In females, a more complex series of gonadal tasks must be accomplished, which include maturation of a single follicle, follicular rupture and ovulation, and corpus luteum formation. The mature female hypothalamic-pituitary-ovarian (HPO) axis must respond dynamically with negative, then positive (bimodal) feedback to rising estradiol, which is secreted by the developing follicle.[2] The positive feedback response, in the form of a luteinizing hormone (LH) surge, must be sufficient to initiate the molecular events of follicle rupture and subsequent luteinization. Optimal functioning of the female reproductive system requires more versatility of the HPO axis and this system is therefore more vulnerable to disruption than is the male.[3]

Pulsatile secretion of hypothalamic GnRH and pituitary LH occurs prior to birth, and continues throughout the prepubertal period.[4] Pulsatile LH secretion has been detected in children and occurs at a normal, adult frequency in boys and girls[5]; however, the amplitude of the signal is miniscule and requires highly sensitive measurement methods to detect. Suppression of pulsatile GnRH-LH secretory amplitude in prepuberty has been attributed to a combination of enhanced sensitivity of the childhood reproductive axis to estradiol, as well as a predominance of inhibitory neural and neuroendocrine signals that ultimately dampen the amplitude of the central GnRH pulse generator.[6] This working model hypothesizes that prepubertally, the HP axis is inhibited, and during puberty the axis is released from this inhibition.

Almost 40 years ago, Frisch and Revelle noted a direct relationship between body weight and age at onset of puberty[7,8] and concluded that a critical amount of body fat was needed for the onset of puberty.[9] These investigators predicted correctly that adipose tissue somehow provided a permissive signal to the reproductive system. The discovery of the endocrine nature of adipose tissue has since led to the identification of a number of adipokines as well as inflammatory factors, and the elucidation of their role in reproduction is emerging rapidly. The best characterized molecule to date is leptin. It has been shown in both animal models[10] and in humans that inadequate leptin is associated with lack of GnRH secretion, and that leptin replacement can restore normal cycles in women who have hypothalamic amenorrhea and hypoleptinemia.[11] However, leptin alone is insufficient to initiate pubertal maturation of the HPG axis and a specific leptin concentration or threshold, above which maturation occurs, has not been identified.[12]

One of the final common pathways that appears to activate GnRH-LH secretion during puberty involves kisspeptin and its cognate receptor, G-protein coupled receptor-54 (GPR54).[13] Kisspeptin appears to act directly on GnRH neurons and amplifies GnRH and consequently LH and follicle stimulating hormone (FSH) secretion. Both kisspeptin and its receptor, GPR54, increase coincident with the onset of puberty. In addition to kisspeptin, there are many other upstream regulators of GnRH that appear to provide redundancy to prevent reproductive failure.

Puberty is also a time during which acquisition of functional networks of neurons and glial cells provide necessary communication pathways through which reproductive signaling can occur. The acquisition of these networks may involve anatomical changes in cell populations or recruitment as well as increases in neuronal connectivity. For example, glial cells long believed to be inert but helpful in providing scaffolding, may play a more direct role in GnRH secretion. Microglia, macrophages that differentiate into glial cells, are necessary for optimal GnRH secretion. CSF-1 knockout mice, who lack adequate macrophage function, do not have adequate microglial development and are deficient in hypothalamic GnRH secretion.[14] The interaction of immunomodulatory molecules with the reproductive neural networking is not yet well characterized.

Much research to date on abnormalities of pubertal maturation has focused on inadequate nutritional states. However, in the U.S. as in most developed nations, these conditions are becoming increasingly rare and are being replaced by reproductive dysfunction due to overweight and obesity. Obesity has been linked to altered pubertal progression and adult reproductive dysfunction. Polycystic ovary syndrome (PCOS) has been well studied in its interactions between obesity and anovulation; however, a far greater proportion of the population is affected by simple obesity. Pubertal onset is earlier in obese girls and boys, but the tempo of puberty is slowed in simple obesity.[15,16] A relative state of hypogonadotropic hypogonadism has been

described in obese women without PCOS.[17] Decreased fecundity, increased reproductive wastage, and a less favorable response to fertility treatments have all been described in association with adult female obesity.[18-21] In men, reduced fertility and a marked increase in bioavailable estrogen have been described.[22,23]

This chapter will discuss the role of overnutrition and undernutrition in relation to reproduction.

Nutrition and Puberty

The known and conjectured mechanisms initiating puberty are reviewed elsewhere in this volume. We will focus on the relevant epidemiology of normal puberty in boys and girls.

Puberty appears to be imminent when hypothalamic-pituitary sensitivity to negative feedback inhibition by estradiol is reduced,[24] and attendant inhibitory neural networks diminish in activity. These processes lead to increased GnRH secretion[25] as well as increased gonadotropin pulsatility, initially at night time, which then extends throughout 24 hours.[26] The result is folliculogenesis in girls, spermatogenesis and testosterone production in boys, and the attainment of fertility milestones. In females, the ultimate maturation of the HPO axis is the manifestation of positive hypothalamic-pituitary feedback to increasing estradiol, which initiates an LH surge and ovulation of the pre-ovulatory dominant follicle.[2]

Sexual maturation is a progressive process, but it is not linear. Girls who have their first menstrual period do not always continue to ovulate monthly thereafter. It is also not certain if menarche routinely reflects an antecedent ovulatory cycle, or if it is merely indicative of a rise and fall in estradiol. Prior epidemiological studies have observed that the attainment of regular menstruation takes from as little as 2[27] to as much as 7 years after menarche.[28-30] However, many such studies were conducted in populations of girls who differ from our modern day U.S. population. There were few young women of color, yet African-American girls are known to attain menarche and to undergo pubertal maturation earlier than Caucasian girls. This has been related in part to increased body mass index (BMI) but may also be due to other factors.[31] It is possible that prior populations studied have been enriched in girls who were nutritionally marginal, certainly so by today's standards. However, weight or BMI data are not available from these earlier studies.[29,32,33] Given the high rates of obesity among adolescents in the current U.S. population, and the increase in body fat in adolescent women in the United States over the past century,[34] it is likely that any restraint that low body weight might place upon the HPO axis is minimally operative in our current society.[35] The virtual elimination of under-nutrition might be expected to reduce the overall age at menarche in population studies. However, this is not uniformly the case. In one 50-year cohort and family study of a population sample in Ohio, higher BMI was not shown to be independently related to an earlier age at menarche.[36] However, this population reflects minimal minority representation, and had a mean age at menarche of 12.7 prior to 1954 and 12.6 after 1954, a minimal decrease. It is possible that overall body composition or BMI in this sample was optimal prior to 1954 (i.e., few girls who were critically underweight were present in this sample).

One study of 112 Caucasian girls, followed every 6 months perimenarcheally, observed elevated urinary progesterone levels consistent with regular ovulation within 1 year of menarche in the majority.[37] However, sampling was only done every 6 months, a frequency inadequate for the detection of monthly ovulatory events. Zhang and colleagues have recently completed a study of perimenarcheal girls using daily urinary sampling, and the findings reinforce the notion that regular ovulation occurs relatively rapidly, within months instead of years after menarche.[38] Metcalf and colleagues[28] and Borsos and colleagues,[39] who did weekly hormonal assessments in perimenarcheal girls, concluded that a mature pattern of ovulatory cycles was established in a majority of girls after the 20th menstrual period. However, even weekly urine sampling may be inadequate for the detection of brief excursions of progesterone, or its urinary metabolite, pregnanediol glucuronide (Pdg). Luteal phases may also be shorter than the adult norm of 14 days when adolescents are observed. Recent data from our group indicates that the capacity to produce an ovulatory LH surge in response to estrogen is present even before the onset of the first menses.[38] These findings indicate that, at least in some girls, a biphasic feedback response to estradiol is not the rate limiting step in attaining regular, ovulatory menses. Irregular menstruation has been associated with low gynecological age, low BMI, chronic nonspecific lung disease or allergic disease, weight loss, and stress.[40]

Rosenfield[41] has hypothesized that low-amplitude hormonal cycles occur in early puberty, with nighttime levels greater than daytime. Indeed, it may be that all the hormonal components of the HPO axis are functioning at an adult frequency, but the hormonal signals are dampened and slowly increase to detectable levels.

The notion that body weight or body fat is a permissive maturational signal to the hypothalamic-pituitary axis in girls is also supported by clinical data demonstrating an increase in leptin associated with puberty. Within developing countries, pubertal timing variations exist between girls in well-off versus underprivileged conditions, with earlier ages at menarche in well-off girls.[42] Whether these differences relate to nutritional status, physical activity or energy expenditure, or perceived environmental stressors is not known. Environmental exposures are also hypothesized to contribute to the trend of earlier puberty in Western societies. Endocrine disruptors contained in plastics are ubiquitous in industrialized societies and most individuals demonstrate appreciable levels of exposure. In general, these compounds bind weakly to sex steroid receptors and their signaling is likely to be overridden by endogenous sex steroids in gonadally intact, reproductive aged males and females. However, at the extremes of reproductive life in women and prepubertally in men, these mixed sex steroid agonists may play a role in providing nonphysiological input to the reproductive axis.

How Does Fat Signal the HPO Axis?

Leptin and Its Effects on the Reproductive Axis

Leptin is a 16kD protein secreted primarily by adipocytes. Initially described as a satiety factor,[43] it conveys an afferent signal to the CNS about body fat stores. Mice deficient in leptin protein (ob/ob) or leptin receptor (db/db)

exhibit hyperphagia, profound obesity, and central hypogonadotropic hypogonadism. It has been proposed that leptin exerts dual regulation of energy balance, based upon the organism's state of energy regulation. When energy input is equal to output, leptin concentrations reflect total body fat mass. Alternatively, in conditions of energy disequilibrium, leptin functions as a sensor of energy imbalance, with low concentrations in states of net weight loss and elevated in positive energy balance states (reviewed in[44]). Leptin has been shown to modulate GnRH pulse frequency *in vitro*.[45] It does not act directly on GnRH neurons, but rather via indirect mechanisms through interneurons secreting hypothalamic neuropeptides, such as neuropeptide Y, galanin-like protein, melanocyte stimulating hormone (MSH), and endogenous opioids.[46]

Leptin's role as a peripheral adipose signal to the central reproductive axis gave rise to speculation that it was the missing link in the Frisch hypothesis[47] and, thus, served as a major trigger of pubertal development. Leptin's role, while necessary, is insufficient to bring about pubertal maturation on its own. Leptin reverses pubertal arrest in leptin-deficient mice.[48] When given to normal prepubertal animals, leptin hastens sexual development as manifested by the advancement of vaginal opening.[49] However, when Cheung and colleagues assessed the temporal sequence of pubertal events in rodents,[50,51] serum leptin was not detectably elevated prior to pubertal development. Moreover, expression of leptin receptor messenger ribonucleic acid (mRNA) in the hypothalami of female mice did not increase with pubertal development. Finally, administration of leptin to starved animals advanced estrus to the same degree as food-restricted untreated controls but first estrus occurred at the same time as mice fed ad libitum. Taken together, these results imply that leptin provides a necessary input of adequacy of energy stores to the brain, thus authorizing but not initiating puberty.

In humans, leptin levels are markedly elevated in obesity[52] and pregnancy,[53] and are overall higher in women than in men.[54] Similar to rodents, deficiencies of leptin or leptin receptor, though rare, are characterized by early-onset obesity and variable degrees of hypogonadotropic hypogonadism.[55,56] Dramatic reversal of pubertal delay with leptin administration in a leptin deficient girl has been described.[57]

In the absence of a deficiency, serum leptin increases during childhood, with the highest concentrations in children who gain the most weight; higher serum leptin concentrations are associated with an earlier menarche.[58] In boys with constitutional delay of puberty, a leptin rise was not required for pubertal progression.[59] Furthermore, two women with lipoatrophic diabetes and chronic hypoleptinemia were reported to have normal menarche and childbearing, suggesting that normal pubertal maturation is possible even with very low serum levels.[60] Thus, while leptin does not act as a "trigger," it somehow conveys an essential metabolical permission for the body to prepare for procreation. Women with amenorrhea of hypothalamic origin, with or without weight loss as a precipitating factor, demonstrate improvement of their menstrual cycles and restoration of regular, pulsatile GnRH-LH secretion in response to leptin.[61,62] Leptin remains the most compelling molecule among currently known adipokines for having a major role in reproduction.

Leptin has numerous affects on peripheral components of the reproductive system in a variety of animals and model systems. On the gonadal level, leptin has been found in ovarian follicular fluid and a leptin receptor has been localized to the human granulosa, theca, and Leydig cells. In a bovine model, leptin decreased gonadotropin-mediated sex steroid production from granulosa and theca cells.[63] In humans, leptin may interrupt normal oocyte maturation[64] and has been correlated with poor implantation potential.[65,66] The predominant effect of leptin's action on the HPG axis has been hypothesized to be dependent upon its concentration, whereby low leptin exerts a negative influence centrally and elevated leptin yields a negative effect peripherally at the gonadal or embryo level.

Other Potential Molecules that Communicate Metabolical Signals to the HPO Axis

Adiponectin, another major adipokine, is the most abundant adipose gene transcript and is the natural peroxisome proliferator-activated receptor gamma (PPAR-gamma) ligand.[67] It plays an overall protective role in the development of insulin resistance and, most notably, is profoundly decreased in obesity and type II diabetes.[68] Adiponectin plasma concentrations reveal a sexual dimorphism, with adult females having significantly higher levels than males[68]; prepubertal children demonstrate no gender difference. Adiponectin levels significantly decrease after puberty,[69] however it remains to be seen if this represents a consequence of the increasing body mass or is a distinct phenomenon.

Ghrelin, a 28 amino acid stomach-secreted peptide, is a ligand for the growth hormone secretagogue receptor.[70] Ghrelin is a central appetite stimulator that regulates hunger and stimulates meal initiation[71] and is another putative link between metabolical and reproductive axes. In cross-sectional studies, ghrelin concentrations decrease from childhood to adolescence,[72] but children with central precocious puberty do not exhibit an increase in serum ghrelin after GnRH agonist treatment.[73] In children evaluated for short stature, administration of exogenous sex steroids decreased serum ghrelin in boys but not girls.[74] While one group related the fall in ghrelin levels to insulin-like growth factor (IGF-I) and IGF binding proteins as a putative mechanism for pubertal growth acceleration,[75] larger longitudinal studies are needed to evaluate ghrelin's role in pubertal development and reproduction.

Inflammatory cytokines, most notably tumor necrosis factor (TNF)-α and interleukin (IL)-6 among others, are produced by adipose tissue, and are elevated in obesity. In monkeys, leptin has been shown to modulate this inflammatory response.[76] Weight loss results in a decrease in macrophage infiltration of adipose tissue and improvement of the cytokine profile.[77] In obese adolescents, C reactive protein is significantly elevated, providing the first indication that low-grade inflammation starts at a young age.[78] It is not currently known whether mediation of this inflammatory response could mitigate some of the reproductive consequences of obesity. On the other hand, the hormonal status of the individual may be linked to the inflammatory cytokine profile. Recent studies have demonstrated an association between vasomotor symptoms and IL-8, implying that low estrogen levels somehow modulate expression of this cytokine.[79,80]

Neuropeptide Y (NPY), a member of the pancreatic polypeptide family, is a 36 amino acid neurotransmitter predominantly found in sympathetic neurons.[81] NPY is one of the most potent orexigenic peptides known and was recently shown to stimulate fat angiogenesis, proliferation and differentiation when administered peripherally.[82] Leptin decreases NPY gene transcription in the arcuate nucleus,[83] thereby sending a signal to decrease food intake. In the male rhesus monkey, antagonism of an NPY receptor led to precocious GnRH release, suggesting a role for NPY in maintenance of the prepubertal brake on the GnRH pulse generator.[84]

Sex Steroid Modulation of Feeding Behavior and Fat Accrual

The arcuate nucleus of the medial basal hypothalamus is a central relay station for body fat signaling and is a site of GnRH release. It functions to converge influences of orexigenic stimuli, such as NPY and agouti-related protein (AGRP), and anorexic stimuli, such as alpha-melanocyte-stimulating protein, corticotropin releasing hormone (CRH), and opioids. In rats, exogenous leptin administration results in down-regulation of NPY and AGRP.[85] Expression of leptin receptors in the arcuate and ventro-medial hypothalamic nuclei suggests this is a site of modulation of leptin effects on the reproductive axis.[64,86] Recent evidence suggests that ER-alpha expressing hypothalamic neurons regulate both feeding behavior and activity. Targeted deletion of ER-alpha in proopiomelanocortin (POMC) neurons leads to hyperphagia alone, but when steroidogenic factor-1 (SF-1) neurons undergo targeted deletion of ER-alpha, hypometabolism and hyperphagia can be created; deletion of ER-alpha in both of these neurons causes hyperphagia, hypometabolism, and abdominal adiposity.[87] Moreover, ER-alpha deletion in these hypothalamic areas causes reproductive dysfunction. These data imply a critical and possibly developmental role for hypothalamic estrogen sensitivity and metabolism.

How Does Body Weight Modify Puberty and Adult Reproduction?

Childhood Nutrition and Modification of Puberty

Reproductive maturation is delayed in a nutritionally deprived environment. The effect of obesity on pubertal timing has more recently been addressed in obese youth with premature pubarche in children with low birth weight and rapid postnatal catch-up in weight, and in boys and girls with simple obesity. These conditions will be addressed in turn, as all inform the pathophysiology of nutritionally associated reproductive disorders in adults.

The Effects of a Nutritionally Deprived Environment

Pugliese and colleagues evaluated 14 boys and girls. Nine boys and 5 girls, ages 9 to 17 years, with growth failure and delay in puberty (7 of the 14) due to malnutrition resulting from self-imposed caloric restriction.[88] In these children, who restricted caloric intake for fear of becoming obese, increased linear growth and pubertal progression resumed with the resumption of age-appropriate caloric intake. Matejek and colleagues studied the relationship between leptin levels, fat stores, and reproductive hormone levels in 13 female juvenile elite gymnasts and 9 adolescent girls with anorexia nervosa.[89] Leptin levels were subnormal and were related to body fat mass in girls with anorexia nervosa, but were lowest in the elite gymnasts. In both groups, estradiol levels were low and menarche was delayed. Catch-up height and weight in immigrant and adopted children, who move from developing to developed countries, is associated with precocious menarche.[90] Taken together, these studies support the notion that a critical body fat mass is necessary for normal pubertal progression.

The Effects of Low Birth Weight and Prematurity

Low birth weight, small for gestational age (SGA) status, and prematurity are all associated with precocious pubarche (appearance of pubic hair earlier than 8 years of age) and early and exaggerated adrenarche in girls.[91-94] Prenatal stress associated with prematurity may have an independent or additive effect on the programming resulting from growth restriction. Low birth weight (LBW) children have higher dehydroepiandrosterone levels compared to children of normal weight, indicating amplified adrenarche,[93,95] and LBW has also been associated with an increased likelihood of developing Polycystic Ovary Syndrome (PCOS). Although a majority of studies indicate that SGA is associated with premature pubarche (PP) and earlier onset of menarche,[96] van Weissenbruch and colleagues observed no differences in the timing and progression of puberty, including age at menarche, between SGA and appropriate for gestational age (AGA) girls[95]; another study found SGA to be associated with pubertal delay.[97] Studies associating birth weight with age at menarche have been similarly variable in outcomes, with findings ranging from no association to lower birth weight being linked to earlier pubertal onset.[98-100] More recent work by Ibanez and colleagues provides further insight into the progression of puberty in girls with precocious pubarche attributed to amplified adrenarche.[101] Followed from birth through adult stature, 187 Northern Spanish girls differed in the timing of the onset of puberty and menarche dependent on the degree of prenatal growth restraint. Girls with precocious puberty and LBW had menarche 8 to 10 months earlier and obtained an adult stature that was as much as 6.5 cm lower than non-LBW girls. While the LBW-PP girls demonstrated an accelerated onset of puberty and progression to adult stature, most of the height loss was observed prior to the onset of puberty and even before the development of PP. Low circulating levels of sex hormone binding globulin (SHBG) (a marker for hyperinsulinemic insulin resistance in nondiabetic girls)[93] and hyperleptinemia may contribute to the accelerated onset and progression of puberty in these LBW-PP girls. The DONALD (Dortmund Nutritional and Anthropometric Longitudinally Designed) Study evaluated 215 children for the effects of early life exposures on the initiation of puberty. The authors found that children weighing between 2500 and 3000 g at birth initiated their growth spurt (defined as the age at minimal height velocity at the onset of the pubertal growth spurt) approximately 7 months earlier than children weighing more than 3000 g at birth.

Children with low birth weight and rapid weight gain in the first 2 years of life displayed initiation of the growth spurt 4 months earlier than those with normal weight gain, indicating earlier pubertal onset. These children also had an earlier age at peak height velocity and earlier menarche in girls, indicating more advanced pubertal maturation. The associations were independent of prepubertal BMI, suggesting that other early life factors may influence the timing of puberty independent of the influences of body fat mass.[102] The findings relating to the relationship between birth weight and puberty initiation are variable and imply that there is not a simple, direct relationship. The variability may in part be explained by the different methods used for documenting pubertal initiation and progression; the use of Tanner staging, initiation of breast development, adrenarche, growth velocity and initiation of growth spurt or menarche, as well as variability in the measures of assessing fat mass or body composition.

The Effects of Simple Obesity in Girls

Longitudinal and cross-sectional data from studies conducted since the 1970s have demonstrated an overall close association between obesity and early pubarche in girls. The majority of members of an expert panel, recently convened to review studies conducted between 1940 to 1994 evaluating the secular trends of pubertal timing, concluded that thelarche and menarche are occurring earlier in U.S. girls.[103] These trends coincide with the rise in obesity prevalence, leading to the hypothesis that increasing adiposity is associated with earlier pubertal development. Several authors have examined the relationship between obesity and early puberty in girls. In comparison with nonobese girls, obese girls have been found to have earlier menarche in Japan (9 months earlier)[104] and Thailand (0.9 year earlier).[105] Early menarche has also been associated with an increased risk of adult obesity, indicating that early menarche may precede the development of obesity.[106,107] Yet other studies have shown that obesity precedes early menarche. Cooper and colleagues found that age at menarche was inversely associated with weight at 7 years[108] and He and Karlberg noted that incremental increases in BMI between the ages of 2 and 8 years were associated with early onset of the pubertal growth spurt in both boys and girls.[109] The Fels longitudinal study examined the relationship between the mean age of menarche and the simultaneous change in BMI from age 3 to 35 years in successive birth cohorts (born from 1929-1990).[110] While the age at menarche in the 1980s cohort was significantly lower than the age in girls born previously, and these findings match the findings of several recent reports which suggest a decline in the mean age at menarche of U.S. girls over the past 20 years, the decline in age at menarche was not associated with a significant concurrent increase in BMI in childhood or adolescence. In a longitudinal study evaluating the relationship between BMI and puberty onset in 354 girls, Lee and colleagues found that an increased BMI as early as age 3 as well as an increased BMI between the ages of 3 and 6 years was associated with an earlier onset of puberty.[111] While these findings do not prove a causal effect, they suggest that increased adiposity precedes early puberty.

Precocious puberty has been linked with childhood obesity[92] and girls with precocious pubarche tend to be hyperinsulinemeic,[93] and insulin resistant,[112-115] especially if there is a history of low birth weight. Yet pubertal progression appears to be alternatively regulated in girls with simple obesity (i.e., obesity that is not complicated by premature adrenarche or PCOS). It is not clear whether early thelarche in overweight girls is related to central activation of the GnRH–gonadotropin axis or whether estrogens from other sources are responsible for the production of breast tissue. Increased estrogen production results from the peripheral conversion of androstenedione by aromatase contained in adipose tissue.[116] Further, the hyperinsulinemia that results in low levels of sex hormone binding globulin and higher levels of free circulating estrogen and androgens has been associated with excess total and central adiposity.[91,113,117] Pharmacological insulin sensitization appears to improve body composition and dyslipdemia, and delays or prevents the progression to PCOS.[114,118] These data favor an association between obesity and subsequent development of PCOS.

The Effects of Simple Obesity in Boys

Data on the association of obesity with pubertal onset timing in boys lacks concurrence. Utilizing Third National Health and Nutrition Examination Survey (NHANES III) survey data, Wang demonstrated that boys with a higher body mass index were more likely to have a later, rather than earlier, sexual maturation.[119] The accuracy of the ascertainment of genital stage in boys in the NHANES III has been called into question based on the observation by Herman-Giddens and colleagues that 25% of 8-year-old boys were at genital stage 2,[120] whereas there had been no concurrent increase in the rate of boys being evaluated for precocious puberty (defined as the onset of testicular enlargement at <9 years). The early staging of puberty (without assessment of testicular volume) is subjective, and inadequate criteria for genital assessment may have resulted in an over-classification of prepubertal boys as having initiated puberty. Evaluation of pubertal onset utilizing testicular size to define pubertal onset has yielded variable results. Increased testicular size was noted in obese prepubertal Chinese boys relative to nonobese age-matched boys (1.18 mL vs. 0.82 mL).[121] Further, morning inhibin B, dehydroepiandrosterone (DHEA), and DHEA-S and bone age were also higher in obese versus non-obese controls. In contrast, Laron demonstrated that while obese Israeli boys were taller and their bone age was more advanced, at all ages, relative to age-matched controls, there was no difference between the two groups in terms of the onset and development of pubarche, voice change, testicular volume, and penile size.[122]

Longitudinal studies have been conducted to further understand the association between obesity and pubertal onset and progression. Recently, in a 10-year longitudinal population-based study on 401 boys enrolled in the National Institute of Child Health and Human Development Study of Early Child Care and Youth Development, Lee and colleagues found that a higher BMI Z-score trajectory during early to middle childhood was associated with a later onset of puberty.[123] A higher proportion (14%) of boys in the highest BMI trajectory were prepubertal (defined as a Tanner stage 1 determined by visual inspection) at 11.5 years old than those in the lowest BMI

trajectory (7.7%), indicating a delay rather than advancement of puberty in obese boys. Vizmanos and colleagues, following 323 boys annually to assess pubertal onset at ages 11, 12, 13, or 14 years old, noted a positive relationship between BMI and age of pubertal onset (defined by testicular volume and genital development according to Tanner stage), yet significant differences in percentage body fat between the different ages at onset of puberty were not noted. These findings suggest that, like girls, boys require a critical fat mass to initiate pubertal maturation.[124] Sorenson and colleagues conducted a cross-sectional study from 1991 to 1993 and a combined cross-sectional and longitudinal study from 2006 to 2008.[125] Measuring pubertal onset based on testicular volume assessments and genital staging in healthy Danish boys, the authors found that age at pubertal onset declined by 3 months from 1991 to 2006 and that age at entry into genital and pubic hair stages 4 and 5 was reached significantly earlier in 2006 than 1991. In addition, BMI increased significantly during that time period. Importantly, earlier age at onset of pubertal testicular growth in 2006 compared with 1991 was no longer significant after adjustment for BMI—indicating that BMI at least partly explained these findings. Juul and colleagues studied pubertal onset in 826 boys and found that while taller compared to youth in 1964, the timing of pubertal onset between 1964 study and the 1991 to 1993 study had not changed.[126] In another 10-year longitudinal study, Juul and colleagues utilized voice change as a marker for later pubertal development in 463 boys and found a downward trend in the age at voice break between 1994 and 2003. Further, boys in the heaviest BMI quartile at 8 years of age had a 1.74-fold risk of early voice break relative to boys in the thinnest quartile.[127] Tracking skeletal maturity and linear growth acceleration throughout infancy, childhood, and adolescence from birth through 18 years old in 521 subjects from the Fels Longitudinal study, Johnson and colleagues found that overweight or obese adults were more skeletally advanced throughout childhood. While BMI-related differences in peak height velocity were observed for both boys and girls, these differences decreased as puberty progressed so that by 18 years old, overweight or obese adults were not significantly taller than their normal weight peers.[128] Overall, the relationship between obesity and timing of puberty appears to differ by the manner in which markers of puberty are defined and documented. There also appear to be differences among different populations.

Recent research on the physiology of puberty in obese girls and boys may shed light on these apparent discrepancies. Bordini and colleagues examined nighttime LH pulsatility in 40 pubertal girls and observed blunted LH secretion in girls with a body weight greater than the 85th percentile.[129] These investigators hypothesize that there is a bimodal female HPO axis response to androgens.[16] As described above, adrenal androgen excess is linked to premature adrenarche and early onset of puberty. This process seems to occur in girls whether or not they are destined to develop PCOS. However, once puberty is initiated in non-PCOS related obesity, the relative hypogonadotropic hypogonadism of obesity retards the process, leading to a normal onset of menarche. The research focus on menarche, as an easily measurable physiological end point of reproductive maturation, may be obscuring the pathophysiological alterations in the stepwise process of puberty in girls.

Adult Obesity and Reproduction

Genetic Predisposition to Simple Obesity: Reproductive Correlates

The latest NHANES study indicates that over 35 percent of all U.S. adults are obese, defined as BMI of greater than 30 kg/m^2.[130] By 2015, 75% of all U.S. adults are projected to be overweight or obese with BMI of at least 25 kg/m^2.[131] Perhaps even more concerning are the recent data on U.S. women born in the 1980s who were found to exhibit up to 21% higher propensity to large body mass when compared to women born in the 1960s.[132] Thus, further escalation of the current obesity rates is likely as the 1980s generation ages and reaches the peak obesity prevalence. Easy availability of high calorie nutrition and low physical activity have been implicated as potential causes for these trends,[133] yet there are few definite answers as to why the obesity epidemic is continuing unabated.

Individuals respond differently to the environmental obesogenic challenges. In twin studies, up to 60% to 70% of total variation in percentage of body fat has been attributed to genetic contribution.[134] In 2007, one genome-wide association study has identified multiple single nucleotide polymorphisms (SNPs) in a gene designated *Fat mass and obesity-related transcript* (FTO) as mediators of the relationship between large body mass and type II diabetes mellitus.[135] This breakthrough in understanding of genetic contribution in patients with simple or nonsyndromic obesity has been replicated by other groups with an underlying theme that specific FTO variants correlate with human obesity. Up to 16% of adults who are homozygous carriers for the risk of FTO allele were reported to weigh 3 kilograms more and have up to 70% increased risk of obesity when compared with noncarriers.[135] The FTO gene was first reported in 1999 in a fused toe (Ft) mouse model.[136] Some of the deleted genes in this model had no known function at the time and one of these genes was serendipitously termed "Fatso (FTO)" because of the large size of the gene.[137] Subsequent studies have shown that FTO primarily functions as a single stranded RNA demethylase[138,139] and is upregulated in rodent hypothalamus after food deprivation.[140] While genes that predispose to adiposity may mediate impaired energy expenditure, FTO genes appear to be important in regulating food intake. In human studies, subjects with some FTO risk alleles were found to have diminished satiety awareness and loss of eating control.[141,142]

Polycystic ovary syndrome (see Chapter 22), is the most common endocrinopathy affecting up to 10% of all reproductive age women. It is frequently accompanied by obesity with some studies reporting up to 50% prevalence in PCOS women.[143] Obesity appears to modulate the risk of PCOS. Studies from Asian populations with lower adiposity as compared to non-Asians, typically report fewer affected subjects, as exemplified by 2.2% PCOS prevalence in a cohort of 915 Chinese women presenting for an annual physical.[144] Similarly, one Spanish study reported 28.3% prevalence of PCOS among consecutively studied obese or overweight women which was markedly higher than

the 5.5% prevalence reported among lean women by the same investigators.[145] A recent study of Polish women suggested that FTO risk alleles may portend a greater impact in women with PCOS as compared to general population.[146] In this study of 136 young women with PCOS, a 10 kg difference in weight was found between the carriers and noncarriers of the FTO risk allele, a difference that is two-to-three times greater than what was reported for the population at large. A recent meta-analysis from 7 studies and 2548 PCOS women has confirmed this trend.[147] This novel relationship between FTO inheritance and PCOS may reflect either an interaction between FTO physiology and environment or multifactorial and polygenic nature of PCOS. The take home message here is that FTO may affect obesity in PCOS or PCOS may, in turn, amplify the effects of FTO on adiposity. While a more complete picture of all genes linked with adiposity and reproductive competence is still emerging, it is imperative to emphasize that the recognition that these exist is important for understanding why some women are particularly vulnerable to obesity and its reproductive consequences.

Women

Adult obesity in women is associated with two reproductive phenotypes. In the presence of marked hyperandrogenism, women who are genetically vulnerable, including those with the newly identified FTO risk alleles, can develop PCOS in association with weight gain. This notion is implied by abundant data on the salutary effects of weight loss on fertility in anovulatory women with PCOS. However, PCOS is only present in 5% to 7% of the adult population, and the current population prevalence of obesity in reproductive aged women is much higher. Therefore, a second pathway must be hypothesized to explain the reproductive phenotype in simple obesity that is unrelated to PCOS. As PCOS will be covered in great detail elsewhere in this volume, this discussion will center on the model for reproductive dysfunction in women with simple obesity.

Increased body size is associated with reduced fecundity in both women and men.[18,148,149] Women who are overweight and obese in their late teens are almost four times more likely never to conceive compared to normal weight women.[20] If they do conceive, obese women experience increased reproductive wastage and worse outcomes with assisted reproductive technologies than do normal weight women.[150] In 162 patients undergoing *in vitro* fertilization (IVF) at one center,[151] obese women required more gonadotropin, yet had lower numbers of oocytes retrieved, and 45% lower fertilization rates compared to women of normal weight. Fedorcsak and colleagues[152] reported similar findings in a sample of over 5000 IVF cycles in 2660 couples. Cumulative live births were 10% lower (41.4% versus 50.3%) in women with a BMI over 30 kg/m^2, compared to women with BMIs between 18.5 and 24.9 kg/m^2, along with increased gonadotropin requirements and reduced oocytes retrieved. Others have reported similar detrimental outcomes for obese women undergoing ART.[153] Among 1293 women who underwent *in vitro* fertilization at one U.S. center, women with a BMI > 40 kg/m^2 had more than double the cancellation rate of normal weight women (and more than triple the cancellation rate when polycystic ovary syndrome was ***not*** present),[154] and a linear trend towards higher risks of preeclampsia and cesarean delivery.

Obesity and Preeclampsia

Preeclampsia complicates 5% to 7% of pregnancies and maternal obesity is a consistent risk factor for preeclampsia, however, the mechanisms involved are not understood.[155,156] The dose-dependent relationship between increasing body mass index (BMI) and the risk of developing pre-eclampsia is well established.[157-160] For instance, an elevated BMI results in an odds ratio of 7.2 for developing preeclampsia as opposed to normal BMI women.[161] Obesity and preeclampsia are both associated with oxidative stress[162-164] as well as circulating markers of inflammation. Plasma levels of C-reactive protein and inflammatory cytokines, tumor necrosis factor-alpha, interleukin-6, and interleukin-8 are elevated in obese individuals and preeclamptic women.[165-168] In addition, obese and preeclamptic women often have comorbidities of dyslipidemia, insulin resistance and impaired endothelial function.[169-171] These shared features suggest that preexisting inflammation may be the link between obesity and preeclampsia.[172,173]

The relative hypogonadotropic hypogonadism of obesity partially explains the fertility impairments. Sherman and Korenman reported longer cycle length, longer follicular phase length, and reduced daily LH, FSH, and progesterone secretion in association with hirsutism in five ovulatory midreproductive aged women who were overweight or obese compared to a larger sample of normal weight women.[174] Grenman and colleagues compared 25 women of very high BMI (mean weight = 120 kg) to 25 normal weight controls and observed decreased estradiol (E2), sex hormone binding globulin (SHBG), androstenedione, and LH in the obese.[175] These changes were partially reversed by acute, modest weight loss (mean loss of 13.2 kg). A number of investigators have reported decreased inhibin B in association with increased weight,[176-178] along with reduced follicular phase gonadotropins and E2.[179,180] The reduced follicular phase gonadotropin and luteal phase progesterone secretion[17] observed in women of large body size can be attributed to relatively insufficient FSH stimulation of folliculogenesis, a model that has been well described in nonhuman primates.[181]

Studies to date in animals and humans[17,174,182] consistently point to a defect in LH pulse amplitude in association with obesity. In obese, leptin resistant Zucker rats—a model similar to human obesity—there is profound suppression of pulsatile LH amplitude but not frequency.[183] It seems likely that some consequence of excess nutrient sensing plays a role in the reproductive dysfunction associated with obesity. Jain and colleagues have observed a dramatically reduced amplitude of endogenous LH pulses in women with a BMI greater than 35 kg/m^2, implying a central defect in reproductive function that may in turn impair ovarian and endometrial function. Women awaiting bariatric surgery who underwent frequent blood sampling had mean LH pulse amplitude of 0.87+/− 0.1 compared to 1.59 +/− 0.15 IU/L in normal weight controls.[17] Post-surgery, after at least 25% of initial body weight had been lost and weight had stabilized, there was a partial return of LH pulsatility and menstrual cycle parameters to those of normal weight controls.[184]

There is also evidence that physiological restraint of FSH secretion is reduced in obese women. Both inhibin B and Müllerian Inhibiting Substance (MIS) or anti-müllerian hormone (AMH) are reduced in obese women,[177,178] despite the lower overall sex steroid secretion throughout the menstrual cycle. These data indicate that FSH is inappropriately low for the given inhibin B levels in obese women. The fact that inhibin B is suppressed in obesity is interesting because inhibin B is widely regarded as a marker of ovarian reserve.[185] However, obese women do not have an earlier menopause.[186] The dual suppression of both MIS and inhibin B in obesity indicates that factors other than low ovarian reserve are operative, perhaps at the level of the ovary.

It is tempting to speculate a role for leptin in this process, because it is a molecule that sends nutritional signals to the CNS component of the reproductive axis. Obesity, because of its associated extreme leptin resistance, might also be construed as a leptin-deficient state.[187] Leptin receptors are not present on hypothalamic GnRH secreting neurons, and leptin is believed to exert effects on GnRH secretion via intermediary molecules,[188] such as neuropeptide Y (NPY) and agouti-related peptide (AGRP). These molecules also affect primarily LH pulse frequency when given intracerebroventricularly.[189,190] An adipokine or nutrient signaling pathway interaction with the hypothalamus that would reduce the amount of GnRH secreted per bolus (which would be reflected in a reduced LH pulse amplitude) would certainly provide a candidate pathophysiological mechanism for these findings.

Mice that experience dietary induced obesity (DIO) have hypogonadotropic hypogonadism,[191,192] and animals who are resistant to DIO-related reproductive dysfunction have reduced central inflammation.[193] Recent studies demonstrating the ability of melanocortin to block pro-inflammatory cytokine or peptide-mediated LH suppression suggest a potential mechanism by which pituitary output of LH and FSH are inhibited in obesity.[192-194] Thus, the emerging relationship between inflammation, nutrient sensing, and reproduction may provide further clues to explain the reproductive phenotype of obese women.

Men

In contrast to the substantial body of literature on the impact of female obesity on reproduction, the corresponding effect of male body mass on the ability to reproduce is only recently coming to the forefront of investigative attention. Obese women with an overweight or obese male partner have up to twofold further loss in fertility as compared to their obese counterparts with normal weight male partners.[195] A dual loss of fertility is more likely, however, because overweight and obese women tend to marry or cohabit with men of similar weight.[196,197] At the population level, several large observational studies have recently demonstrated an inverse relationship between male obesity and fecundity. Data from U.S. farmers indicated that each 3-unit rise of male BMI was associated with a 12% increase in prevalent infertility in affected couples.[198] Similarly, Danish National Birth Cohort records indicated a dose-response relationship between male adiposity and involuntarily delayed pregnancy.[195] Further, data from over 26,000 Norwegian pregnancies revealed that the detrimental influence of male obesity on fertility was demonstrated even when coital frequency data was included in the analysis, suggesting that this effect was not mediated by sexual dysfunction.[199] More recently, adiposity of the male partner has been associated with decreased chance for clinical pregnancy after ART.[200,201]

Male obesity may affect spermatogenesis and has been linked with reduced semen parameters. A study of over 500 infertile couples from Utah has shown that large body mass tripled the relative risk of oligospermia and reduced total motile count in men with BMI over 30 kg/m^2.[202] Similar associations were reported in healthy young Danish men reporting for a pre-army physical[203] and Egyptian infertility patients.[204] In further support of these observations, increased DNA fragmentation has been demonstrated in sperm from obese men.[205-207] Recently, a meta-analysis based on data from over 9700 men from 14 studies worldwide demonstrated that, as compared to men of normal BMI, both overweight and obese men were more likely to have oligospermia and azoospermia.[208] Remarkably, men with BMI over 30 kg/m^2 had approximately 40% higher chance of presenting with oligospermia and up to 80% higher chance of presenting with azoospermia. While the precise mechanisms underlying these associations remain uncertain, adiposity leads to reproductive hormonal changes that may alter spermatogenesis.

Similar to obese women, decreased LH pulse amplitude but unaffected LH pulse frequency have been reported in obese men.[209] The endocrine profile of hypogonadotropic hypogonadism in obese men leads to significantly depressed testosterone and is often, but not always, associated with erectile dysfunction.[210] A dose-response relationship between hypoandrogenism and the degree of obesity has also been reported and is thought to occur in concert with a corresponding increase in circulating estrogens.[211] On the pathophysiological level, obstructive sleep apnea (OSA), commonly diagnosed in obese men, has been linked with reproductive hormone alterations. In 1989, Grunstein and colleagues demonstrated reduced overnight secretion of testosterone in obese men with OSA.[212] Treatment of OSA has been demonstrated to result in the increase of serum testosterone with either surgical (uvulopalatal resection) or medical (nasal continuous positive airway pressure, nCPAP) management.[212,213] In obese men with OSA, pituitary gonadotropins have been demonstrated to be significantly reduced as compared with normal weight controls and partially reversed by correction of hypoxia.[214,215] Taken together, the potentially reversible hypogonadism associated with OSA and the decreased fertility and semen parameters associated with male obesity make a strong case to explore the mechanistic implications of symptomatic sleep disruption on sperm production. This is especially relevant given the recently shown significant improvement of male fertility with antioxidants[216] and findings in the murine diet-induced obesity model of *de novo* induced oxidative stress, sperm DNA damage, and decreased fertilizing ability.[217]

Impact of Weight Loss and Bariatric Surgery on Reproductive Function

In normal weight women, weight loss of as little as 10% to -15% induces reversible deficits in reproductive function that

include altered LH pulsatility.[218] In overweight and obese women, small improvements of fertility have been reported with diet-achieved moderate weight loss.[219] Increased regularity of menstrual cycles and improved fertility has been reported in women who maintain weight loss.[220] However, conservative weight reduction is difficult to achieve and is notoriously reversible when dieting is not aggressively maintained. Bariatric surgery produces dramatic and long lasting weight loss with the reductions up to 60% of presurgical body weight.[221] Most bariatric patients experience an initial, dramatic weight reduction of 3 to 4 pounds per week for the first 6 months, followed by a plateau phase within a year. The chief mechanism for weight loss is thought to be consumption of smaller meals due to the decreased volume of the stomach. In women who have undergone bariatric surgery, improvement in menstrual irregularities and higher fertility rates have been reported.[222] Most of the literature support a favorable risk-benefit ratio in support of bariatric surgery in patients with a BMI greater than 40 kg/m^2. In men, with the exception of one case report of unexplained spermatogenic arrest after bariatric surgery-induced weight loss,[223] there is a paucity of studies assessing the impact of weight loss on male reproductive success, assisted or unassisted. In small case series, obese men showed an overall improved reproductive hormone profile following weight loss, with an increase in SHBG and testosterone and decrease in estrogen after bariatric surgery.[224,225] Given the increasing utilization of bariatric surgery for morbid obesity as well as recently advocated indication for type II diabetes mellitus,[226] comprehensive studies on the impact of surgical loss on male and female reproductive potential are urgently needed.

Nutrition in the Young Oncology Patient

The American Cancer Society estimates there will be over 12,000 newly diagnosed pediatric cancer patients in 2012.[227] However, improvements in chemotherapy, radiation and surgery, and supportive care have dramatically increased survival and cure rates. Pediatric and young adult cancer patients represent a special population in which nutrition and metabolical status may be intimately associated with disease status.

Nutritional Status at Cancer Diagnosis

Although weight loss may be a presenting sign of malignancy in adults, pediatric patients appear to have good baseline nutritional status. In fact, Carter and colleagues[228] found a low incidence of malnutrition in 277 children with newly diagnosed tumors. When malnutrition was detected, it was primarily due to inadequate caloric intake, not protein deficiency. Pediatric cancer patients with advanced disease or those undergoing intensive chemotherapeutic protocols are at high risk of losing weight and becoming malnourished.[229] In addition, certain treatments such as abdominal radiation, surgery, frequent chemotherapy, severe infection, or pain may predispose patients to nutritional depletion.[230] Brain tumors are the most common solid tumors in children.[227] While some tumors are surgically managed, many brain tumors require a combination of surgery, radiation, and chemotherapy—resulting in nutritional compromise. Additionally, long-term steroid use is common and may lead to nutrition complications such as obesity, with associated depleted protein reserves and metabolical abnormalities including hyperglycemia, myopathy, and osteoporosis.[231,232]

Malnutrition has been associated with a number of adverse clinical outcomes, including reduced quality of life, decreased response to treatment, increased risk of chemotherapy-induced toxicity and reduction in overall survival.[233-236] Specific sequelae include inability to tolerate aggressive chemotherapy or radiation, frequent delays in therapy due to myelosuppression, increased risk of infection, and decreased overall survival. In fact, malnutrition and weight loss are associated with overall decreased median survival and decreased performance status in adult patients.[237,238] In pediatric oncology, children who are underweight or overweight at diagnosis have poorer outcomes compared to children who are well nourished at diagnosis.[239-242] Furthermore, a direct relationship between nutritional status and disease-free interval has been observed in children with solid tumors.[238]

Nutritional Concerns of Childhood Cancer Survivors

While many nutritional issues during childhood cancer treatment are associated with weight loss or poor growth, more recently, the risk of obesity, excess body fat, and metabolic syndrome is an emerging issue for survivors. Cranial radiation and hematopoietic stem cell transplant (HSCT) are two primary risk factors that have been associated with the development of these late effects. For example, obesity is a common finding in survivors of acute lymphoblastic leukemia (ALL) treated with cranial radiation, which correlates with significant reduction in physical activity and energy expenditure.[44,243-246] The Childhood Cancer Survivor Study, a multicenter, retrospective cohort study of pediatric cancer survivors, reported a higher prevalence of overweight and obesity in cancer survivors who received high-dose cranial radiation (≥20 Gy) compared to healthy sibling controls, particularly in female patients.[247] Hypothalamic-pituitary-adrenal and gonadal axis impairment can lead to a variety of hormone deficiencies and downstream effects. The association between cranial radiation and obesity is due, in part, to radiation-induced growth hormone insufficiency and leptin insensitivity.[248-250] In addition, a significant association between hypogonadism and metabolical syndrome has been noted.[251,252] However, even survivors treated without radiation (which represent the majority of children with ALL treated in the current era) may be at risk for obesity as a consequence of their cancer therapy.

Features of metabolical syndrome, including insulin resistance and dyslipidemia, have been found in ALL survivors, however, prevalence appears to be most common in patients who received HSCT.[253,254] Oudin and colleagues examined the prevalence and risk factors associated with metabolical syndrome in 184 young adult survivors of childhood leukemia and found an overall prevalence of 9.2%.[251] In addition, HSCT with total body irradiation (TBI) was associated with elevated triglycerides (OR = 4.5, P = .004), low high-density lipoprotein cholesterol (OR = 2.5, P = .02), and elevated fasting glucose (OR = 6.1, P = .04). Beyond serum abnormalities, cancer survivors are at risk of vascular

disease. For example, the Childhood Cancer Survivor Study demonstrated that cancer long-term survivors were 8.2 times more likely to die of cardiac-related events and were more likely to develop coronary artery disease and cerebrovascular events than healthy siblings (RR = 10.4 and 9.3, respectively).[255-257]

Summary

From the time of diagnosis through the survivorship period, nutrition should be integrated in the care and recovery of the pediatric cancer patient. Children must maintain growth and development during extended treatment courses as well as post-treatment. Malnutrition should not be accepted as an inevitable consequence of malignancy or its treatment. Likewise, effort should be made to prevent obesity and metabolical chronic diseases in long-term cancer survivors. Complications of oncological treatment, including obesity, metabolical dysfunction, and hypogonadism, need further evaluation and management.

Conclusions

Amplification of the pulsatile GnRH signal appears to be the obligatory final pathway for pubertal maturation; however, the many inputs into the hypothalamus imply that there are numerous concurrent factors that act as permissive signals. Moreover, it is likely that these signaling pathways are redundant, as it is in the best interests of the survival of the human species to assure that reproductive maturation will take place. It is now clear that environmental modifiers of the pubertal transition also contribute to the process. Psychological stressors, xenoestrogens, and body fat are but three such factors. It appears that none of these factors bears a simple relationship to the pubertal process, in that both accelerations and delays are associated with various stressors and increasing body fat.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 20

Environmental Factors and Reproduction

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Documented adverse trends in reproductive health in the U.S. include a decline in the age of onset of puberty[1]; declines in fertility and fecundity[2,3]; increased rates of poor birth outcomes such as babies born prematurely,[4,5] small for gestational age,[6] and with certain birth defects[7]; increased rates of childhood diseases such as autism,[8] certain types of cancer,[9] and obesity[10]; and declines in life expectancy, with some communities having life expectancies already well behind those of the best-performing nations.[11] Due to the time course of these changes they cannot be explained by changes in genetic makeup alone.[12] A possible explanation is exposure to environmental chemicals, which has increased over sixteenfold over the past 70 years.[13] There are currently over 80,000 chemicals in commerce including over 3000 high production volume chemicals—those imported or manufactured in excess of 1 million pounds each per year.[14,15] The vast majority of chemicals in commerce have not been fully tested for reproductive and/or developmental health effects.[16]

In recognition of the scientific evidence linking ubiquitous exposure to environmental chemicals to adverse health impacts, leading scientists, clinicians, and their professional societies have called for timely action to prevent harm.[17-20]

Of particular importance to endocrinologists is a statement published in 2009 by the Endocrine Society based on a review of the scientific evidence.[21,22] The Endocrine Society review concluded that environmental chemicals that disrupt the endocrine system (i.e., "endocrine disrupting chemicals" [EDCs]) have been found to have an effect on male and female reproduction, breast development and cancer, prostate cancer, neuroendocrinology, thyroid, metabolism and obesity, and cardiovascular endocrinology. The Statement concluded that, "The Endocrine Society is concerned that the public may be placed at risk because critical information about potential health effects of endocrine disrupting chemicals to which Americans are exposed is being overlooked in the development of federal guidelines and regulations," and "Until such time as conclusive scientific evidence exists to either prove or disprove harmful effects of substances, a precautionary approach should be taken in the formulation of EDC policy.[21]" A recent position statement by The Endocrine Society emphasizes the importance of the developmental stage when considering EDCs and that there is no "safe" dose[23] and a 2012 review of EDCs also concluded that these chemicals can act at low doses and in a non-linear fashion, and echoed the Endocrine Society's call for improved methods of chemical testing and prevention of exposure to EDCs.[24]

This chapter summarizes key themes related to the reproductive and developmental health impacts of exposure to environmental chemicals and the role of clinicians in prevention.

What Is Reproductive Environmental Health

Reproductive environmental health addresses the exposure to environmental contaminants, such as synthetic chemicals and metals, and the effect to reproductive health and development. Exposure to environmental chemicals can have an impact during all periods of human reproduction including fertility, conception, pregnancy, child and adolescent development, and adult health.[18]

Classic Examples of Thalidomide and Diethylstilbestrol

Two key principles of reproductive environmental health are supported by evidence from human exposure to the drugs Thalidomide and Diethylstilbestrol (DES).

The Fetus Can Be Uniquely Sensitive to Chemical Exposures: Thalidomide

In the 1960s, the drug Thalidomide was given to pregnant women to prevent morning sickness. Women who took the drug experienced no ill health effects; their children however, experienced a high rate of congenital limb and gastrointestinal malformations, particularly when the drug was taken during the 28th through 42nd day postconception.[25-27] In the 46 countries where the drug was approved, more than 10,000 children in 46 countries were born with deformities as a consequence of their mothers using the drug during pregnancy.[28]

Intergenerational Harm Can Result from *In-Utero* Chemical Exposures: Diethylstilbestrol

Diethylstilbestrol (DES) was prescribed in up to 10 million pregnancies from 1938 to 1971 to prevent miscarriage. DES

was subsequently found to be a "transplacental carcinogen." When the sons and daughters of DES-exposed mothers reached puberty they began presenting with both benign and malignant reproductive tract abnormalities. These abnormalities, which, in contrast to Thalidomide only appeared decades after exposure, were found to be causally linked to the mothers exposure to DES during pregnancy.[29] The established health impacts of DES in females exposed *in utero* include: vaginal clear cell adenocarcinoma, vaginal epithelial changes, reproductive tract abnormalities (e.g., gross anatomical changes of the cervix, T-shaped and hypoplastic uteri), ectopic pregnancies, miscarriages, premature births, and infertility.[30] Likewise, males exposed *in utero* also had delayed adverse health effects including reproductive tract abnormalities such as epididymal cysts, hypoplastic testis, and cryptorchidism.[30]

Although DES has not been prescribed for over 40 years, evidence of the delayed health consequences of exposure continues to emerge. Women exposed *in utero* to DES have a higher risk of breast cancer at age 40 than women who were not exposed.[31] A 2011 study found that women exposed to DES *in utero* have a higher lifetime risk of multiple adverse health outcomes compared to women who were not exposed, including infertility, spontaneous abortion, preterm delivery, early menopause, and breast cancer.[32] Studies have also shown that the delayed health impacts of DES also extend to the mothers who took the drug, as they too have an increased risk for breast cancer.[30] Animal studies support the evidence of the health effects of DES that have been documented in humans and predict that inter-generational impacts will extend to the next generation among the granddaughters of DES-exposed women.[33]

Key Scientific Concepts

Environmental Chemicals

Environmental chemicals include synthetic chemicals and metals found in our homes, workplaces, and communities. Common pathways of exposure to environmental chemicals include air, water, soil, dust, consumer products, and food. Paradoxically, the industrialized food system contributes a large proportion of environmental contaminants from food production, packaging, and transport including Bisphenol-A (BPA), perfluorooctanate (PFOA), phthalates, and certain organophosphate pesticides.[34]

Table 20.1 presents examples of reproductive and/or developmental health impacts of prenatal exposure to environmental contaminants.

Extent of Exposures

Exposure to environmental chemicals is widespread among the U.S. population. "Biomonitoring" is the study of human samples, usually blood, urine, or breast milk, to measure the amount of chemicals and their metabolites in people's bodies. It can also be used to evaluate the rate of placental transfer of chemicals by correlating levels across various biological matrices such as maternal blood, cord blood, amniotic fluid, meconium, placenta, and fetal tissue.

Biomonitoring data have documented ubiquitous exposure to environmental chemicals among the U.S. population.[64,65] Studies have also found multiple chemicals measured in human tissues in Europe and in populations in the Arctic indicating the wide range of global of exposure.[66]

Examples of environmental chemicals for which exposure is widespread include persistent organic pollutants (POPs), pseudo-persistent compounds, and metals. POPs are chemicals that do not break down in the environment; for example, the pesticide dichloro-diphenyl-trichloroethane (DDT) and its metabolite dichloro-diphenyl-dichloroethylene (DDE), and polychlorinated biphenyls (PCBs). These chemicals have been banned in the U.S. for over 30 years yet they are still found in virtually all of the population; other POPs such as some of the perfluorinated compounds have been phased out more recently but are still found in over 98% of the U.S. population; and polybrominated diphenyl ethers (PBDEs) are found in over 95% of the U.S. population.[67,68]

Pseudo-persistent compounds are those that can be metabolized and removed from the body, but because exposure is so consistent that they are almost always found in human samples. Examples of pseudo-persistent compounds include certain phthalates, polyaromatic hydrocarbons (PAHs), and phenols such as BPA and perchlorate.[68]

Metals are also common environmental contaminants and include mercury, lead, and arsenic. Due to public policy initiatives that removed lead from gasoline, paint, food cans, and other products, the levels of lead have dropped precipitously in the U.S. However, there remain populations both within and outside the U.S. with high blood lead levels, often from exposure to lead contaminated paint.[9] A recent CDC report summarizes new findings suggesting that the adverse health effects of blood lead levels less than 10 μg/dL in children extend beyond cognitive function to include cardiovascular, immunological, and endocrine effects.[39] The effects do not appear to be confined to lower socioeconomic status, and the report concludes that there is no identified blood lead level without deleterious effects, underscoring the critical importance of primary prevention.[39]

Pregnancy Exposures

A study examining chemical exposures among pregnant women sampled as part of 2003-2004 National Health Examination and Nutrition Survey (NHANES) found that virtually all pregnant women in the U.S. were exposed to at least 43 different chemicals.[69]

Among pregnant women, several classes of chemicals were particularly well represented. For example, 13 different phthalates were measured in the population and 9 were measured simultaneously in the average individual. In addition, specific PCBs, organochlorine pesticides, perfluorochemicals (PFCs), phenols, PBDEs, phthalates, PAHs, and perchlorate were detected in 99%–100% of pregnant women. Many chemicals were measured at levels similar to the levels found in epidemiological studies that were found to have adverse reproductive and developmental outcomes. Furthermore, virtually all women were exposed to multiple chemicals. Exposure to multiple chemicals that affect the same health endpoint can result in a greater risk than by exposure to a single chemical—the U.S. National Academy of Sciences recommends that health risk assessments account for such simultaneous exposures to multiple chemicals.[70]

Table 20.1 Examples of Reproductive and Developmental Health Impacts of Prenatal Exposure to Environmental Contaminants

Chemical	Exposure Sources and Pathways	Reproductive/Developmental Health Impact
Bisphenol A (BPA)	Chemical intermediate for polycarbonate plastic and resins. Found in consumer products and packaging. Exposure through inhalation, ingestion, and dermal absorption.	Recurrent miscarriage[36] Aggression and hyperactivity in female children[37]
Lead	Occupational exposure occurs in battery manufacturing/recycling, smelting, car repair, welding, soldering, firearm cleaning/shooting, stained glass ornament/jewelry making; non-occupational exposure occurs in older homes where lead-based paints were used, in or on some toys/children's jewelry, water pipes, imported ceramics/pottery, herbal remedies, traditional cosmetics, hair dyes, contaminated soil, toys, costume jewelry.	Alterations in genomic methylation[38] Intellectual impairment[39] Increased likelihood of allergies[40]
Mercury	Mercury from coal fired power plants is largest source in the U.S. Primary human exposure by consumption of contaminated seafood.	Reduced cognitive performance[41,42] Impaired neurodevelopment[43,44]
Polybrominated diphenyl-ethers (PBDEs)	Flame retardants that persist and bioaccumulate in the environment. Found in furniture, textiles, carpeting, electronics, and plastics which are mixed into, but not bound to foam or plastic.	Impaired neurodevelopment[45] Premature delivery, low birth weight and stillbirth[46]
Polychlorinated Biphenyls (PCBs)	Used as industrial insulators and lubricants. Banned in the 1970s, but persistent in the aquatic and terrestrial food chains resulting in exposure by ingestion.	Development of ADHD associated behavior[47] Increased BMI[48] Reduced IQ[49]
Perfluorochemicals (PFCs)	Widely used man-made organofluorine compounds with many diverse industrial and consumer product applications. Examples are perfluorooctane sulfonate (PFOS) and perfluorooctanate (PFOA), which are used in the manufacture of non-stick Teflon® and other trademark cookware products and in food-contact packaging to provide grease, oil, and water resistance to plates, food containers, bags, and wraps that come into contact with food. They persist in the environment. Occupational exposure to workers and general population exposure by inhalation, ingestion, and dermal contact	Reduced birth weight[50]
Perchlorate	Used to produce rocket fuel, fireworks, flares, and explosives and can also be present in bleach and in some fertilizers. Primary pathway for exposure is through drinking water due to contaminated runoff.	Altered thyroid function[51]
Pesticides	Applied in large quantities in agricultural, community, and household settings. In 2001, over 1.2 billion pounds of pesticide active ingredients were used in the U.S. Pesticides can be ingested, inhaled, and absorbed by the skin. The pathways of pesticide exposure include food, water, air, dust, and soil.	Impaired cognitive development Impaired neurodevelopment[52,53] Impaired fetal growth[54] Increased susceptibility to testicular cancer[55] Childhood cancers[56]
Phthalates	Synthetically derived, phthalates are used in a variety of consumer goods such medical devices, cleaning and building materials, personal care products, cosmetics, pharmaceuticals, food processing, and toys. Exposure occurs through ingestion, inhalation, and dermal absorption.	Reduced masculine play in boys[57] Reduced anogenital distance[58] Shortened gestational age[59] Impaired neurodevelopment in girls[60]
Toluene	Exposure occurs from breathing contaminated workplace air, in automobile exhaust, some consumer products, paints, paint thinners, fingernail polish, lacquers, and adhesives.	Decreased fetal and birth weight[61] Congenital malformations[62,63]

From Sutton P, et al: Toxic environmental chemicals: the role of reproductive health professionals in preventing harmful exposures. *American Journal of Obstetrics and Gynecology,* 207(3):164-173, 2012.

Fetal Exposures

Maternal body burden of chemicals during pregnancy can lead to the potential for fetal exposure. Chemicals that have been found in the fetal environment include pharmaceuticals, illicit drugs, alcohol, tobacco, metals, pesticides, PCBs, PBDEs, and phytoestrogens among other chemicals.[71]

The placental transfer of chemicals is well established and varies depending on the chemical structure and composition; for example some chemicals may have a higher or lower affinity for crossing the placenta. Several studies highlight the ability and correlation of maternal-fetal doses from exposure to common environmental contaminants.

In a birth cohort study from the Faroe Islands, where the population is exposed to high levels of environmental chemicals through their marine-based diet, the authors measured the concentration of 87 environmental pollutants in mother-child samples and found that virtually all substances that were found in the mothers were also present in fetal tissues and cord blood, however, the degree of transplacental passage and correlation with

maternal serum varied depending on structure and size of the chemical.[72] For example, amongst the five perfluorinated chemicals measured, shorter chain PFCs and PFCs with sulfonic acid as the active group transferred more readily than long chain PFCs or PFCs with carboxylic acid as the active group. Similarly PBDEs that are more highly brominated were detected at lower concentrations than those with fewer bromines, supporting the evidence that brominated substances are less efficiently transferred into cord blood.[72]

A 2011 study by Gützkow and colleagues measured perfluorinated alkyl acids (PFAAs) in 123 paired samples of maternal and fetal plasma from a subcohort of the Norwegian Mother and Child Cohort Study. Strong and highly significant correlations were found between maternal and cord blood concentrations on fresh weight basis for PFOA, PFOS, and three other PFAAs.[73] Compared to the maternal samples, cord plasma had a 1.4 to fourfold lower median concentration of all the PFAAs measured. Placental transfer was found to be selective with a higher proportion of shorter chained PFAAs in cord blood together with a higher amount of branched isomers of PFOS indicating specific fetal exposures.

Balakrishnan and colleagues (2010) investigated the placental transfer of the pseudo-persistent chemical BPA at environmentally relevant concentrations. Within 3 hours of exposing the placenta to BPA, about 27% of BPA was detected in the fetal compartment, demonstrating that BPA at low concentrations can cross the human placenta mainly in the unconjugated or active form.[74]

In addition to crossing the placental barrier, some environmental chemicals are capable of bioaccumulating in the fetus resulting in greater exposure during this sensitive period of development. For example, fetal methyl mercury levels have been shown to be about 1.7 times greater than maternal levels.[75] Higher blood pressure and maternal alcohol use have been shown to be associated with relatively greater fetal blood lead concentration compared to maternal concentrations while other factors appear to reduce the degree of bioaccumulation.[76]

Mechanisms of Action

There are many environmental contaminants that can affect reproductive health,[18] and they can do so through diverse biological mechanisms. Traditionally the concern with exposures to chemicals has focused on genotoxic or mutagenic effects—i.e., those that can damage or interfere with the integrity of genetic material. However, exposure to chemicals can act in other dangerous and subtle ways, for example, by epigenetic and/or by affecting the production and transport of hormones.

Mutagenic Mechanisms

Mutagenic mechanisms are caused by exposures that induce DNA damage and can adversely affect reproduction and development. A well-documented example is radiation-induced cancer due to exposure to ionizing radiation, which is the rationale for the growing concern about the sharp rise in the use of computed tomography scans in medicine due to the non-negligible radiation exposures involved, particularly for pediatric patients.[77] It is generally believed that complex forms of DNA double-strand breaks are the most biologically important type of lesions induced by ionizing radiation, and these complex forms are likely responsible for subsequent molecular and cellular effects.[78]

Epigenetic Mechanisms

Epigenetic, in contrast to mutagenic, mechanisms act by altering the gene activity without changing the DNA sequence. This is often accomplished through methylation of the DNA and chromatin modification that can lead to changes in chromatin folding and gene expression.[79] Recent animal studies have demonstrated that a single exposure to the commonly used fungicide vinclozolin affected the physiology, behavior, and metabolical activity in offspring three generations removed through the inheritance of epigenetic changes.[80]

Endocrine Disrupting Mechanisms

Endocrine disrupting compounds (EDCs) are chemicals that act by perturbing the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior.[81] Mechanisms of EDCs (e.g., estrogenic, anti-estrogenic, anti-androgenic) involve binding to receptors and having effects on enzymes or transport proteins (Table 20.2A, 20.2B). EDCs can bind to hormone receptors producing agonist effects, those that mimic endogenous hormones[21] (for example, experiments have demonstrated that BPA can act additively on ovarian estrogens[23]), or antagonist effects (i.e., those that inhibit receptor activity such as vinclozolin that interferes with the androgen receptor).[21,82] EDCs can also act at the level of steroid biosynthesis or metabolizing enzyme[83]—for example, PCBs can interfere with thyroid hormone action and it has been known to cause a decrease in circulating total and free T_4 levels.[23] Hormone action can also be prevented by enzymatic conversion of the hormone from the active to the inactive form.[23] Furthermore, EDCs can disrupt neural and immune signaling pathways[84-86]; and alter the regulation of gene expression by way of DNA methylation, RNA stability, protein degradation (reviewed in Edwards and Myers[87]).

Common EDCs include BPA, phthalates, and certain pesticides. Often the molecular structure of EDCs contains a central ring that mimics the structure of steroid hormones. Thus, these compounds generally affect estrogen, androgen, and thyroid signaling, which are essential for normal embryonic development and reproductive activity in all vertebrates studied to date.[89-91]

EDCs are clinically relevant because they are associated with a wide range of effects on male and female reproduction including breast development and cancer, prostate cancer, neuroendocrinology, thyroid, metabolism and obesity, and cardiovascular endocrinology.[21] EDCs can have multiple hormonal effects, for example, DDT is an estrogen disruptor, while its metabolite, dichlorodiphenyl dichloroethylene (DDE), is an androgen antagonist[21]; and BPA perturbs both estrogen and thyroid hormones.[92] EDCs may exert dose-response curves that are not linear,[21] and as described in the next section, low dose exposure during critical and sensitive periods of development may be more potent than higher dose exposures.[24]

Table 20.2 Mechanisms of Action of Endocrine Disrupting Chemicals

A. Binding to Receptors

Substances	Mechanism	Major Effect	Biological Effects
Agonists			
Bisphenol-A Phthalates Polyphenols including isoflavones and genistein Some UV-screens (benzophenone 2; cinnamate; camphor derivatives)	Activation via ERE	Estrogenic	Adverse effects on reproduction; uterus, mammary gland
Antagonists			
Pesticides, fungicides, herbicides (linurone, procymidone, vinclocolin) dioxin	Various mechanism often inhibition of ARE mediation	Antiandrogenic	Adverse effects on reproduction

B. Effects on Enzymes or Transport Proteins

Substances	Mechanism	Affected Hormone(s) Biological Effect
Fungicides (azoles)	Synthesis inhibitors: Inhibition affected step of synthesis (sterol dementhylase and chromatase)	All steroids; inhibition of adrenal steroids is particularly important; life-threatening reduction of adrenal corticoids
Isoflavones	Inhibition of thyroid peroxidase	Thyroxin synthesis (hypothyroidism)
Polyphenols (isoflavones, genistein)	Sulfatase increased Decreased sulfo-transferase Effects on proteins transport	Free estrogen increased
Isoflavones, polychlorinated diphenols	Transthyretin (the transport protein of thyroid hormones)	Altered amounts of free thyroid hormones

EDs, Endocrine disruptors; ERE, Estrogen Response Elements; ARE, Androgen Response Elements.
From Wuttke W, Jarry H, and Seidlova-Wuttke D, *Definition, classification and mechanism of action of endocrine disrupting chemicals.* Hormones (Athens). **9**(1):9-15, 2010.

Critical and Sensitive Windows of Susceptibility

Exposure to chemicals during pregnancy and development are of particular import since these are times of DNA demethylation-methylation during gametogenesis and embryogenesis, rapid growth, and differentiation.[93] The nature and extent of the health consequences of exposure to environmental chemicals are influenced by the timing of exposure, i.e., the "window of susceptibility." A critical window of susceptibility is when exposures to environmental contaminants can disrupt or interfere with the physiology of a cell, tissue, or organ[19]; exposures that occur during this time can lead to permanent and lifelong health effects and can often be passed down to future generations. Sensitive windows of susceptibility can still cause disease but with less intensity and severity as compared to those occurring in the critical window.[94] Critical and sensitive windows occur during times of rapid development and cellular differentiation such as periconception, pregnancy, infancy, childhood, puberty, and lactation.

Exemplary of the concept of windows of vulnerability, studies have found that ethylene oxide can induce skeletal effects when administered to mice at the zygote stage of development—at a time that is long before skeletogenesis begins.[95,96] The skeletal effects that are observed after exposure at the zygote stage are different than those observed after exposure during organogenesis. Another example of how timing of exposure matters is the neurotoxic impacts of the pesticide chlorpyrifos. The conventional view of the mechanism for neurotoxicity from chlorpyrifos exposure involves the irreversible inhibition of acetylcholinesterase. However, animal studies demonstrated that the effects of chlorpyrifos on the developing brain are subtle, widespread, and can occur below the threshold for any signs of exposure and even without any detectable cholinesterase inhibition.[97,98] Moreover, these studies have shown that chlorpyrifos disrupts rat brain development through a variety of cellular and molecular mechanisms, with both the mechanism and the outcome changing with the progression of cell differentiation.

Of note are recent data regarding differences in DNA methylation in placentae and cord blood from children conceived by IVF vs. naturally conceived children. DNA methylation was investigated at 1536 CpG sites (700 genes) in placenta and cord blood. IVF was associated with lower mean methylation at CpG sites in placenta and higher mean methylation at CpG sites in cord blood. Differences were observed in imprinted and nonimprinted genes, and several of these expressed genes have been implicated in chronic metabolical disorders (e.g., obesity, type II diabetes). The reasons for this are unclear, but environmental influences in culture conditions or on parental loci have been postulated.[99]

Developmental Basis of Disease and Dysfunction

The Developmental Basis of Adult Disease and Dysfunction describes the interaction between the *in utero* environment, the external environment, an individual's genes, and the propensity to develop disease or dysfunction later in life.[21] The central nervous system, the cardiovascular system, the endocrine system, and the immune system appear to be particularly vulnerable to adverse effects during development, and specific diseases, such as particular forms of cancer, may well be initiated before birth or during early postnatal life.[19]

Box 20.1 Features of "Developmental Origins of Disease and Dysfunction" Paradigm Common to Both Nutritional and Environmental Exposure Studies

TIME SPECIFIC (VULNERABLE WINDOW) AND TISSUE-SPECIFIC EFFECTS MAY OCCUR WITH BOTH NUTRITIONAL AND ENVIRONMENTAL CHEMICAL EXPOSURES
The initiating *in utero* environmental insult (nutritional or environmental chemical) may act alone or in concert with other environmental stressors. That is, there could be an *in utero* exposure that would lead by itself to pathophysiology later in life or there could be *in utero* exposure combined with a neonatal exposure (same or different environmental stressor[s]) or adult exposure that would trigger or exacerbate the pathophysiology.

THE PATHOPHYSIOLOGY MAY MANIFEST AS: THE OCCURRENCE OF A DISEASE THAT OTHERWISE WOULD NOT HAVE HAPPENED; AN INCREASE IN RISK FOR A DISEASE THAT WOULD NORMALLY BE OF LOWER PREVALENCE; OR EITHER AN EARLIER ONSET OF A DISEASE THAT WOULD NORMALLY HAVE OCCURRED; OR AN EXACERBATION OF THE DISEASE
The pathophysiology may have a variable latent period from onset in the neonatal period, to early childhood, to puberty, to early adulthood, to late adulthood depending on the environmental stressor, time of exposure, and tissue/organ affected.

EITHER ALTERED NUTRITION AND/OR EXPOSURE TO ENVIRONMENTAL CHEMICALS CAN LEAD TO ABERRANT DEVELOPMENTAL PROGRAMMING THAT PERMANENTLY ALTERS GLAND, ORGAN, OR SYSTEM POTENTIAL. THESE STATES OF ALTERED POTENTIAL OR COMPROMISED FUNCTION (REGARDLESS OF THE STRESSOR—NUTRITIONAL OR CHEMICAL EXPOSURE) ARE LIKELY TO RESULT FROM EPIGENETIC CHANGES (E.G., ALTERED GENE EXPRESSION DUE TO EFFECTS ON IMPRINTING, AND THE UNDERLYING METHYLATION-RELATED PROTEIN-DNA RELATIONSHIPS ASSOCIATED WITH CHROMATIN REMODELING). THE END RESULT IS AN INDIVIDUAL THAT IS SENSITIZED SUCH THAT IT WILL BE MORE SUSCEPTIBLE TO CERTAIN DISEASES LATER IN LIFE
The effect of either developmental nutrition or environmental chemical exposures can be transgenerational, affecting future generations.

WHILE THE FOCUS OF NUTRITIONAL CHANGES DURING DEVELOPMENT HAS BEEN ON LOW BIRTH WEIGHT, EFFECTS OF *IN UTERO* EXPOSURE TO TOXIC ENVIRONMENTAL CHEMICALS OR NUTRITIONAL CHANGES MAY BOTH OCCUR IN THE ABSENCE OF REDUCED BIRTH WEIGHT. THE LACK OF A SPECIFIC EASILY MEASURABLE BIOMARKER FOR THESE EFFECTS THAT IS SIMILAR TO BIRTH WEIGHT MAKES IT MORE DIFFICULT TO ASSESS DEVELOPMENTAL EFFECTS. THUS, FOR BOTH EXPOSURES, NEW, MORE SENSITIVE BIOMARKERS OF EXPOSURE ARE NEEDED
Extrapolation of risk from both nutritional studies and environmental exposures may be difficult because effects may not follow a monotonic dose–response relationship. Nutritional effects which result in low birth weight are different from those that result in high birth weight. Similarly, low dose effects of environmental chemicals may not be the same as the effects that occur at higher doses. Also, the environmental chemical and/or nutritional effects may have an entirely different effect on the embryo, fetus, or perinatal organism, compared to the adult.

EXPOSURE OF ONE INDIVIDUAL TO AN ENVIRONMENTAL STRESSOR (ENVIRONMENTAL CHEMICAL OR NUTRITIONAL OR COMBINATIONS) MAY HAVE LITTLE EFFECT, WHEREAS ANOTHER INDIVIDUAL WILL DEVELOP OVERT DISEASE OR DYSFUNCTIONS DUE TO DIFFERENCES IN GENETIC BACKGROUND INCLUDING GENETIC POLYMORPHISMS

From Environmental Impacts on Reproductive Health and Fertility, edited by Tracey J. Woodruff, Sarah J. Janssen, Louis J. Guillette, Jr, and Linda C. Giudice. Copyright (c) 2010 Cambridge University Press. Reprinted with permission.

The influence of perinatal exposures and their effect on adult disease was first described in the field of nutrition when it was seen that "low birth weight" resulting from poor maternal nutrition is a cause of disease and illness later in life such as obesity, coronary heart disease, type II diabetes, and metabolical dysfunction among others.[100] This concept was later independently developed in the field of developmental toxicology (Box 20.1).[33] Animal studies have demonstrated that the *in utero* and neonatal development periods are a "critical window" for nutrition as well as for exposure to environmental chemicals and can have an impact on developing regulatory systems.[101] For example, there is some evidence that environmental endocrine disrupting chemicals can act as "obsesogens" and can permanently alter the developing regulatory systems required for body weight homeostasis.[101,102]

Identification of Chemicals with Reproductive and Developmental Toxicity

The information available to clinicians in the field of environmental health science differs from that available for decision-making in the clinical health sciences. Clinicians are accustomed to pharmaceuticals going through comprehensive *in vitro* and *in vivo* testing as mandated by regulatory agencies. However, in regards to environmental exposures, clinicians cannot assume that the safety of products has been established and must recognize that most patient exposure occurs in the absence of information on reproductive, developmental, and other toxicities.

Randomized control trials (RTCs) are considered the "gold standard" for establishing the risk-benefit of clinical decision-making. However, ethical considerations preclude RCTs in the assessment of the health impacts of exposure to environmental chemicals. In the clinical arena, RTCs are conducted to assess whether the benefits of exposure to an exogenous chemical outweigh the risks. With environmental chemicals the benefits are often unrelated to patient's health and exposures are often unavoidable and highly variable.

Therefore, clinicians must rely on the available animal and epidemiological data to shape patient recommendations and prevention strategies related to environmental chemicals. Animal studies of reproductive and developmental toxicity are recognized as reliable predictors of human health effects.[103-106] Since 1984, a technical report by the National Center for Toxicological Research, as well as other studies, have established the concordance of developmental and reproductive effects between animal and human studies

and concluded that the humans are as sensitive as or more sensitive than even the most sensitive animal species.[95,105]

Epidemiological studies provide the most direct link between an environmental exposure and increased risk of an adverse human health outcome. However, these studies require waiting a long time after exposures have occurred to assess harm, as it can take decades for the health impacts of exposure to be manifest. Thus, sole reliance on epidemiological studies squanders the option of preventing exposures before such harm has occurred.

The Role of Clinicians in Prevention

Clinicians can serve as a science-based source of guidance for their patients on recognizing, avoiding, and preventing harmful exposures to environmental chemicals.[107,108] For example, clinicians can provide information on the science and areas of uncertainty about environmental chemicals so that patients can make informed choices based on their values and preferences. Science-based, patient-centered educational materials to support patient counseling activities can be found at: http://www.prhe.ucsf.edu/prhe/toxicmatters.html. Patients who are pregnant or who are planning on becoming pregnant are often highly motivated and interested in finding out about chemical exposures or unaware about the risks caused by exposure.

When counseling patients it is important that clinicians have the capacity to:

- Understand the patient risk from exposure to environmental chemicals. Patient risk is a function of the toxicity and dose of the exposure. Routes of exposure include dermal, inhalation and ingestion and exposure is determined by the concentration, frequency and duration, and patient vulnerability, including any underlying health conditions.[109]
- Advise all patients on how to prevent exposures at home, work, and in the community. Women often do not know they are pregnant until after organogenesis has already begun so intervening in the periconception period to identify and address patient exposure is a way to maximize the potential to prevent harm.
- Understand socioeconomic and racial disparities can exacerbate the amount of environmental exposures experienced by an individual and understanding the environment of the patient population can help target high risk exposures.[110]
- Identify patients with hazardous exposures by taking an exposure history. Workplace exposures can be a significant source of exposure to toxic chemicals for pregnant women and legal workplace limits are not created to protect pregnant women.[111] Women of reproductive age with occupational exposures to substances with reproductive and developmental toxicity are at high risk and susceptible to adverse reproductive outcomes.[112]

While taking an exposure history is a key first step, many environmental exposures are not actionable by individuals, for example, air and water pollution. There are also many legal, economic, and social justice issues interwoven into the distribution of environmental chemicals throughout our society that cannot be addressed by individual action alone. Thus society-wide actions to prevent exposures are essential to patient and population health. Harnessing the voice of health professionals to policies to prevent exposure to environmental chemicals can be a powerful contributor to improved patient health.[20,34,111,113]

Summary and Conclusion

Adverse trends in indicators of reproductive and developmental health have occurred over roughly the same time frame as vast increases in population exposure to environmental chemicals. Currently, exposure to toxic environmental chemicals is ubiquitous and linked to a myriad of adverse reproductive and developmental health impacts that can occur across the lifespan of individuals. Exposures that occur during critical and sensitive windows of development (i.e., preconception, prenatal, infant, child, and adolescence) are of particular concern because the health impacts of these exposures can occur at environmentally-relevant levels of exposure and be permanent. Health professionals can play a critical role in patient health by intervening in clinical and policy arenas to prevent developmental exposure to environmental chemicals.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 21

Physiological and Pathophysiological Alterations of the Neuroendocrine Components of the Reproductive Axis

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Reproductive dysfunction of central etiology can result from any process that disturbs the tightly regulated hypothalamic–pituitary system. The reproductive axis itself relies on pulsatile release of gonadotropin-releasing hormone (GnRH) from a collection of neurons that are distributed diffusely throughout the hypothalamus rather than within a discrete nucleus.[1] Control of GnRH secretion is complex, including the kisspeptin ligand-receptor system described in detail below. Neuropeptide Y (NPY) facilitates GnRH release and gonadotropin responsiveness to GnRH. Substance P, beta-endorphin, leuenkephalin, and other endogenous opiates inhibit hypothalamic GnRH release, both acutely and tonically (in the case of beta-endorphin).[2,3] Stress-related surges in corticotrophin-releasing hormone (CRH) suppress GnRH gene transcription as well as GnRH release, as do inflammatory cytokines, such as tumor necrosis factor-α (TNF-α).[4] This CRH-induced suppression may be mediated by endogenous opiates.[5,6] The effects of catecholamines (norepinephrine, epinephrine, dopamine) are more controversial and likely depend on the prevailing endocrine milieu and the stage of sexual maturation.[2] Neurotransmitters, such as gamma-aminobutyric acid (GABA), have been shown to have both excitatory[7,8] and inhibitory[9,10] effects in animal studies, and use of the GABA-ergic drug valproic acid (VPA) in women did not affect hypothalamic–pituitary hormones.[9] Such conflicting data on the role of GABA in modulating GnRH release illustrate the challenges inherent in using rodent models to understand GnRH regulation in humans.[2]

Integration of these multiple signals ultimately results in neuronal release of GnRH, which then travels via the hypothalamic–hypophyseal portal circulation to the anterior pituitary gland, where it binds to the GnRH receptor 1 and up-regulates synthesis and secretion of two pituitary hormones—luteinizing hormone (LH) and follicle-stimulating hormone (FSH). GnRH secretion is pulsatile, with discrete bursts of GnRH effecting the release of pulses of LH and FSH by the pituitary. Moreover, the frequency of GnRH secretion has been shown in animal models to differentially affect the synthesis of LH and FSH, with rapid-frequency GnRH pulses (1 pulse/hour) favoring LH synthesis and slow-frequency GnRH pulses favoring the synthesis of FSH.[10] The secretion of LH and FSH is also regulated by direct negative feedback from gonadal products (estradiol [E_2], inhibin A and B). Once released from the pituitary, LH and FSH travel through the systemic circulation to stimulate sex steroid production and gametogenesis by the gonads. The patterns of hypothalamic, pituitary, and gonadal activity are responsible for the transition from childhood to adulthood, as well as for normal ovulatory cycles in postmenarchal women and normal sexual function in males.

The presence of appropriate quantities of these hormones is not enough to maintain a normal reproductive axis. Frequent sampling of plasma hormone concentrations has led to the belief that most hypothalamic hormones are secreted episodically, and often in a circadian rhythm. Hormonal release may be linked primarily to the sleep–wake cycle, synchronized with food ingestion, or related to the dark–light cycle. Thus, integrity of the hypothalamic suprachiasmatic nucleus, which maintains both the circadian rhythm and the sleep–wake cycle, is an important factor for regulating gonadal function in men and women.

Patients with abnormalities of this complex hypothalamic–pituitary axis often show undetectable or inappropriately low levels of pituitary gonadotropins (FSH and

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LH) in combination with low sex steroid levels (testosterone <100 ng/dL in males and E_2 <20 pg/mL in females). This combination of laboratory findings defines the condition *hypogonadotropic hypogonadism*. In contrast, patients with primary gonadal pathology have elevated FSH and LH levels in combination with low testosterone or E_2 levels, a disorder referred to as *hypergonadotropic hypogonadism*. The clinical presentation of hypogonadotropic hypogonadism varies with age of onset and degree of hormonal deficiency. Males may present with micropenis and cryptorchidism. Testicular function can be completely impaired, with Leydig cell atrophy, or testicular size can approach normal, with only mild Leydig cell impairment. The phenotype of hypogonadotropic hypogonadism in women is also quite variable, ranging from a classic eunuchoid appearance in some women (lower body segment [floor to *pubis*] >2 cm longer than the upper body segment [*pubis* to crown] and arm span >2 cm longer than height) to moderate breast development in others.[11] Amenorrhea, whether primary or secondary, frequently is the reason why women seek medical attention. The degree of ovarian follicular development depends on the duration of gonadotropin deficiency; in cases of congenital hypogonadotropic hypogonadism, few follicles beyond the primordial stage may be present.[12] The administration of exogenous gonadotropins (or exogenous pulsatile GnRH, when it was available) can effect ovulation and pregnancy.[13]

Although some patients with hypogonadotropic hypogonadism have no evidence of pulsatile LH secretion, others have detectable LH secretory pulses that are of insufficient frequency and amplitude to trigger gonadal steroid synthesis. A small number of patients show nearly normal patterns of LH secretion, but the LH molecules are inactive and consist only of the uncoupled α subunit.[10] This heterogeneity of basal LH secretion in patients with hypogonadotropic hypogonadism is mirrored in their responses to exogenous GnRH stimulation. Complete unresponsiveness to GnRH is rare,[14] but many patients show subnormal increases of FSH and LH. In other cases, FSH or LH levels may rise in isolation, or both may increase normally.[13,15]

Hypogonadotropic hypogonadism may be physiological, as occurs during puberty and lactation, or it may be a manifestation of congenital or acquired pathology. Congenital hypogonadotropic hypogonadism usually occurs in the absence of other pituitary hormonal deficits, whereas acquired GnRH deficiency may be caused by tumors, infiltrative diseases, infection, trauma, or radiation—all of which can also cause other hormonal deficiencies. Epilepsy is frequently associated with reproductive dysfunction, often polycystic ovary syndrome (PCOS). Abnormalities of other hormones (i.e., thyroid hormone, cortisol, prolactin [PRL]), overnutrition and undernutrition, exercise, stress, and medications can also cause functional hypogonadotropic hypogonadism. Hypogonadotropic hypogonadism commonly occurs with pituitary disease due to tumors and apoplexy, as well as other autoimmune and inflammatory disorders.

Hypothalamic Dysfunction

Reproductive integrity requires an intact hypothalamus, which secretes GnRH from neurons that are distributed diffusely throughout the hypothalamus rather than in a discrete nucleus.[1] As discussed earlier, GnRH is secreted in a pulsatile fashion, the frequency of which differentially affects the pulsatile secretion of the gonadotropins. Moreover, the frequency of GnRH secretion is known to affect gene expression, with rapid-frequency GnRH pulses (1 pulse/hour) favoring LH synthesis, and slow-frequency GnRH pulses favoring FSH synthesis. Pituitary LH and FSH secretion are also regulated by direct negative feedback from gonadal products (E_2, inhibin A and B). The patterns of hypothalamic, pituitary, and gonadal activity are responsible for the transition from childhood to adulthood, as well as for normal ovulatory cycles in postmenarchal females and sexual function and spermatogenesis in males. Disruption of hypothalamic GnRH secretion at any point during development can have transient or permanent effects on fertility. Such disturbances can result from congenital, structural, or functional abnormalities of the central nervous system (CNS)-hypothalamic component of the reproductive axis.

Congenital Disease of the Hypothalamus

Hypogonadotropic hypogonadism resulting from abnormal development of GnRH neurons is relatively rare, affecting 1:10,000 males and 1:50,000 females.[16] GnRH neurons originate during embryonic life outside of the CNS in the nasal placode. Olfactory neurons have a similar embryonic origin in humans and many other vertebrate species. Migration of olfactory axons toward the olfactory bulb anlage is required for normal olfactory bulb (OB) development.[17,18] GnRH neurons then migrate across the cribriform plate toward the OB in two phases—one before OB formation and one in association with OB formation.[19] The latter wave of migration occurs in association with olfactory, vomeronasal, and terminal nerves as well as neural cell adhesion molecules, ultimately resulting in GnRH neuronal cell dispersion in the arcuate nucleus of the medial basal hypothalamus by 14 weeks of gestation.[20,21] The mature GnRH axonal neuron network eventually coordinates pulsatile secretion of GnRH into the hypothalamic–hypophyseal portal circulation.[22] Disruption of this complex series of events can result in varying degrees of hypothalamic dysfunction, which manifests clinically as hypogonadotropic hypogonadism.

Congenital hypogonadotropic hypogonadism is usually divided into two categories: that which is accompanied by olfactory deficits (anosmic idiopathic hypogonadotropic hypogonadism [IHH] or Kallmann syndrome [KS]) and that which is associated with an intact sense of smell (normosmic IHH). Exciting progress has been made in identifying genetic causes for both anosmic and normosmic IHH,[23] as summarized in Figure 21.1 and Table 21.1. Some genes are necessary for the correct embryonic differentiation of the GnRH-secreting neurons (*fibroblast growth factor receptor 1 [FGFR1], fibroblast growth factor 8 [FGF8], negative elongation factor [NELF], heparin sulfate* 6-O-*sulfotransferase [HS6ST1]*), others are essential for the migration of the GnRH neurons to the hypothalamus (*KAL1, Prokineticin 2/Prokineticin 2 receptor 2[PROK2/PROKR2], chromodomain-helicase-DNA-binding protein 7 [CHD7]*),[24] and others are involved in the upstream signals which participate in the activation

of the GnRH neuron *(tachykinin 3/tachykinin 3 receptor [TAC3/TACR3], Kisspeptin1/Kisspeptin1 receptor [KISS1/KISS1R]*). The candidate genes include also GnRH, GnRH receptor, and the gonadotropins. However, most cases of GnRH deficiency in humans are sporadic. In the cohort of 106 patients with GnRH deficiency with and without anosmia reported by Waldstreicher and colleagues only 30% of cases were familial.[25] Because IHH predominantly impairs fertility, families with multiple affected members are few, but the availability of gonadotropin therapy to induce fertility in both affected males and females may increase vertical transmission of the condition.

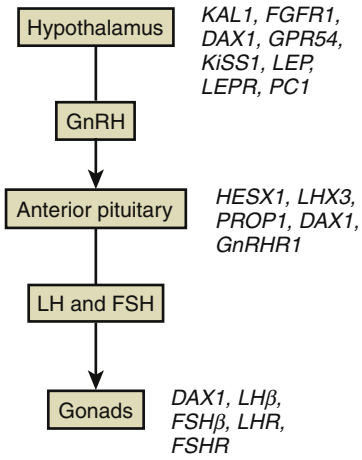


FIGURE 21.1 Schematic diagram of the hypothalamic–pituitary–gonadal axis showing genetic mutations that are responsible for some cases of hypogonadotropic hypogonadism. FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone. *(Modified from Cadman SM, Kim SH, Hu Y, Gonzalez-Martinez D, Bouloux P. Molecular pathogenesis of Kallmann's syndrome.* Horm Res *67:231, 2007. With permission from S. Karger AG, Basel.)*

Anosmic Idiopathic Hypogonadotropic Hypogonadism

KAL 1 *Mutation*

The pathological association between IHH and olfactory dysfunction was originally recognized in the 19th century, but Kallmann and Schoenfeld first suggested a genetic basis for this group of patients in 1944.[26] In 1989, Schwanzel-Fukuda and colleagues discovered a tangle of GnRH neurons on the surface of the cribriform plate in a human fetus with X-linked KS.[27] The finding of the neurons in this location rather than in the typical location in the hypothalamus established defective GnRH neuronal migration as the cause of hypogonadotropic hypogonadism in KS.[27,28] Because the initial differentiation and migration were normal, research then focused on factors affecting axonal growth, pathfinding, and maturation. The *KAL1* gene on the X chromosome (Xp22.3) was subsequently identified in 1991 by two groups, and both deletions and point mutations have since been found in patients with KS.[29,30] The product of *KAL1*, anosmin-1, is transiently expressed in numerous tissues of the developing human fetus, including the OB and along the migratory pathway for GnRH neurons. In the absence of anosmin-1 (with the *KAL1* mutation), OB differentiation and early olfactory axon navigation are impaired. Without olfactory nerve synaptogenesis in the OB, GnRH neurons have no migratory pathway to follow to the forebrain, resulting in the anosmia and hypogonadotropic hypogonadism typical of KS.[31] *KAL1* expression also occurs in the mesonephric tubules, ureteric bud, and corticospinal tract by 7 weeks of age, and in the retina and kidney by 11 weeks of age.[32] This tissue-specific expression accounts for the high prevalence of renal, neurological, and midline facial abnormalities in patients with KS. Unilateral renal agenesis is believed to occur because of developmental failure of the collecting duct system in the absence of mesonephric tubule anosmin-1. Synkinesia ("mirror movements," or

Table 21.1 Genes and Idiopathic Hypogonadotropic Hypogonadism

Clinical Features	Condition	Gene	Localization	Inheritance	Site of GnRH Impairment
HH + anosmia	Kallmann syndrome	*KAL1*	Xp22.3	X-linked	Hypothalamus (incomplete GnRH neuronal migration)
HH ± anosmia ± midline facial defects	Kallmann syndrome	*KAL2*	8p11.2	Autosomal dominant	
HH + anosmia	Kallmann syndrome			Autosomal recessive	
HH		*GnRH-R*	4q21.2	Autosomal recessive	Pituitary (↓ GnRH-R activity)
HH		*GPR54*	19p13	Autosomal recessive	Hypothalamus
HH + adrenal insufficiency	Adrenal hypoplasia congenital	*AHC*	Xp21	X-linked	Hypothalamus Pituitary
HH		*LHβ*	19q13.3	Autosomal recessive	Pituitary
HH		*FSHβ*	11p13	Autosomal recessive	
Optic atrophy + midline CNS abnormalities + HH	Septo-optic dysplasia	*HESX1*	3q21.1-21.2	Autosomal recessive	Pituitary
Obesity + HH	Obesity syndrome	*Leptin*	7q31.3	Autosomal recessive	Hypothalamus
Obesity + HH	Obesity syndrome	Leptin receptor	1q31	Autosomal recessive	
Combined hormone deficiencies (short stature, hypothyroidism, HH)		*PROP1*	5q	Autosomal recessive	Pituitary

AHC, adrenal hypoplasia congenital; CNS, central nervous system; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; GnRH-R, gonadotropin-releasing hormone receptor; HH, hypogonadotropic hypogonadism; LH, luteinizing hormone.
Modified with permission from Hay C, Wu F. Genetics and hypogonadotropic hypogondism. *Curr Opin Obstet Gynecol* 14:303, 2002.

the involuntary movements of one extremity when an individual is asked to perform rapid, repetitive motions with the other extremity) results from abnormal fast–conducting ipsilateral corticospinal tract projections.[3] Absence of anosmin-1 expression in the facial mesenchyme leads to a high incidence of cleft palate in patients with *KAL1* mutations. Renal agenesis and synkinesia were present in 31% and 85% of patients, respectively, of a British cohort of patients with KS.[33] These abnormalities were not present in any patients with KS without the *KAL1* mutation or in patients with normosmic IHH.

As is the case with all forms of hypogonadotropic hypogonadism, KS is much more common in males, with prevalence estimated at 1:7500 males versus 1:70,000 females.[34] The *KAL1* mutation accounts for only 3% to 15% of cases of anosmic IHH[16,35] and no more than one third of familial cases of IHH,[25] but it is highly penetrant. No cases of *KAL1* mutations have been described in female patients or in normosmic males.[35] Patients with KS usually do not undergo puberty, but a minority may have some degree of testicular growth or breast development, suggesting that GnRH deficiency is not always complete. Additional reproductive features may include cryptorchidism and microphallus. As is the case with other genetic defects discussed later, there is no clear genotype-to-phenotype relationship in those with a *KAL1* mutation,[34] illustrating the importance of modifier genes and environmental factors.

KAL 2 *Mutation*

Mutations in the gene encoding fibroblast growth factor receptor 1 *(FGFR1)*, a tyrosine kinase receptor, also referred to as *KAL2*, appear to be inherited in an autosomal dominant pattern and are seen in 7% to 10% of individuals with KS.[35] Fibroblast growth factor signaling is involved with the formation, growth, and shaping of a variety of tissues and organs, including the OB.[36] Fibroblast growth factor (FGF) receptor-1 *(FGFR1)* has been detected in both the nasal placode and the developing OBs of mice as well as along the migratory pathway for GnRH neurons and in the mature hypothalamus. Studies in rodents with targeted abolition of *FGFR1* signaling resulted in aplasia of the OB. Both *FGFR1* and anosmin-1 are coexpressed in the olfactory placode of human embryos by 4.5 weeks' gestation, and again at 8 weeks in the terminal nerve portion that guides GnRH neuronal migration to the hypothalamus.[28] Studies suggest that *FGFR1* is a ligand for anosmin-1.[24] Loss-of-function mutations in the *FGFR1* gene located on chromosome 8p11.2 can cause IHH, with or without anosmia.[37,38] At least 12 missense mutations have been identified in this gene thus far, and loss of function of the isoform *FGFR1c* is present in approximately 10% of male and female patients with KS.[22] Isoform *FGFR1c* is also important for palate morphogenesis as well as olfactory development.[39]

Unlike *KAL1* mutations, *KAL2* mutations are not always associated with a severe reproductive phenotype. A minority of patients may have delayed puberty or normal reproductive function, suggesting that the *KAL2* mutation may not prevent GnRH migration entirely, but may affect neuronal maturation in the hypothalamus. This variable expressivity of *KAL2* suggests a role for other genes or the environment in modifying the phenotypic expression of this mutation. It also raises the possibility that *KAL2* mutations account for other reproductive phenotypes associated with hypothalamic dysfunction, such as hypothalamic amenorrhea and delayed puberty.[37]

Congenital hypogonadotropic hypogonadism (CHH) is less severe in patients with *KAL2* muations than in those with *KAL1* mutations, and mean LH levels are significantly lower in *KAL1* patients compared to *KAL2* patients.[40] Identification of *KAL2* mutations and the relationship between FGF signaling and anosmin-1 may explain the male predominance of hypogonadotropic hypogonadism despite the low prevalence of the X-linked form of the disease. Females have two copies of the *KAL1* gene and, therefore, higher levels of anosmin-1, which may compensate for inadequate *FGFR1* function in the presence of a *KAL2* mutation. Thus, women with *KAL2* mutations often have a milder, if not a normal, reproductive phenotype. On the other hand, males have a lower level of *KAL1* or anosmin-1 expression and are unable to compensate for impaired FGF signaling in the presence of a *KAL2* mutation. Thus, their reproductive phenotypes tend to be more severe.[22]

Other Candidate Genes

As is the case for IHH as a whole, most cases of KS are sporadic and cannot be traced to familial transmission. Autosomal recessive transmission of KS does exist, but the responsible gene has yet to be cloned. Multiple other candidate genes that may be involved in cases of anosmic IHH without *KAL1* mutations have been identified in the past decade. Mutations of *CHD7*, which can cause CHARGE syndrome (coloboma, congential heart disease, choanal atresia, mental and growth retardation, genital hypoplasia, and ear malformations or deafness) have been seen in KS patients. Heterozygous mutations in *NELF* have been reported in a few patients with KS; the murine form of *NELF* is involved in olfactory and GnRH neuronal migration.[41] Mutations in *Pkr2*, which encodes a G-protein–coupled receptor critical for OB and reproductive organ development in mice.[25] have been found, as have mutations in the heparan sulfate 6-O-sulfotransferase 1 *(HS6ST1)* gene. The enzyme is involved in the modulation of extracellular matrix components, such as heparin sulfate polysaccharides, thought to modulate cell-cell communication, which is required for neural migration.[42] Heterozygous mutations of WD repeat domain 11*(WDR11)* have been found in a small number of patients with Kallmann syndrome. The *WDR11* gene encodes a protein which interacts with the transcription factor EMX1. The interaction between both factors is important for the development of olfactory neurons.[43] FGF receptor mutations have also been identified in normosmic individuals with IHH, suggesting that anosmic IHH and normosmic IHH are part of a spectrum of disease rather than distinct clinical entities.[22,38]

Normosmic Idiopathic Hypogonadotropic Hypogonadism

Not all patients with hypogonadotropic hypogonadism have an impaired sense of smell. Those with a normal sense of smell are given the diagnosis of IHH. In the American cohort reported by Waldstreicher and colleagues, approximately half of patients with hypogonadotropic hypogonadism were

normosmic.[25] Nasal mucosal biopsy specimens from patients with normosmic IHH showed immunologically recognizable GnRH, providing evidence that the GnRH gene itself was intact and that defective GnRH synthesis was not the cause of IHH in these patients.[21]

GnRH Mutations

There are also patients with IHH manifesting with severe hypogonadism who do have isolated GnRH deficiency. It was not until 2009 that a homozygous frameshift mutation in the GnRH1 gene in such patients was reported. GnRH1 encodes the preprohormone of GnRH. These patients had severe hypogonadism, suggesting that isolated GnRH deficiency can be a rare cause of IHH.[44,45] Other etiologies for IHH have since been identified in this group of patients, in whom the frequency of cryptorchidism[33] and nonreproductive phenotypes (synkinesia, palate abnormalities, hearing loss) is much less common than in anosmic IHH.[37]

GnRH Receptor Mutations

Attempts to identify congenital defects that would explain normosmic IHH have led to the discovery of loss-of-function missense mutations in the gene encoding the GnRH *receptor* (*GnRH-R*) on chromosome 4q21.2. These mutations are transmitted in an autosomal recessive pattern. The GnRH receptor is a G-protein–coupled transmembrane receptor involved with signal transduction. Mutations in this protein were initially considered unlikely because patients with normosmic IHH showed increased FSH and LH secretion in response to exogenous GnRH stimulation. However, in the study by de Roux and colleagues,[46] the index cases both had partial IHH and likely responded normally to GnRH stimulation because the exogenous dose was high enough to overcome their partial receptor defects. *GnRH-R* mutations are estimated to occur in 40% of autosomal recessive cases of normosmic IHH and in 10% to 15% of sporadic cases.[47,48] Heterozygotes are unaffected.[28]

Identical mutations can produce different clinical phenotypes, suggesting that gonadotroph function depends on other genes as well. The clinical presentation of patients with *GnRH-R* mutations can include microphallus, cryptorchidism, gynecomastia, and delayed puberty in males, and primary amenorrhea, incomplete thelarche, and delayed puberty in females.[48] Phenotypes can also be milder, as in men with normal adult-sized testes as well as intact spermatogenesis, but impaired LH-dependent Leydig cell testosterone secretion ("fertile eunuch syndrome").[49] It is now believed that GnRH secretion in these cases is sufficient to trigger enough Leydig cell testosterone production locally to maintain spermatogenesis and testicular growth, but insufficient to generate systemic testosterone levels capable of achieving full virilization.[50]

Screening for *GnRH-R* mutations at the time of diagnosis of IHH is not clinically useful unless fertility is an immediate treatment goal. In these cases, knowledge of a *GnRH-R* mutation would guide one toward gonadotropin therapy and away from pulsatile GnRH therapy (when it was available for clinical use), as the latter was less effective in restoring reproductive function. As with other confirmed genetic abnormalities, identification of a *GnRH-R* mutation would also allow screening and treatment of family members.[48]

GPR54 Mutations

Homozygous deletions in the gene *GPR54* on chromosome 19 (19p13) were first reported in 2003 in a large consanguineous family with multiple members affected by IHH but with normal GnRH receptors. *GPR54* encodes an orphan G-protein–coupled receptor that is expressed widely across the reproductive axis, including the hypothalamus, pituitary, gonads, and placenta.[51] The ligand for *GPR54* is kisspeptin (formerly metastin), which is derived from kisspeptin-1. Kisspeptin was originally discovered because of its ability to inhibit metastatic melanoma and breast cancer cell lines. Continued study of this protein suggests that it may play an important role in regulating central puberty through its ability to signal through *GPR54* and stimulate GnRH release. *GPR54* mutations account for 2% to 5% of normosmic IHH.[52] Both male and female *GPR54*-deficient mice had a hypogonadotropic hypogonadal phenotype (males with small testes, females with delayed vaginal opening and delayed follicular maturation), but had normal levels of hypothalamic GnRH and responded to exogenous GnRH.[46,53] Similarly, spermatogenesis and successful ovulation have been achieved in *GPR54*-deficient patients treated with exogenous GnRH. *GPR54* does appear to have tissue-specific effects, because the *in vitro* administration of kisspeptin metastin did not have the same stimulatory effects on pituitary gonadotrophs as it did on the hypothalamus, and homozygous mutations in *GPR54* did not affect spermatogenesis or ovarian steroidogenesis.[51] The exact mechanism by which *GPR54* regulates puberty remains the subject of ongoing investigation.

TAC3 Mutations

Homozygous mutations in tachykinin 3 (TAC3), which encodes neurokinin B and its heptahelical transmembrane G-protein-coupled receptor *TACR3*, have been described in patients with normosmic IHH. Patients with this mutation have very low basal LH secretion with a nonpulsatile pattern, while pulsatile GnRH treatment normalizes LH release.

Gonadotropin Mutations

Both FSH and LH are composed of a common α subunit and a β subunit that is unique to FSH, LH, thyroid-stimulating hormone (TSH), and human chorionic gonadotropin (hCG). Alpha subunit mutations have not been reported and likely are not compatible with life.[28] However, beta subunit mutations in both the LH[54] and FSH molecules[46] have been identified and are associated with delayed puberty.

Pituitary Transcription Factor Mutations

Mutations in various homeobox transcription factors involved with normal adenohypophyseal development have been reported. Homozygous mutations in *LHX3* have been described in two consanguineous families who presented with combined pituitary hormone deficiencies, including deficits in LH and FSH.[55] LHX3 encodes a protein which is required for pituitary development and motor neuron specification. Mutations in *PROP1* are also associated with variable degrees of FSH and LH insufficiency and other pituitary hormone deficiencies.[56,57] PROP1 has both DNA-binding and transcriptional activation ability. Its expression leads to ontogenesis of homozygous and heterozygous

mutations of *HESX1* and has been implicated in some cases of septo-optic dysplasia, a disease that includes optic nerve hypoplasia, pituitary gland hypoplasia, and midline CNS anomalies. Children with septo-optic dysplasia can have either precocious puberty or pubertal failure.[58,59]

Hypogonadotropic Hypogonadism and Adrenal Failure

Normosmic hypogonadotropic hypogonadism can occur in association with adrenal failure due to loss-of-function mutations in *DAX1* (adrenal hypoplasia congenital). *DAX1* encodes an orphan nuclear receptor on chromosome Xp21 and is critical for the development and function of both the adrenal gland and the hypothalamic–pituitary–gonadal axis. In the presence of *DAX1* mutations, adrenal failure usually presents in male infants or boys, and is followed by impaired pubertal development due to failure of hypothalamic and pituitary gonadotropin production. Males with this mutation are often resistant to gonadotropin stimulation because of impaired spermatogenesis. Steroidogenic factor 1 (SF1), another nuclear receptor that regulates gene transcription in both the adrenal gland and the gonads, caused both adrenal and gonadal agenesis when knocked out in a murine model.[60] Human SF1 mutations were associated with XY sex reversal and adrenal failure.[61] It is now thought that *DAX1* represses transcription of nuclear receptors, including SF1, and that loss of *DAX1* function causes a variety of adrenal, pituitary, and hypothalamic abnormalities.[50]

Hypogonadotropic Hypogonadism and Obesity Syndromes

Mutations in leptin[62] and the leptin receptor[63] have been identified in morbidly obese patients with normosmic IHH. It is leptin's effects on the release of NPY that are likely responsible for its effects on GnRH secretion.[64] Mutations in prohormone convertase 1 (PC1) also cause morbid obesity and hypogonadotropic hypogonadism, as well as hypocortisolemia (with elevated proopiomelanocortin) and hypoinsulinemia (with elevated proinsulin).[65]

Adult-Onset Idiopathic Hypogonadotropic Hypogonadism

An acquired form of GnRH deficiency was described in 1997 in 10 men who presented with decreased libido, impotence, and infertility at a mean age of 35 years, after undergoing normal puberty. None had features of congenital GnRH deficiency (anosmia, synkinesia, cleft palate, or a family history of GnRH deficiency), and none had a tumor, a history of radiation, infection, or infiltrative disease that might have explained their abnormal gonadotropin and sex steroid levels. Treatment with pulsatile GnRH reversed the hypogonadism and restored fertility in the subset of men who received long-term therapy.[66] Recent data show that some men with IHH, carry a mutation in the *FGF8* gene, and have normal reproductive function before onset of hypogonadism[67] suggesting a combination of genetic and environmental infuences.

Diagnosis of Idiopathic Hypogonadotropic Hypogonadism

Hypogonadotropic hypogonadism should be included in the differential diagnosis for any patient with absent or incomplete pubertal development. In males, this can include microphallus, cryptorchidism, absence of facial hair, and small testes; and in women, incomplete or absent breast development, amenorrhea, or a eunuchoid body habitus. Obtaining a family history can also be helpful when considering a diagnosis of IHH. Whereas the incidence of delayed puberty in the general population is less than 1%, the incidence of delayed puberty among relatives of a series of 106 patients with IHH was 12%.[50] Because the GnRH pulse generator is quiescent until the onset of puberty (after an initial period of activation during infancy), the diagnosis of hypogonadotropic hypogonadism is usually not made until the age of 18 years, when such physical findings might prompt laboratory evaluation. Characteristic findings include low sex steroid levels (testosterone <100 ng/dL; $E_2 < 20$ pg/mL) in association with low or inappropriately "normal" gonadotropin levels. Assessment of other pituitary hormones should also be performed when hypogonadotropic hypogonadism is initially discovered. To exclude structural causes of GnRH deficiency, magnetic resonance imaging (MRI) is indicated in the evaluation of such patients. When laboratory studies and negative findings on imaging studies offer no other explanation for hypogonadotropic hypogonadism, a diagnosis of IHH or isolated GnRH deficiency is appropriate.

Management of Idiopathic Hypogonadotropic Hypogonadism

Therapy for patients with IHH depends on the degree of sexual maturity and the desire for fertility. For men with severe gonadotropin deficiency who have yet to complete puberty, hCG is used as a surrogate for LH to stimulate Leydig cell testosterone secretion, typically using an intramuscular dose of 2000 IU three times weekly. Plasma testosterone and sperm counts are followed every 4 to 8 weeks, and hCG doses are adjusted to maintain normal testosterone levels. Recombinant FSH (75-150 IU three times weekly) is often required to improve the quality of the sperm produced, although it may not be required for initiation of spermatogenesis in men with partial IHH and those with postpubertal onset of hypogonadism. Inability to achieve a normal sperm count after 6 months of hCG treatment alone and declining sperm count despite hCG are indications to add FSH.[68-70] Success with this combination is possible, regardless of previous exposure to testosterone, although individuals differ in the timing of their response to induction of spermatogenesis.[71,72] Restoration of fertility occurs as early as 4 months in some men, but can take more than 1 year in others. Even if spontaneous pregnancy does not occur, adequate sperm are usually present after combined hCG and FSH therapy such that assisted reproductive techniques, including *in vitro* fertilization with intracytoplasmic sperm insertion, are good options. Subcutaneous or intravenous administration of GnRH via pump has been shown to achieve spermatogenesis more rapidly than treatment with gonadotropins, but it is no longer available.[73] Once pregnancy is achieved, hCG but not FSH should be continued to maintain normal serum testosterone and some spermatogenesis to facilitate future pregnancies. Men who only desire virilization can be given intramuscular or topical testosterone, depending on their preferred mode of administration.[35]

Cyclic estrogen and progesterone should be given to female patients to induce normal sexual development and

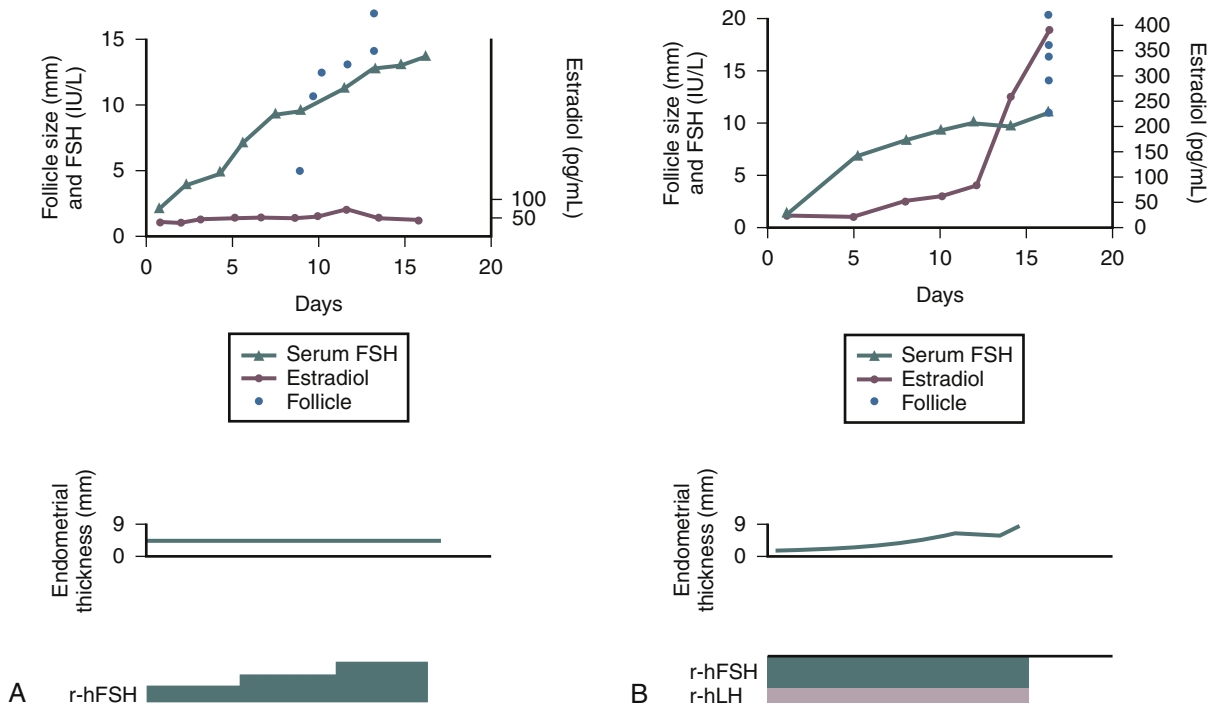


FIGURE 21.2 Ovarian response to recombinant follicle-stimulating hormone (r-hFSH) **(A)** or a combination of recombinant FSH and luteinizing hormone (r-hLH) **(B)** in women with severe hypogonadotropic hypogonadism. Follicle size and endometrial thickness were monitored by transvaginal ultrasonography. *(Modified from Shoham Z, Mannaerts B, Insler V, Coelingh-Bennink H. Induction of follicular growth using recombinant human follicle-stimulating hormone in two volunteer women with hypogonadotropic hypogonadism.* Fertil Steril *59:738, 1993.)*

preserve normal bone mineral density (BMD), although progesterone should not be added until breast development is complete.[48] Women who desire fertility should receive exogenous gonadotropins to stimulate ovulation. Recombinant FSH alone can induce follicular growth, as shown in Figure 21.2A, and has been shown to be as effective as highly purified FSH in achieving pregnancy, with a lower risk of ovarian hyperstimulation.[74] However, women with severe hypogonadotropic hypogonadism require some source of LH to generate adequate androgen precursors for aromatization to E_2, as illustrated in Figure 21.2B.[75] E_2 is required for endometrial proliferation and creation of a uterine environment optimal for implantation.[76,77]

Treatment with hCG (75 IU daily) may be used in such cases to maintain adequate E_2 concentrations. In women with baseline LH levels of greater than 1.2 IU/L, FSH alone may be sufficient to stimulate follicular growth as well as ovarian steroidogenesis. Once adequate follicular development and E_2-induced endometrial proliferation have occurred, exogenous recombinant hCG (250 μg) can be used to induce ovulation and is as effective as urinary hCG.[78] Recombinant formulations of LH are available, but their shorter half-life and higher cost preclude their widespread clinical use. Provision of luteal-phase progesterone is also recommended. When using FSH and hCG in combination, a woman's chance of conception is approximately 70% after six cycles. Such treatment does carry a risk of ovarian hyperstimulation and multiple gestations, and these risks are higher than those associated with pulsatile GnRH therapy when it was available.[79] For this reason, close monitoring with transvaginal ultrasound is required in women receiving gonadotropin therapy. In addition, pregnancies in women with hypopituitarism should be considered high risk because they are associated with higher rates of spontaneous abortion, possibly reflecting a uterine environment devoid of important endocrine growth factors.[80]

In addition to issues surrounding hormone replacement therapy and fertility treatment, there are other considerations when treating patients with IHH. Those with anosmic IHH and a family history suggestive of an X-linked mode of inheritance should have an abdominal-pelvic ultrasound at the time of diagnosis to assess for possible renal agenesis, given the relatively high frequency of this nonreproductive abnormality in patients with a *KAL1* mutation. Genetic testing is not routinely employed in patients with IHH because the majority of cases are sporadic. However, limited genetic testing is available for patients suspected of carrying a *KAL1* mutation. Research laboratories can also investigate for point mutations in *KAL1* or *FGFR1* and *GnRHR* gene mutations.[35]

Prognosis for Recovery

Until recently, it was believed that lifelong hormone therapy was required to maintain secondary sex characteristics and sexual function in men with IHH, but recent reports of testicular growth in men receiving maintenance androgen replacement therapy led to the identification of a minority of patients (10%) in whom reversal of IHH occurs. Such reversal can occur in men who underwent partial puberty or who were prepubertal at the time of diagnosis, and in men with either anosmic or normosmic IHH. It is believed that exposure to sex steroids promotes plasticity of the GnRH neuronal network, such that gradual reversal of IHH is possible, even in the face of genetic defects in men who

are treated with androgen replacement therapy. Thus, men being treated for IHH should have their hypothalamic–pituitary axis reassessed periodically so that such reversal can be identified.[81,82]

Structural Disease of the Hypothalamus

Mass lesions in the hypothalamus can also disrupt the secretion of GnRH into the hypothalamic–hypophyseal portal circulation and cause hypogonadotropic hypogonadism. Such cases usually cause other pituitary hormone abnormalities as well. Distinguishing hypothalamic mass lesions from pituitary mass lesions requires MRI. Dynamic testing with hypothalamic-releasing hormones is not used clinically, with the exception of CRH, which is used in combination with inferior petrosal sinus sampling (IPSS) to determine whether hypercortisolemia is of pituitary or ectopic origin.

Tumors

Craniopharyngioma

Craniopharyngiomas are rare, almost always benign, epithelial tumors arising from remnants of the craniopharyngeal duct that occur with an annual incidence of 0.13 cases per 100,000 person-years.[83,84] They are the most common lesions involving the hypothalamic–pituitary region in children, and account for 5.6% to 15% of the intracranial neoplasms in this population (2% to 5% in adults).[85,86] Craniopharyngiomas affect men and women at equal rates, and no genetic susceptibility has been identified.[83] They are histologically benign lesions, usually cystic or mixed (84% to 99%) rather than solid (1% to 16%).[87] Two subtypes have been identified adamantinomatous and papillary. The adamantinomatous type is more common in children and often contains calcification. Although tumor margins are usually sharp, adamantinomatous craniopharyngiomas tend to generate significant reactive gliosis in the adjacent normal brain tissue, making complete surgical resection difficult. Cysts may be multiloculated and contain viscous fluid rich in desquamated squamous epithelial cells and cholesterol.[84] Because of its sometimes brown-green appearance of this cystic fluid, the term "motor oil" has been used to describe it. A recent preliminary study showed that nuclear accumulation of b-catenin is present in adamantinomatous craniopharyngiomas, but not in papillary craniopharyngiomas.[88] The strong beta-catenin expression indicates the reactivation of the Wnt signaling pathway, which is involved in the development of several neoplasms.[89] The papillary subtype is seen almost exclusively in adults and is rarely associated with calcification. Papillary craniopharyngiomas tend to be solid or mixed rather than purely cystic, and they are less likely to infiltrate surrounding normal brain tissue, making surgical resection easier.[90] Papillary craniopharyngiomas usually lack the peripheral palisading of epithelial cells, cystic change, calcification, and the cholesterol depots found in the adamantinomatous type.

Craniopharyngiomas almost always have a suprasellar component and are rarely purely intrasellar,[91] as the images in Figure 21.3 show. Because of their proximity to vital neural structures and their significant size at the time of diagnosis (58% to 76% are between 2 and 4 cm), they frequently present with headache, nausea, vomiting, visual disturbances, growth failure (in children), and hypogonadism (in adults), although the rate of tumor growth also determines the severity of symptoms.[84,90] Bitemporal hemianopsia is the most common visual complaint, occurring in almost 50% of cases. Hydrocephalus can be present, occurring more frequently in children than in adults for unclear reasons (41% to 54% versus 12% to 30%).[84] With the exception of hydrocephalus and its associated symptoms, there was no difference in symptom duration or extent of hormone deficiency between children and adults with craniopharyngiomas.[91]

Both computed tomography (CT) and MRI can be helpful in diagnosing craniopharyngiomas. CT can show calcification that is characteristic of the adamantinomatous subtype, whereas MRI with gadolinium enhancement provides better structural analysis. The appearance of the craniopharyngioma depends on the proportion of solid and cystic components and the content of the cyst.[92] The solid tumor part is iso-or hypointense compared to the brain on precontrast T1 sequences and shows enhancement after contrast. On T2 sequences the signal is mixed, hypo- or hyperintense.[93] The cystic component is hypointense on T1 and hyperintense on T2. If there is protein, cholesterol, or methemoglobin present, then there may be a high T1 and a low T2 signal.[94] The cyst can show a peripheral contrast-enhancing rim in the T1 postcontrast images.[93] When the findings of imaging studies are consistent with craniopharyngioma, surgical resection is usually the initial therapy, although radiation therapy (RT) has been used for small lesions not causing pressure-related visual, neurological, or endocrinological damage. The surgical approach depends on tumor size, location, and degree of calcification, and the experience of the surgeon, but often involves craniotomy and sometimes a two-stage process. Preoperative drainage of large cystic components in the days before surgery can relieve pressure-related symptoms and make surgical resection easier, although cyst cavities refill if surgery is delayed by weeks. Gross total resection is the goal in all cases, but it often cannot be achieved because of tumor size and adherence to vital neurovascular structures.[84]

Unfortunately, craniopharyngiomas often recur even after gross total resection has been achieved and confirmed radiographically. Recurrence rates at 10 years vary from 0% to 62%, but improve with use of adjuvant RT. Recurrence-free survival correlates best with extent of surgical removal; age, sex, location (intrasellar versus suprasellar), and size of the tumor at the time of surgical resection do not predict recurrence. Some reports suggest that recurrent craniopharyngiomas show a higher microvessel density, lower levels of galectin-3 and macrophage migration inhibiting factor, and lower levels of retinoic acid receptor beta and higher levels of retinoic acid receptor gamma.[84] Management of recurrent disease usually involves RT rather than repeated surgery because of the perioperative morbidity and mortality associated with the latter. In fact, surgery for recurrent disease is only recommended in the event of acute pressure-related symptoms.[84] The 10-year progression-free survival rate from the time of the first recurrence was 72% in a series of 25 patients who received RT.[95] Other options for the management of recurrences include brachytherapy (for predominantly cystic lesions), bleomycin installation via an Ommaya reservoir, and stereotactic radiosurgery.[84,96]

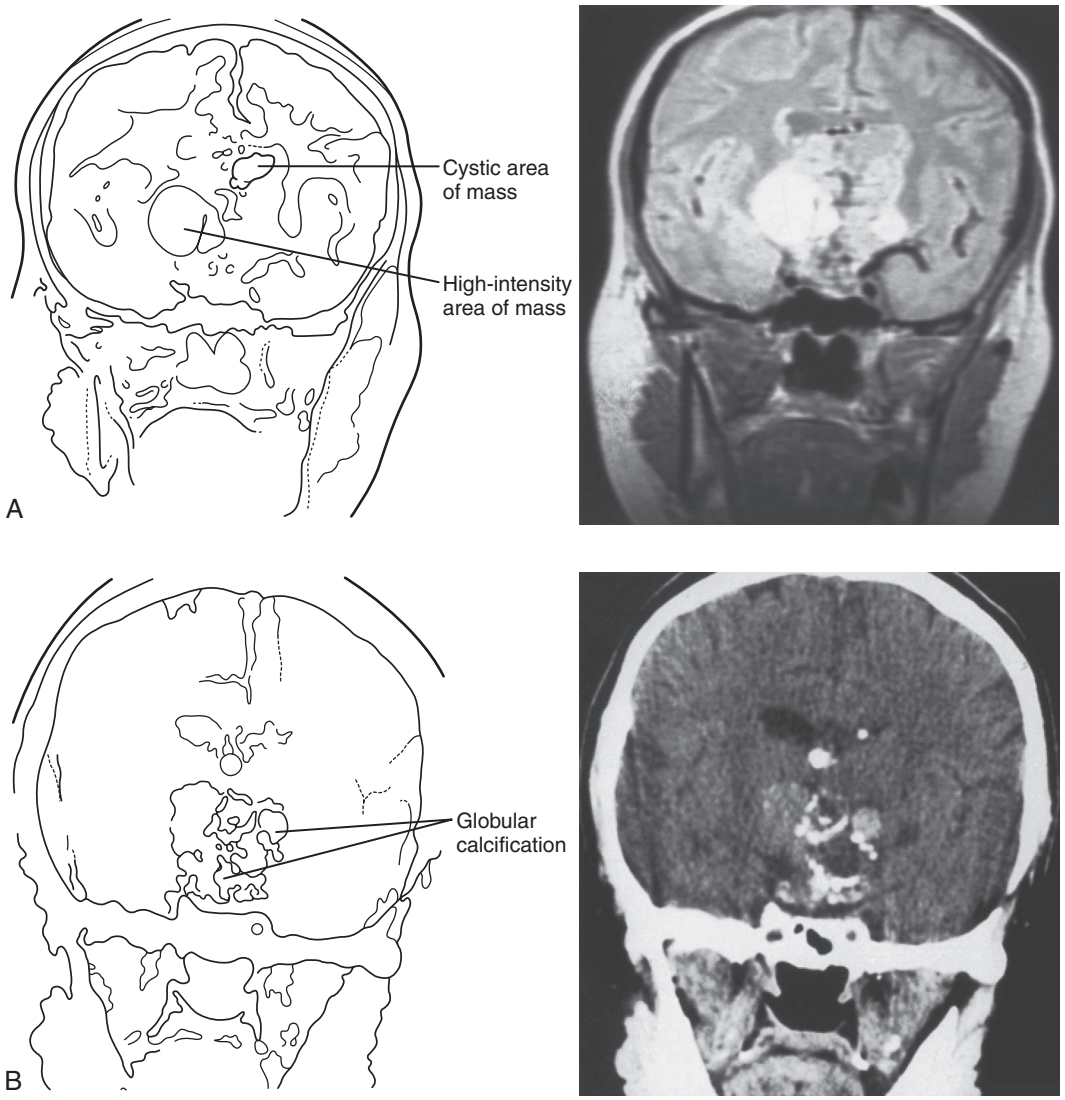


FIGURE 21.3 **A,** Schematic and proton-density–weighted coronal magnetic resonance imaging showing a cystic area and a high-intensity signal area in a large sellar-suprasellar mass, typical of craniopharyngiomas. **B,** Characteristic globular calcification is apparent on a coronal computerized tomography image of the same patient. *(Modified from Peebles T, Haughton VM. Neuroradiology and endocrine disease. In Besser GM, Thorner MO [eds].* Comprehensive Clinical Endocrinology, *3rd ed. London, Elsevier, 2002.)*

The endocrine, visual, and neuropsychological morbidities associated with craniopharyngiomas are significant. At the time of presentation, 35% of the patients in Van Effenterre's cohort had one to three hormone deficits.[97] After treatment, the frequency of individual hormone deficits varies significantly across studies (88% to 100% for growth hormone [GH], 80% to 95% for FSH and LH, 55% to 88% for adrenocorticotropic hormone [ACTH], 39% to 95% for TSH, and 25% to 86% for antidiuretic hormone [ADH]) and is not affected by the type of therapy. Unlike pituitary adenomas, it is very unusual for patients with pretreatment hormone deficits to experience restoration of those axes after treatment, but pituitary hormones can be replaced relatively easily. Visual defects (i.e., quadrantanopia or worse) are present in the majority of patients after surgery with or without adjuvant RT.[98] Potentially more disabling are the consequences of hypothalamic damage that occur so commonly in patients with craniopharyngiomas. Obesity secondary to hyperphagia affects 26% to 61% of patients who undergo surgery with and without adjuvant RT.[84] Diabetes insipidus (DI), with an impaired sense of thirst resulting in water and electrolyte imbalances, was present in 14% of a pediatric cohort.[99] Neuropsychological and cognitive functions often decline in patients with craniopharyngiomas. At follow-up 7 years after initial treatment, 16% of the adults and 26% of the children in Van Effenterre's cohort were not living independently and had not returned to their previous jobs or schools.[97] Mortality rates three to six times higher than those of the general population have been reported in patients with craniopharyngiomas.[84]

Rathke Cleft Cyst (RCC)

RCCs are the most common incidentally discovered sellar lesion. They are found in 13% to 33% of normal pituitary glands. They are discovered usually later in life with a peak between the 4th and 6th decades, which is most likely due to the fact that they grow slowly[100-102] and symptoms occur later in life. RCC originates from embryonic remnants of

the Rathke pouch which extends cranially to form the craniopharyngeal duct. The pouch is derived from the ectodermal stomadeum (primitive oral cavity). The Rathke pouch cells proliferate and form the pars anterior of the pituitary and the cells from the posterior wall differentiate into the pars intermedia. The pouch involutes and the craniopharyngeal duct obliterates. If the obliteration of the lumen does not occur, then a cyst develops and the remnants in the craniopharyngeal canal develop into cystic lesions ranging from simple RCCs to complex craniopharyngiomas.[102] RCCs are benign lesions which can range from a few milimeters to 5 cm. The content of the cyst is mostly thick and can consist of cholesterol and proteins. The cyst wall is lined by cuboid or columnar epithelium and occasionally with goblet cells secreting mucous into the cyst. RCCs and craniopharyngiomas have overlapping pathological features and are sometimes difficult to distinguish. Co-existence of RCCs with pituitary adenomas has been shown in 0.5% to 1.7%. Of these adenomas, GH secreting pituitary adenomas and prolactinomas are the most frequently described coexisting with RCCs. Location of RCCs are usually in the pars intermedia sometimes with suprasellar extension. Symptoms usually do not occur until the cyst is large enough to put pressure on the surrounding structures. The most common symptom includes headaches. The presence of headaches does not correlate with the cyst size, but seems to be related to either high or isointense content on T1-weighed MRI, mucous cyst content or cystic wall inflammation. Major visual field defects are present in up to 75% of cases and anterior pituitary hormone deficits are present in 19% to 81% of patients. Hypogonadism because of hyperprolactinemia is the most frequent hormonal manifestation. MRI often shows a hypointense lesion with rim enhancement and a hyperintense signal in the T-2 weighted images. Hyperintensity on T1-weighted images and isointensity on T2-weighted images suggests mucoid material is present in the cyst; hyperintensity in both T1- and T2-weighted images suggests the presence of blood. Cysts with high protein content show a high T1 signal intensity.[102] Only 13% of RCCs show calcification on CT. Asymptomatic, small RCCs are treated conservatively without surgery. If symptomatic, the aim is to drain the cyst content and to remove as much of the capsule as possible. Radiation is usually not used routinely for treatment with the exception of recurrent cysts. The 4-year recurrence rate has been reported to be 48%; the presence of squamous metaplasia in the cyst wall is associated with a higher risk of recurrence.

Germ Cell Tumors

Germ cell tumors (GCTs) are believed to result from malignant transformation and abnormal migration of primordial germ cells. They are characterized by their secretion of hCG and alpha-fetoprotein (αFP). Intracranial GCTs occur most commonly in the pineal region (50%) and in the anterior hypothalamus (30%).[103] They account for 2.9% of all intracranial tumors in children and have a peak incidence between ages 10 and 14 years old.[104] Presenting symptoms depend on tumor location: nausea, vomiting, diplopia, and paralysis of upward gaze (Parinaud syndrome) occur with pineal gland tumors, whereas suprasellar lesions can cause hypopituitarism, DI, and visual deficits.[103] Histologically, GCTs are often cystic and are classified as germinomatous GCT (GGCT) or nongerminomatous GCT (NGGCT). NGGCTs account for the minority of intracranial GCTs and include choriocarcinomas, teratomas, embryonal carcinomas, and yolk sac tumors. Serum hCG and AFP levels tend to be elevated in NGGCTs, whereas only a minority of patients with GGCTs have an abnormal serum hCG.[105] Stereotactic biopsy is indicated when the diagnosis is unclear from imaging studies and serum hCG and AFP levels. If a diagnosis of GGCT is made, therapy usually consists of radiation rather than resection, given the radiosensitivity of GGCTs and the neurological morbidity associated with surgery. Chemotherapy is an alternative therapy, but is associated with a higher recurrence rate when used in the absence of RT.[106] NGGCTs are less radiosensitive and, therefore, carry a poorer prognosis. However, platinum-based chemotherapy, in combination with craniospinal RT, is often used, followed by surgical resection if a residual mass persists.[107,108]

Other lesions occurring in the pineal region include pineal parenchymal tumors, astrocytomas, meningiomas, metastases, cysts, and vascular malformations. Hypothalamic–pituitary function can be disturbed by any of these.

Infiltrating Diseases

Sarcoidosis

Sarcoidosis is a multisystem disease of activated T lymphocytes and noncaseating granulomas that commonly affects the lungs, lymph nodes, eyes, and skin, but can involve the CNS and peripheral nervous system in 10% to 20% of cases. Neurological symptoms can be the presenting sign of disease in 50% of the latter group of patients and can increase morbidity and mortality. CNS lesions can appear as subdural plaques or infundibular plaques, infiltrating intraparenchymal pseudotumoral masses, or as multiple nodules on MRI studies. Although hypothalamic–pituitary lesions may not be evident on MRI, 25% to 33% of patients with neurosarcoidosis have DI and autopsy studies in such patients show extensive granulomatous inflammation of the hypothalamus, with little pituitary involvement. DI results from vasopressin deficiency and destruction of neurons in the neurohypophysis, but damage to the osmoreceptor controlling thirst also contributes to polyuria and polydipsia. Other symptoms of hypothalamic disease include somnolence, body temperature dysregulation, hyperphagia, and obesity.[109,110] Impaired secretion of anterior pituitary hormones also occurs; GH and gonadotropin deficiencies occur more commonly than TSH or ACTH deficiency.[110] In Murialdo and Tamagno's Italian series of 91 patients, 39% had hypogonadotropic hypogonadism, and 59% of the women in the study ($n = 46$) reported amenorrhea.[109]

As with other forms of sarcoidosis, confirmation of neurosarcoidosis requires histological evidence of noncaseating granulomas and lymphocyte infiltration of affected tissue, but obtaining nervous system tissue can be difficult. However, a probable diagnosis can be made with the proper clinical picture and imaging studies (i.e., MRI; Fig. 21.4). PRL elevation can be a useful screen for hypothalamic involvement. Results of cerebrospinal fluid (CSF) analysis are abnormal in 80% of patients with CNS sarcoid, and can include elevations in protein, lymphocytes, or angiotensin-converting enzyme levels.[109]

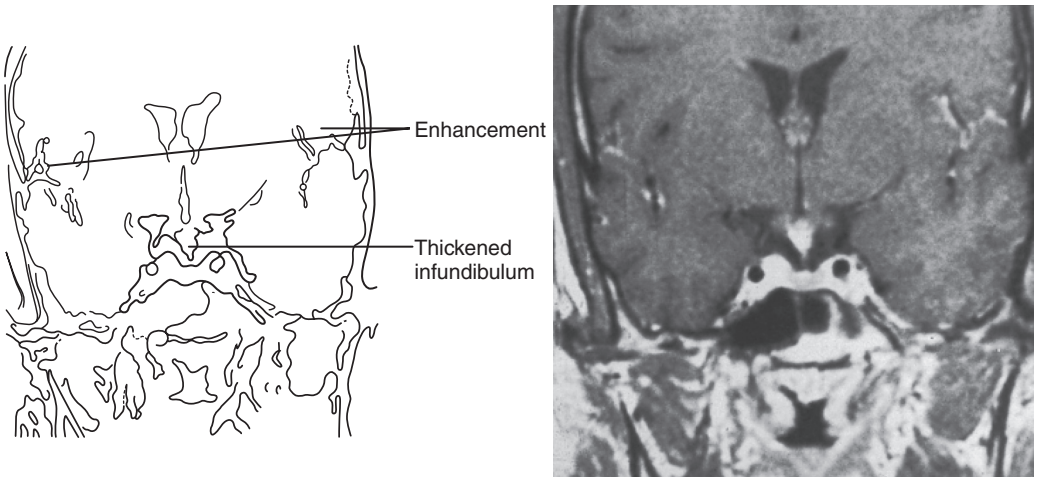


FIGURE 21.4 Schematic and T1-weighted magnetic resonance imaging after contrast administration showing a thickened, enhancing infundibulum typical of granulomatous disease, such as sarcoidosis. Enhancement in the sylvian fissure on the *left* and near the inner table on the *right* identifies leptomeningeal involvement. *(Modified from Peebles T, Haughton VM. Neuroradiology and endocrine disease. In Besser GM, Thorner MO [eds].* Comprehensive Clinical Endocrinology, *3rd ed. London, Elsevier, 2002.)*

Prednisone (up to 40 to 80 mg daily) is the mainstay of treatment for neurosarcoidosis, as it is for other forms of the disease, although therapy is often intensified in CNS disease. Cyclosporine A (4 to 6 mg/kg) is often added to minimize glucocorticoid requirements. RT is employed in patients who do not respond to oral medications. Unfortunately, anterior pituitary deficiencies usually are not restored, despite treatment. Dopaminergic agents can be used to decrease PRL levels, but they will not restore fertility if hypogonadotropic hypogonadism is also present.[109]

Sarcoidosis can also infiltrate the epididymis and testes, resulting in painless epididymitis or a scrotal mass, both of which respond to glucocorticoids. Granulomatous involvement of the uterus and fallopian tubes has also been reported in women with sarcoidosis who present with amenorrhea or menorrhagia.[110] Thus, reproductive dysfunction in patients with sarcoidosis can be both primary and secondary to CNS disease.

Langerhans Cell Histiocytosis

Langerhans cell histiocytosis (LCH) is another multisystem disease of unknown etiology that has a predilection for the hypothalamus and pituitary. It is characterized by the clonal accumulation and proliferation of abnormal dendritic histiocytes, with accompanying lymphocytes, eosinophils, and neutrophils. These cellular infiltrative lesions can destroy a variety of tissues, including skin, bone, lymph nodes, liver, spleen, lungs, and bone marrow. Known previously as *Letterer-Siwe disease, Hand-Schüller-Christian disease, eosinophilic granuloma,* and *histiocytosis X,* LCH primarily affects children between the ages of 1 and 3 years old. It can remit spontaneously or disseminate, even resulting in death in rare cases.[111] Adults make up fewer than 30% of all reported cases of LCH, with an incidence of 1.8 cases per million people. LCH in adults more commonly affects the skin, lung, and bones.[112]

The hypothalamic–pituitary axis is involved in 5% to 50% of children with LCH,[94] with 15% to 50% of these children presenting with DI.[113,114] The incidence of DI increases over time. It is usually seen within 5 years of diagnosis, and occurs more often in the setting of multisystem disease.[115] DI was also the earliest hormonal abnormality in the adult cohort reported by Kaltsas and colleagues, 33% of whom presented with DI.[112] Anterior pituitary hormone deficiency occurs in approximately 20% to 35% of patients and is usually associated with DI. In a large pediatric cohort, the estimated 5- and 10-year risks of pituitary involvement with LCH were 22% and 24%, respectively. GH deficiency is the most common pituitary endocrinopathy, occurring in 10% of patients with LCH and increasing in frequency over time. Ear, nose, and throat involvement increases the risk of GH deficiency.[114] GH deficiency in the absence of DI is unusual,[113] but 54% of patients with DI who were followed for 10 years ultimately had GH deficiency.[114] GH replacement effectively improves the final height in children with GH deficiency, although midparental height is usually not achieved because children have fairly severe growth restriction at the time therapy is initiated.[113,114] Importantly, GH therapy did not increase the risk of LCH disease activity.[114] GH replacement in adults with LCH has not been well studied.

Gonadotropin deficiency also occurs in LCH, almost always in association with other anterior pituitary hormone deficiencies. Because most patients are prepubertal at the onset of disease activity, those with gonadotropin deficiency require exogenous sex steroids to induce and maintain puberty.[113] Fifty percent of the cohort of 12 patients reported by Kaltsas and colleagues had gonadotropin deficiency at a median of 7 years old after diagnosis of LCH, and exogenous gonadotropins successfully restored fertility in 1 male patient.[112]

Findings on MRI are abnormal in the majority of patients with LCH and endocrinopathy. Loss of the posterior pituitary bright spot on T1-weighted images and infundibular enlargement are the most common abnormalities, with the former observed in 100% of patients with DI.[113] As shown in Figure 21.5, other less common radiological abnormalities include a thickened infundibular stalk, a partially or completely empty sella, and lesions in the hypothalamus.[111]

Both children and adults with LCH and DI are at high risk for anterior pituitary deficits and should be followed

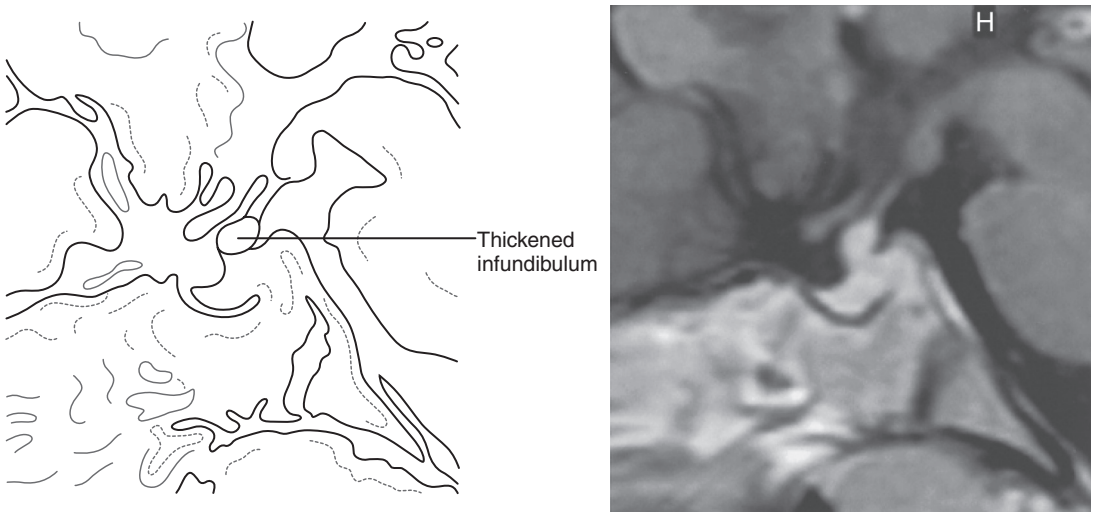


FIGURE 21.5 Schematic and sagittal T1-weighted contrast-enhanced magnetic resonance imaging showing a thickened enhancing infundibulum from known Langerhans cell histiocytosis (H). *(Modified from Peebles T, Haughton VM. Neuroradiology and endocrine disease. In Besser GM, Thorner MO, [eds].* Comprehensive Clinical Endocrinology, *3rd ed. London, Elsevier, 2002.)*

closely. Unfortunately, dynamic evaluation of pituitary function was not a useful predictor of later endocrinopathies.[116] DI and other hormonal deficiencies tend to be permanent once established, despite treatment of LCH lesions with radiation therapy, chemotherapy, or both. RT may be helpful for controlling localized disease and mass effects, however. Chemotherapy was not helpful in long-term disease management in adults.[112]

Other infiltrating diseases that can cause hypothalamic infiltration with resulting reproductive dysfunction include hemochromatosis (which more commonly affects the pituitary; discussed later in the chapter),[117] leukemia,[118,119] lymphoma,[120-122] and Wegener granulomatosis.[123-125] Central DI is often the presenting feature, and treatment of the underlying condition sometimes results in resolution of this and other associated hormonal abnormalities.[124,125] When associated with malignancies (leukemia, lymphoma), hypothalamic involvement is a poor prognostic feature and usually does not respond to treatment.[126]

Infections

Infections are a rare cause of hypothalamic–pituitary disease affecting the reproductive axis. They usually present acutely with fever, headache, meningismus, and change in mental status or seizure, and can be identified using culture, polymerase chain reaction, or detection of antibodies to viral antigens. Tuberculosis and atypical mycobacterial infections, viral encephalitis, mycotic infections, and bacterial meningitis can all cause infiltration of the hypothalamus or pituitary and result in hormonal deficiencies. Box 21.1 summarizes the classification of infiltrative, infectious, and other nontumoral diseases involving the hypothalamus that can cause hypogonadotropic hypogonadism.

Box 21.1 Classification of the Infiltrative, Infectious and Other Nontumoral Diseases Involving the Hypothalamus Causing Hypogonadotropic Hypogonadism

INFILTRATIVE OR AUTOIMMUNE DISEASES
- Sarcoidosis
- Langerhans' cell histiocytosis
- Lymphomatous diseases
- Castelman disease
- Wegener granulomatosis
- Vasculitis

INFECTIOUS DISEASES
- Tuberculosis
- Atypical mycobacterial infections
- Bacterial meningitis
- Encephalitis (viral)
- Mycotic infections

OTHERS
- Trauma
- Pituitary stalk section
- Radiation therapy
- Hydrocephalus and pseudotumor cerebri

Modified from Murialdo G, Tamagno G. Endocrine aspects of neurosarcoidosis. *J Endocrinol Invest* 25:650, 2002.

Head Trauma

Head trauma has been recognized as a cause of neuroendocrine dysfunction for many years, but assessment of hypothalamic–pituitary function during acute and long-term management of patients with traumatic brain injury (TBI) occurs infrequently. TBI is most common in males between the ages of 11 and 29 (5:1 male-to-female ratio), and is the leading cause of death and disability among young adults.[127] Road accidents account for 75% of cases of TBI.[128] Most patients who have post-head trauma hypopituitarism (PHTH) were comatose for a period after their injuries, and all had Glasgow Coma Scale scores of less than 13. PHTH can result from structural injury to the hypothalamus or pituitary, but a vascular insult leading to cerebral swelling is the most likely mechanism in most cases.[129] Such localized edema increases intracranial pressure in the sellar or suprasellar regions and can temporarily impair hypothalamic–pituitary function. PHTH may also be an adaptive mechanism to the acute illness often associated with TBI.[127,130]

One third of patients studied 3 months to 23 years after head injury had at least one pituitary hormonal deficiency: 18% had an inadequate GH response to insulin tolerance testing, and 20% to 25% had insufficient responses to GnRH stimulation.[129] The frequency of gonadotropin deficiency was even higher (80% to 90%) when defined by low early morning testosterone or E_2 levels and inappropriately low FSH and LH levels.[127] TBI-associated neuroendocrine dysfunction only presents with DI in the minority of cases (25% to 30%). PRL elevation occurs in approximately 50% of patients and correlates negatively with Glasgow Coma Scale score, likely because it is a marker of pituitary stalk or hypothalamic damage.[127,128]

Unfortunately, PHTH is usually not diagnosed during hospitalization unless a patient has acute ACTH deficiency or DI. Because pituitary hormone deficiency eventually recovers in approximately 60% of patients with moderate to severe head injury, some deficiencies (i.e., GH, gonadotropin) may never be recognized.[129,130] Gonadotropin secreting cells appear to be the most sensitive to TBI, and patients with PHTH often present after hospital discharge with amenorrhea, erectile dysfunction, and infertility.[128] FSH and LH in such patients may be inappropriately low. Most hormonal deficits (75%) appear within 1 year after injury, but diagnosis can be delayed by 5 or more years in 15% of patients.[128] In patients with a known history of TBI whose anterior pituitary function is initially normal, repeat evaluation during follow-up care may be indicated, given the delayed appearance of hypopituitarism in some cases.

Radiaton Therapy

Hypothalamic–pituitary dysfunction after cranial irradiation has long been recognized to occur in survivors of childhood cancer and in patients who receive high-dose RT for nasopharyngeal carcinoma[131,132] or pituitary disease.[133] However, not until recently has the high frequency of endocrine dysfunction in adult survivors of nonpituitary brain tumors who receive RT been recognized.[134] This frequency is only likely to increase with the improvements in prognosis for both children and adults with primary brain malignancies. Of a cohort of 56 patients with primary brain tumors who had received their last dose of RT at least 1 year before the time of study, 23 (41%) showed evidence of hypopituitarism with multiple (25%) or single (16%) hormone deficiencies. GH deficiency was most common, followed by gonadotropin, ACTH, and TSH deficiency.[135] DI is rarely associated with radiation-induced hypopituitarism. The risk of hormonal deficiency depends on the total dose, fraction size, number of fractions received, and total duration of RT. Panhypopituitarism can occur when the total dose exceeds 60 gray (Gy), but this risk can be reduced by administering more fractions of smaller dose, thereby extending treatment time.[136]

The rates of gonadotropin and GH deficiency in adults are significantly lower than those observed in pediatric studies, in which 20% to 50% of patients eventually have partial or severe gonadotropin deficiency[134,137] and GH deficiency occurs in 80% to 100%.[135] It is thought that the hypothalamic–pituitary axis is more radiosensitive in children than in adults.[135,138,139] Doses exceeding 50 Gy in childhood can cause gonadotropin deficiency, but smaller doses can result in precocious puberty. In low-dose cranial irradiation used prophylactically in children being treated for acute lymphocytic leukemia, girls are more susceptible to early puberty than boys, but this sexual dichotomy disappears when higher doses (25 to 50 Gy) are used to treat primary brain tumors.[136] In a pediatric cohort with preexisting GH deficiency, the relationship between age at time of irradiation and age at onset of puberty was linear.[140] The severity of gonadotropin deficiency varies in postpubertal subjects, from low normal to frankly low sex hormone levels.

The site of radiation-induced injury is the hypothalamus, and postradiation studies show normal responses to hypothalamic-releasing hormones, including GnRH.[141] The mechanism of this radiation-induced damage is unclear, but may involve direct damage to hormonally active cells or to their surrounding microvasculature. Alternatively, radiation may damage the vascular connections between the hypothalamus and the pituitary.[134,135] Hormone deficiencies may not become evident for many years,[142] because the radiation-induced damage takes its toll on the slowly dividing cells of the hypothalamus and secondary pituitary atrophy occurs after previous hypothalamic damage.[134-136] The delayed appearance of these deficits mandates that such patients be followed annually for these late effects of radiation.

Gonadal damage is also a concern in patients treated for primary brain tumors, but usually occurs as a result of adjuvant chemotherapy or spinal RT. Alkylating agents, procarbazine, cisplatin, and vinblastine are chemotherapeutic agents often associated with gonadal toxicity, whereas gonadal damage secondary to spinal RT occurs from radiation scatter.[136] Sixty-four percent of girls who received craniospinal irradiation but no chemotherapy had evidence of primary ovarian failure over 8 years of follow-up.[143] Sex steroids and germ cells are both lost in female patients treated with RT or chemotherapy, whereas males may present with infertility with normal levels of testosterone as a result of a differential effect of cancer therapy on Leydig cell function and spermatogenesis.[136]

Treatment for radiation-associated infertility secondary to disruption of the hypothalamus or pituitary requires replacement of gonadotropins. Physiological exogenous pulsatile GnRH therapy restored ovulation in the majority of women when it was clinically available.[144] Theoretically, cryopreservation of embryo, sperm, oocyte, or ovarian tissue can be used to preserve fertility when cancer treatment is expected to cause gonadal damage. However, each has significant drawbacks and none is routinely employed.[145,146]

Seizure Disorders

Epilepsy is a relatively common condition, with a prevalence of 5:1000 to 9:1000 and has been associated with subfertility in numerous epidemiological studies. The background rate of menstrual dysfunction (50% to 60%) far exceeds that of the general adult female population.[147] PCOS is the most common form of reproductive dysfunction in this population and affects up to 25% of epileptic women, although hypothalamic amenorrhea and luteal phase deficiency (LPD) can also occur.[148] The diagnosis of PCOS was unrelated to the type of epilepsy.[148]

Both seizures themselves and the antiepileptic drugs (AEDs) used to treat them have been implicated as the cause for reproductive dysfunction in patients with epilepsy. Epileptiform discharges transiently increase PRL and may disrupt GnRH pulsatility, a hypothesis suggested by observations of altered LH pulse frequency in regularly menstruating women with epilepsy who were not taking AEDs.[148,149] The similar rates of PCOS among medicated and unmedicated women with epilepsy also provide evidence that seizure activity itself interferes with normal reproductive function.[147,148] In addition, the use of AEDs (specifically, VPA) in normal women and those with other conditions (bipolar affective disorder) does not increase the risk of PCOS or affect baseline LH pulse dynamics, gonadal hormones, or rates of oligomenorrhea,[150-152] arguing for the position that epilepsy itself is responsible for interfering with reproductive function in some women.

Antiepileptic drug therapy, especially VPA, appears to worsen the central reproductive dysfunction caused by epilepsy itself. VPA has been shown to augment LH secretion in response to GnRH in healthy women, presumably via its stimulatory effects on glutamic acid decarboxylase and its inhibitory effects on GABA transaminase, ultimately leading to an increase in brain GABA activity.[150,153] No association between PCOS and the use of any specific AED was apparent in a relatively lean cohort of epileptic women reported by Bilo and colleagues,[154] but polycystic ovaries and hyperandrogenism were observed in 70% of a Finnish cohort receiving VPA monotherapy.[155] The frequency of menstrual disorders, polycystic ovaries, and hyperandrogenism was increased in both lean and obese women, but was higher in the latter.[156] Anovulatory cycles occurred in 43% of women who were currently using VPA or had done so within the previous 3 years, whereas only 10% of cycles were anovulatory in women with generalized epilepsy who had never taken VPA.[157] Although VPA is associated with weight gain, the changes observed in serum androgens (testosterone, androstenedione) occurred as early as 1 to 3 months after initiation of VPA and before any significant changes in body weight.[158] These observations suggest that VPA-associated PCOS in women with epilepsy is likely multifactorial in origin, with genetic and environmental factors playing some role.[148,159,160] In addition, the initiation of VPA before the age of 20 years was a risk factor for future development of PCOS.[155] Although VPA has never been shown to increase LH secretion, it may have direct ovarian effects, which in combination with the GnRH pulse generator dysfunction described earlier, ultimately lead to the hyperandrogenemia characteristic of many epileptic women receiving VPA.[160]

Ovulatory dysfunction related to AEDs may also arise as a result of alterations in cytochrome P450 activity. Phenytoin, phenobarbital, and carbamazepine induce cytochrome P450 activity, thus increasing sex hormone–binding globulin (SHBG) synthesis and reducing bioavailable testosterone and E_2 levels.[160] VPA inhibits cytochrome P450 enzyme activity and increases androgen concentrations.[157] However, these changes in bioavailable hormone levels are not always significant enough to alter reproductive function, and women taking carbamazepine have never been shown to have a higher risk of menstrual dysfunction, hyperandrogenemia, or PCOS.

Unfortunately, anovulatory cycles can be the only sign of epilepsy-associated reproductive dysfunction. Thus, an awareness of the effect of the disease and its treatment on patients' reproductive health should be part of routine care for women with epilepsy.[157] Menstrual cycle duration and body weight should be monitored regularly in women taking AEDs, especially VPA, so that reproductive abnormalities are not overlooked.[160] When such changes develop, switching women to other AEDs, such as lamotrigine, in consultation with their neurologist, can reverse the adverse reproductive and metabolical phenotype associated with AED treatment.[161]

Men with epilepsy also have reproductive endocrine abnormalities and are 36% less likely than unaffected male siblings to be biological fathers.[162] Approximately one third of men with temporolimbic epilepsy have hypogonadism,[163] and reductions in sperm count or motility as well as abnormal sperm morphology are observed in 8% to 90% of men, regardless of AED use.[164,165]

Functional Alterations and Disorders of the Hypothalamus

Physiological Hypogonadotropic Hypogonadism

Pubertal Transition

Hypogonadotropic hypogonadism can be physiological during certain phases of life. GnRH neurons are temporarily activated during infancy, producing midpubertal levels of either testosterone or E_2. After 6 months of age, however, GnRH neurons become quiescent, and gonadotropin levels and sex steroid levels fall. This *physiological hypogonadotropic hypogonadism of prepuberty* persists until approximately 8 to 9 years of age, when serum LH, FSH, and E_2 levels begin to rise, primarily overnight.[50,166] Over the course of puberty, mean LH increases 116-fold, FSH increases 7-fold, and E_2 increases twelvefold.[167] These changes are shown in Figure 21.6.

These gonadotropin changes directly reflect hypothalamic GnRH release because they can be simulated by exogenous GnRH administration,[168] can be blocked by GnRH antagonist administration,[166] and can occur in the absence of functioning gonadal tissue.[169] The factors mediating this reactivation of the GnRH pulse generator in early puberty are still unclear,[169] as are the factors permitting the progressive increase in daytime LH that occurs during puberty. Because hypogonadotropic hypogonadism is normal during puberty, distinguishing it from pathological causes of hypogonadotropic hypogonadism becomes difficult during adolescence. Both are characterized by absent or incomplete sexual maturation, low gonadotropin concentrations, low sex steroid levels (testosterone <100 ng/dL; $E_2 <20$ pg/mL), and no evidence of other hypothalamic–pituitary axis abnormalities. The α subunit response to GnRH injection was used in the past to separate these two conditions; adolescents who ultimately had the former condition had little α subunit production after GnRH, indicating pituitary disease.[170] However, the current unavailability of GnRH forces clinicians to rely on clinical features and family history rather than laboratory data to distinguish constitutional delay of growth and puberty (a normal variant characterized by hypogonadotropic hypogonadism) from more sinister causes of delayed puberty.

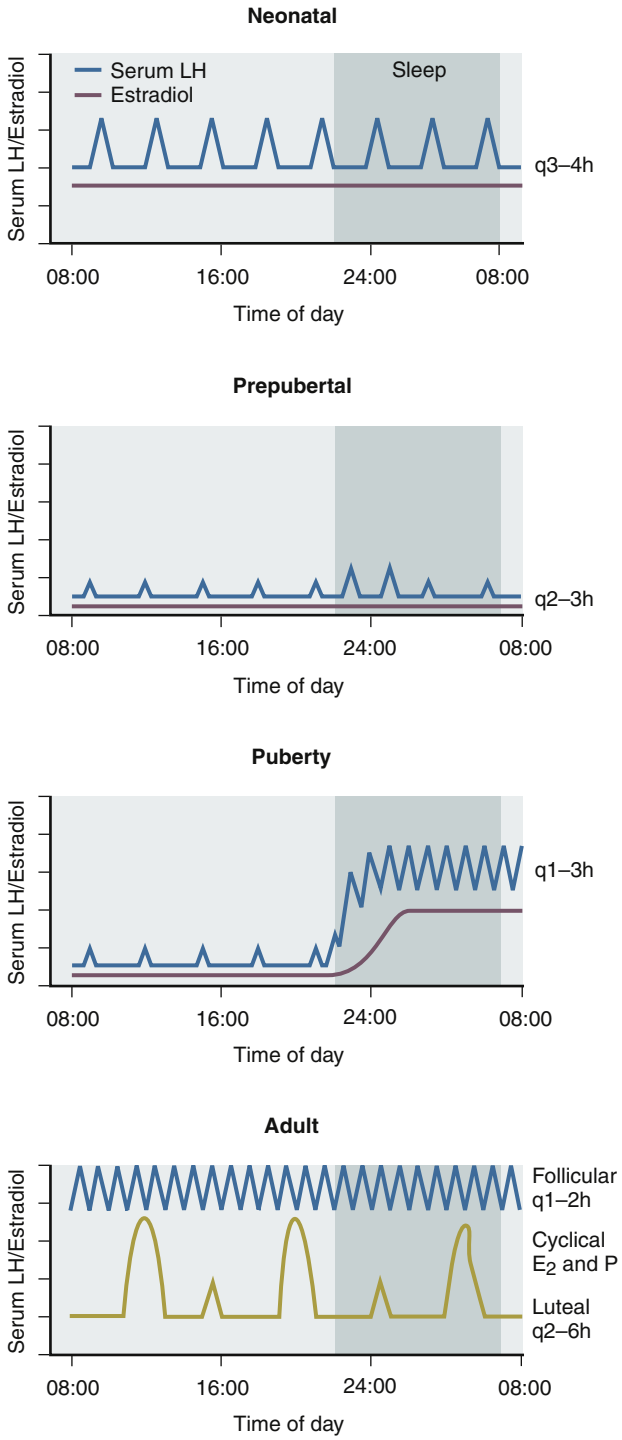


FIGURE 21.6 Patterns of serum luteinizing hormone (LH) during pubertal maturation in females. The duration of sleep is shown by the *bar*. E_2, estradiol; P, progesterone. *(Modified from Marshall JC. Control of pituitary hormone secretion: role of pulsatility. In Besser GM, Thorner MO [eds].* Comprehensive Clinical Endocrinology, *3rd ed. London, Elsevier, 2002.)*

Postpartum Period

The postpartum period is another time in life in which hypogonadotropic hypogonadism is physiological. Amenorrhea occurs in all women after childbirth. High levels of E_2 and progesterone during pregnancy are initially responsible for this hypothalamic–pituitary suppression. However, amenorrhea persists for varying durations into the puerperium and is prolonged further in women who breastfeed.[171] FSH levels remain low (similar to those seen in the early follicular phase), mean LH and LH pulse frequency are reduced, and serum E_2 and progesterone are low.[172] The mechanisms responsible for this continued suppression of the hypothalamic–pituitary–gonadal axis are unknown.[173,174] PRL has been implicated because it is universally elevated in the immediate postpartum period and suppresses GnRH release. However, PRL declines long before menstrual cyclicity returns in lactating women, arguing that mechanisms other than hyperprolactinemia must be responsible for lactational amenorrhea.[175] The suckling stimulus itself increases PRL, as shown in Figure 21.7, but also increases the sensitivity of the GnRH pulse generator to the negative feedback effects of E_2, such that GnRH and LH pulse activity are nearly absent in the immediate postpartum period.[176] Both of these effects of suckling diminish with time, however.

Once they reappear, LH pulse patterns are initially erratic and are augmented by sleep, similar to the pattern observed in early puberty.[173,176] Follicle growth and associated E_2 production often return to normal before ovulation resumes because suckling impairs the normal positive feedback of E_2 that is required for the preovulatory LH surge. Thus, menses may return, but cycles may be anovulatory until the suckling stimulus decreases further and normal ovulatory cycles resume.[171,176] Additional evidence suggesting a hypothalamic etiology for lactational amenorrhea comes from studies in lactating women exposed to pulsatile exogenous GnRH. Midcycle LH surges occurred in these women, luteal phases were normal, and FSH declined appropriately in response to increasing levels of ovarian steroids.[177] The hypothalamic hypogonadism of lactation can persist for more than 12 months, depending on the frequency and intensity of suckling.[176] This endogenous contraception allows women to cope with the physiological demands of the postpartum period before considering additional pregnancies. Both puberty and the postpartum period illustrate the neuroendocrine control mechanisms that govern GnRH, and abnormalities can be seen in a number of pathophysiological states.

Pathophysiological Hypogonadotropic Hypogonadism

Hypogonadotropic Hypogonadism Associated with Altered Secretion of Thyroid Hormone, Cortisol, and Prolactin

The neuroendocrine components of the reproductive axis are highly sensitive to disturbances in other hormones, including thyroid hormone, cortisol, and PRL. Within this context, menstrual irregularity occurs in approximately 25% of women with any degree of *thyroid hormone deficiency*, although the prevalence increases with the severity of hypothyroidism. Oligomenorrhea and menorrhagia are the most common menstrual disturbances, and sexual dysfunction (hypoactive sexual desire, erectile dysfunction, and delayed ejaculation) is observed in the majority of men with hypothyroidism.[178,179] Men with hypothyroidism often have low free testosterone levels that improve with initiation of thyroid hormone replacement.[180] Hypogonadotropic

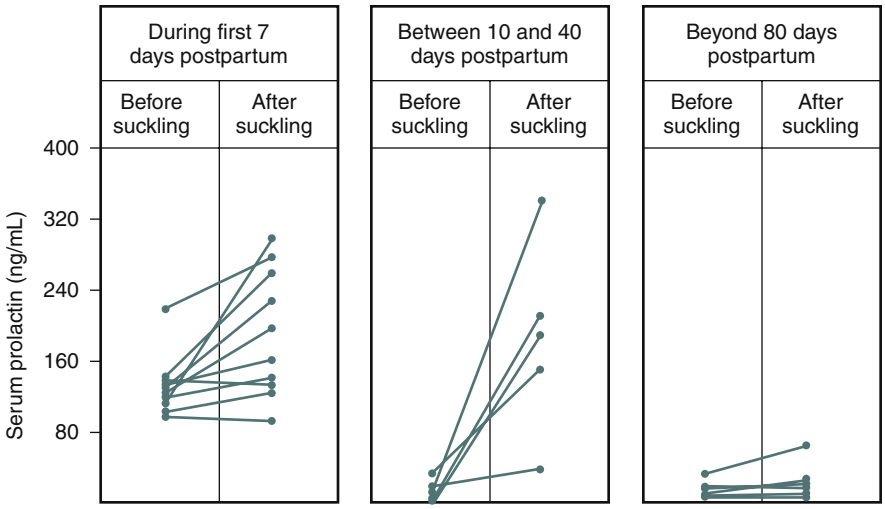


FIGURE 21.7 Serum prolactin concentrations basally and in response to suckling as a function of time after delivery. Within the first week after delivery, the basal value is high relative to the nonpregnant state, and there may be a further increase in response to suckling. Several weeks after delivery, the basal value is close to that of the nonpregnant state, but there is still a pronounced increase in response to suckling. Three months after delivery, the basal value is similar to that of the nonpregnant state, and there is a minimal response to suckling, if any. *(From Tyson JE. Studies of prolactin secretion in human pregnancy.* Am J Obstet Gynecol *113:14, 1972.)*

hypogonadism (low FSH and LH) may be present, but the clearance rate of the gonadotropins and sex steroids is reduced in the presence of hypothyroidism.[180-183] Pituitary sensitivity to GnRH may be reduced in hypothyroid children, but appears normal in hypothyroid women.[181,183] Fewer than 10% of patients with newly diagnosed hypothyroidism (TSH > 4 mU/L) have associated hyperprolactinemia that resolves with initiation of thyroid hormone replacement. However, correction of hyperprolactinemia in these patients does not correct the menstrual dysfunction. Only with restoration of a euthyroid state does menstrual function normalize, assuming it was secondary to thyroid dysfunction originally.[184]

Thyroid hormone excess, as occurs in Graves disease, is also associated with menstrual irregularities, anovulation, and infertility in women and sexual dysfunction in men.[179,185] Amenorrhea may be present in severe hyperthyroidism. Excess thyroid hormone increases gonadotropin sensitivity to GnRH[186-188] and may impair aromatase activity.[189] Hepatic SHBG production is also increased in hyperthyroidism, resulting in higher total testosterone and E_2, but normal free testosterone.[186-188] This increase in E_2 can cause gynecomastia in men with severe hyperthyroidism. Spermatogenesis may be impaired in men with thyroid hormone excess, but normalizes after restoration of a euthyroid state.[187,188]

Reproductive dysfunction frequently accompanies *abnormalities of cortisol secretion*. Amenorrhea develops in 25% of patients with *adrenocortical insufficiency*, although the etiology of such a severe reproductive disturbance is likely multifactorial, with chronic illness, weight loss, or autoimmune ovarian failure contributing.[190] Appropriately treated patients with autoimmune adrenal insufficiency (Addison disease) have basal LH levels and LH pulse patterns similar to those of normal healthy adults. During experimental periods with low cortisol replacement, patients with Addison's disease showed elevated CRH and ACTH levels, but normal sex steroid levels (testosterone and E_2). Gonadotropin alterations were gender-specific; mean LH concentrations and LH pulse amplitudes were suppressed in men in the setting of insufficient cortisol replacement for 47 hours, whereas similar conditions had no effect on LH dynamics in premenopausal and postmenopausal women. These differences may reflect higher opioid receptor activation in men than in women because opioids mediate the inhibitory effects of glucocorticoids on LH release.[191] Responses to low-dose GnRH stimulation did not vary in either sex under low cortisol conditions,[191] although higher doses of GnRH did alter stimulated FSH and LH concentrations.[192] These experimental conditions do not replicate the effects of long-term hypocortisolism, however, and clinical observations would suggest that maintaining physiological cortisol levels is crucial for preserving a normal hypothalamic–pituitary–gonadal axis.

Menstrual irregularities (amenorrhea, oligomenorrhea, polymenorrhea) are present in the majority of women with *cortisol excess* (Cushing syndrome), with only 20% reported to have normal menses in one large cohort.[193] GnRH neurons possess cortisol receptors,[194] and hypercortisolemia blocks both GnRH release and the action of gonadotropins on the gonad.[193] Gonadotropin levels are low or inappropriately normal in the presence of glucocorticoid excess, although GnRH testing showed normal or slightly increased gonadotropin reserve in hypercortisolism.[193,195,196] Thus, impaired GnRH release from the hypothalamus rather than pituitary dysfunction likely explains the reproductive pathology seen in Cushing syndrome. This reduction in gonadotropin secretion explains the radiographic appearance of the ovaries in women with Cushing syndrome, which tend to be smaller, with few primordial follicles and stromal hyperplasia.[197] Plasma E_2 and free and total testosterone are low in hypercortisolism, consistent with hypogonadotropic hypogonadism.[193,198,199] E_2 correlates inversely with the degree of cortisol elevation.[193] This hypogonadism can be reversed by restoration of normal serum cortisol levels.[196,198]

Although excess cortisol itself could explain these abnormalities of the hypothalamic–pituitary–gonadal axis, other hormones have been implicated, including *corticotrophin-releasing hormone*, *PRL*, and *androgens*. CRH is an important inhibitor of gonadotropin release in settings of acute stress, but it is suppressed by negative feedback in states of cortisol excess and is therefore unlikely to play a role in menstrual dysfunction in patients with Cushing disease. Similarly, hyperprolactinemia is present in the minority of patients with Cushing disease, but does not correlate with the severity of the reproductive disturbance and is unlikely

to be causative. Androgens were long believed to mediate Cushing-associated menstrual abnormalities. However, in a cohort of 45 premenopausal women with newly diagnosed Cushing disease, serum testosterone and androstenedione concentrations were elevated in only 29% and 39%, respectively, and there was no correlation between androgen levels and cortisol.[193] Metyrapone treatment of women with Cushing syndrome restores regular menses despite the increase in androgen levels associated with this drug.[200,201] These data suggest that hypothalamic GnRH release is suppressed by excess cortisol itself rather than by its adverse effects on other hormonal parameters.

Hyperprolactinemia is a common cause of reproductive dysfunction in premenopausal women, and is usually caused by pituitary lactotroph adenomas that are less than 1 cm in diameter (microadenomas). Hyperprolactinemia accounts for 10% to 20% of cases of amenorrhea (after pregnancy is excluded). Menstrual disturbances of varying degrees were present in 87% of a cohort of premenopausal women with hyperprolactinemia, whereas galactorrhea, another manifestation of hyperprolactinemia, was only present in 47%.[202] The severity of menstrual disturbance is related directly to the degree of hyperprolactinemia. Mild hyperprolactinemia (20-50 ng/mL) may impair progesterone secretion by the corpus luteum, resulting in a shortened luteal phase, but no menstrual irregularities. Moderate increases in PRL (50-100 ng/mL) can result in oligomenorrhea, whereas amenorrhea and overt hypogonadism occur when PRL is significantly elevated (>100 ng/mL). Any degree of hyperprolactinemia can contribute to infertility, however.[203,204] The severity of reproductive dysfunction in men also relates to the degree of hyperprolactinemia and can include decreased libido, infertility, gynecomastia, and galactorrhea.[205,206] The diagnosis of hyperprolactinemia is often delayed in postmenopausal women because they are already hypoestrogenic; such patients often present with compressive symptoms related to PRL-secreting adenomas, including headache, visual symptoms, or abnormalities of other pituitary hormones.

The mechanism of PRL-associated amenorrhea is suppression of hypothalamic GnRH release, resulting in low or inappropriately normal gonadotropin levels and low sex steroid levels. This reduction in hypothalamic GnRH release results in decreased LH pulse frequency, but LH pulse amplitude is higher.[207,208] The sleep-associated slowing in LH pulse frequency that occurs in normal adults is also lost in those with hyperprolactinemia. These effects of PRL on the hypothalamus are not mediated by increased hypothalamic opioid activity, and can be overcome with provision of exogenous pulsatile GnRH, which has resulted in pregnancy.[209,210] These observations and others show that hyperprolactinemia, whether due to normal physiological states (lactational amenorrhea) or pathophysiological causes, does not interfere with pituitary–gonadal feedback mechanisms or with the actions of LH and FSH on the ovary.[209,210] Restoration of normal PRL levels, either medically or surgically, allows LH pulse frequency and amplitude to recover, thereby normalizing menses, correcting hypogonadism, and restoring fertility.[202,211]

Evaluation of any woman with menstrual dysfunction or any man with symptoms consistent with hypogonadism should include measurement of a serum PRL level. Mild increases in PRL can occur with intense breast or chest wall stimulation, strenuous exercise, and emotional or physical stress. Thus, mild elevations should be confirmed before further evaluation for a cause of hyperprolactinemia. PRL secretion is normally inhibited by tonic hypothalamic dopamine secretion; thus, use of any drug that inhibits hypothalamic release or transport of dopamine to the pituitary or that blocks pituitary dopamine receptors can cause hyperprolactinemia. Such agents include metoclopramide, phenothiazines, butyrophenones, risperidone, verapamil, and rarely, the selective serotonin reuptake inhibitors. Discontinuation of potentially offending agents should be attempted, if possible, for at least 72 hours to determine whether hyperprolactinemia is drug-induced. In the absence of drug-induced hyperprolactinemia, hypothalamic lesions (craniopharyngiomas, granulomatous infiltration) must also be considered in the differential diagnosis. Such processes can cause hyperprolactinemia by compressing the pituitary stalk and thus interfering with dopamine transport. PRL levels greater than 200 ng/mL should also be repeated after dilution to avoid the "hook" effect, which can artificially lower actual PRL values by interfering with both immunoradiometric and chemiluminescent assays.[212] Once the presence of non–drug-induced hyperprolactinemia is confirmed, pituitary imaging with MRI is preferred for identification of adenomas.

Effects of Nutrition on the Neuroendocrine Components of the Reproductive Axis

States of Undernutrition: Effects of Fasting

Nutritional status is an important regulator of reproductive health. Individual and species survival are at stake when food is scarce; thus, reproductive potential is appropriately reduced in the setting of undernutrition. The effects of undernutrition on the reproductive axis have been studied experimentally by fasting normal subjects. These studies have suggested that the hypothalamic–pituitary axis in men may be more sensitive to the effects of starvation than that in women. Mean LH and FSH decrease within 48 hours of fasting in men.[213,214] Although the mechanisms involved remain unclear, similar reductions observed in animal studies did not appear to be opioid-mediated.[215] The data on LH pulse frequency and amplitude are less consistent, however. Within this context, Veldhuis and colleagues did not detect any change in LH pulse frequency in healthy men during a 5-day fast, although LH pulse amplitude was attenuated.[214] In contrast, the results of Cameron and colleagues in healthy men who fasted for 48 hours showed a significant reduction in LH pulse frequency, but no change in pulse amplitude.[213] Serum testosterone decreased in both studies.[213,214] The GnRH pulse generator in men may be more sensitive to the effects of fasting because of the sensitizing effects of gonadal steroids.[216] Older men, whose sex steroid levels have declined with age, do not show the same fasting-induced changes in LH pulsatility that are seen in younger men.[217] Animal studies have also shown that these effects of sex steroids on neuroendocrine function may vary with the type of stressor.[218-220]

The GnRH pulse generator in women appears less susceptible to the effects of acute fasting. Despite minor perturbations in LH pulse frequency and mean LH, at the end of a 72-hour follicular-phase fast, follicle development in fasting women was similar to that in women who were fed, as illustrated in Figure 21.8 and Figure 21.9.[221-223]

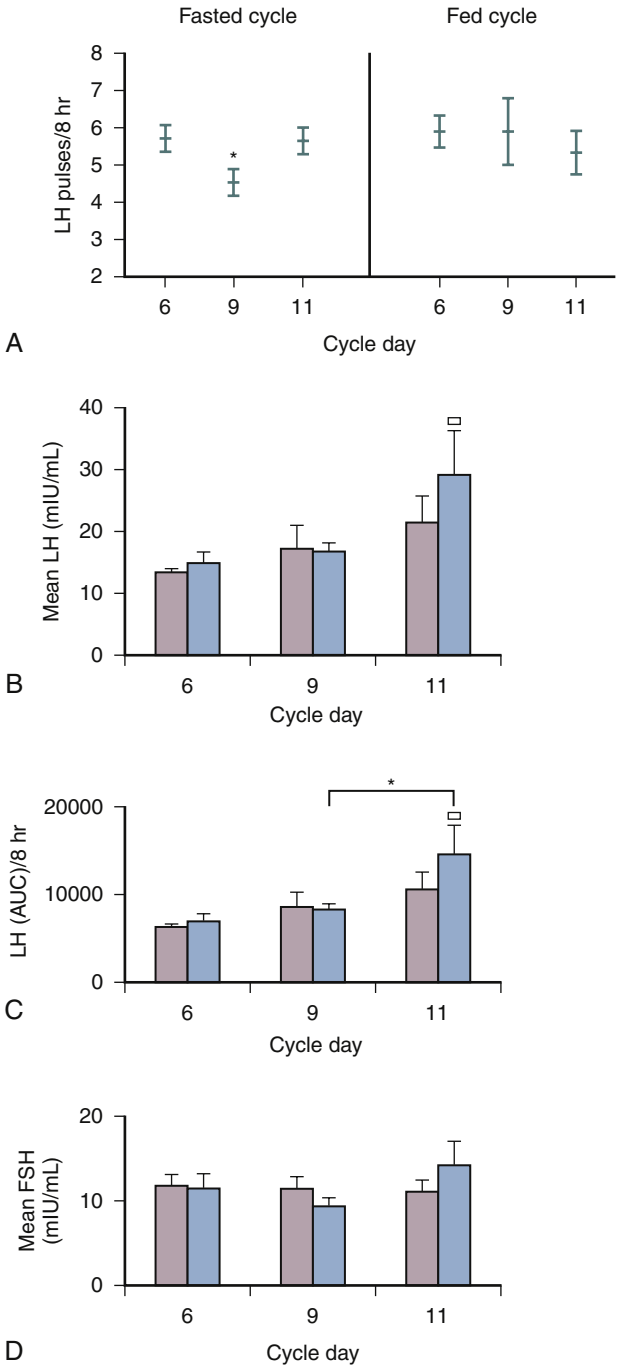


FIGURE 21.8 Cumulative 8-hour luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion on cycle days 6, 9, and 11 in women fasted on cycle days 7 to 9 (purple bars, $n = 10$) or fed (blue bars, $n = 7$). **A,** Mean number of LH pulses for each woman during the study for fasted *(left)* and fed *(right)* cycles. **B,** Mean LH value for the groups. **C,** LH area under the curve (AUC). **D,** Mean FSH values. The *rectangle* indicates the effect of time within a group of $P < 0.05$ by repeated-measures analysis of variance. *Asterisks* indicate $P < 0.05$ significance from day 6 in part A and differences detected between days within a group in part C. *(Adapted from Olson BR, Cartledge T, Sebring N, Defensor R, Nieman L. Short-term fasting affects luteinizing hormone secretory dynamics but not reproductive function in normal-weight sedentary women.* J Clin Endocrinol Metab *80:1187, 1995. With permission from The Endocrine Society.)*

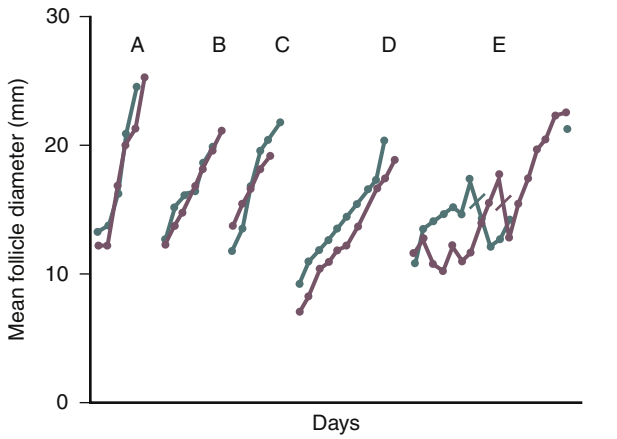


FIGURE 21.9 Patterns of daily follicle growth in five women during fed *(green)* and fasted *(purple)* cycles beginning on cycle day 6 and lasting until the day before the collapse of the dominant follicle. The *interrupted line* in subject E in both fed and fasted curves represents the loss of the largest measured follicle not associated with a luteinizing hormone surge. A 5-day hiatus in measurements occurred before the last point in the fed cycle. *(Adapted from Olson BR, Cartledge T, Sebring N, Defensor R, Nieman L. Short-term fasting affects luteinizing hormone secretory dynamics but not reproductive function in normal-weight sedentary women.* J Clin Endocrinol Metab *80:1187, 1995. With permission from The Endocrine Society.)*

This resistance of the GnRH pulse generator to the acute effects of fasting occurs despite documented changes in cortisol and melatonin, both of which have been proposed to be critical regulators of the GnRH axis.[221] Body weight may mediate the effect of fasting on GnRH drive, and LH pulse frequency declined by 20% in lean women compared with normal weight women.[224] Attempts to determine the effects of acute undernutrition on GnRH activity during the luteal phase in women have proven more difficult, and exogenous administration of E_2 and progesterone to simulate the luteal phase also suppresses LH. As in their male counterparts, however, women showed alterations in both cortisol and melatonin when fasted during a simulated luteal phase, with dramatic increases in cortisol and duration of nocturnal melatonin secretion with fasting, evidence that this nutritional stress was not without some hormonal perturbation.[221]

Fasting can only be maintained in healthy subjects for short periods. The effects of more sustained caloric deprivation have been assessed in healthy women maintained on 800 to 1100 kcal/day diets for one menstrual cycle. Such caloric restriction was sufficient to cause weight loss[225,226] and resulted in anovulation in some women.[207,209] Dieting reduced follicular-phase LH secretion as well as follicular-phase LH pulse frequency, but luteal-phase LH patterns and FSH were unaffected.[225] Sex steroids (testosterone, androstenedione) were decreased, SHBG increased, and dehydroepiandrosterone sulfate did not change with dietary restriction.[226] These data in healthy, regularly ovulating women suggest that cortisol and melatonin may be the first hormones affected by short-term fasting,[221,227] but that continued caloric deprivation reduces GnRH activity within weeks. Amenorrhea can develop with loss of approximately 10% of body weight, regardless of initial body weight.[227]

States of Undernutrition: Anorexia Nervosa

Anorexia nervosa (AN) is an extreme example of the effects of undernutrition on the reproductive axis. It is difficult to separate the neuroendocrine effects of this nutritional deprivation itself from the hormonal alterations caused by the comorbid psychiatric illnesses that are frequently associated with AN. However, the effects of long-term caloric deprivation alone are also difficult to discern in other states of chronic undernutrition, such as protein–calorie malnutrition in hospitalized patients. Thus, AN has been best studied as a model of the reproductive consequences of severe caloric restriction. AN is defined by weight loss of greater than 15% for height, an intense fear of gaining weight, a disturbance in one's body image, and amenorrhea for at least 3 months. It is estimated to affect 0.5% of young women, with an even lower prevalence in males. Both primary and secondary amenorrhea can occur in AN, depending on the time of onset of the condition. A loss of 10% to 15% of normal weight for height is enough to delay the onset of menarche (primary amenorrhea), and a similar amount of weight loss disrupts normal menstrual cycles in postmenarchal girls (secondary amenorrhea). However, amenorrhea precedes weight loss in a significant percentage of patients with anorexia,[228,229] evidence that psychological stress and excessive exercise can also play a role. Other organ systems affected by AN include the cardiovascular and peripheral vascular (hypotension, arrhythmias), hematological (anemia), dermatological (lanugo hair), and renal systems (hypokalemia).[229]

Menstrual disturbances in anorexia result from hypothalamic rather than pituitary or ovarian dysfunction. Mean serum LH concentrations are lower than those of regularly menstruating women studied in the follicular phase,[230] and the LH secretory pattern is prepubertal, even in previously menstruating adolescents.[231] Responses to exogenous GnRH are blunted; the degree of response is greater in women who are closer to ideal body weight and in those who will ultimately resume normal menses after restoration of body weight.[232-234] Positive feedback from E_2 on the hypothalamus is also impaired in anorexic patients, as shown by studies with clomiphene citrate and exogenous estrogen that did not augment gonadotropin secretion.[235,236] The reduction in gonadotropin stimulation of the ovaries results in low estrone (E_1), E_2, progesterone, testosterone, and androstenedione levels.[232] Reduction in aromatase activity as a result of low body fat also contributes to the hypoestrogenism seen in anorexia, as does the preferential synthesis of catecholestrogen over E_2. Catecholestrogen is an endogenous antiestrogen that is capable of binding to estrogen receptors without having any biological action. Catecholestrogen also competes with dopamine for the enzyme catechol-O-methyltransferase, thus increasing dopamine levels, further inhibiting GnRH pulsatility, and potentiating hypoestrogenism.[228] These alterations in E_2 metabolism result from changes in body weight and are not specific to anorexia.[237] Additional evidence that anorexia-associated amenorrhea is the result of hypothalamic dysfunction is the induction of menses in anorexic women with the use of pulsatile GnRH.[238]

The biochemical factors responsible for the hypothalamic amenorrhea in anorexia are unclear. Increased opioid tone is unlikely to be the only operative mechanism because administration of naloxone increased LH secretion in some,[239] but not all, studies.[233,240] Women who had reached ideal body weight and those with the highest basal LH levels had the most significant responses to opiate antagonists (naloxone).[233,239] Dopamine can also inhibit LH release, but the use of the dopamine agonist metoclopramide did not result in a significant increase in LH in a cohort with anorexia.[241] Metabolites of serotonin, such as 5-hydroxyindolacetic acid, which stimulate hypothalamic GnRH release and increase pituitary sensitivity to GnRH,[242] are low in patients with anorexia and increase during recovery, as shown in Figure 21.10.[243]

Selective serotonin reuptake inhibitors, which restore CNS serotonin levels, have been shown to be effective in reversing abnormal eating behaviors in some patients with anorexia. Thus, disturbance of the serotonin system may also play some role in anorexia-associated hypogonadotropic hypogonadism.[228,243] Another potential factor is leptin, which is secreted by adipocytes and is critical for energy homeostasis. Leptin receptors have been identified in the hypothalamus, pituitary, endometrium, ovary (granulosa, theca, and interstitial cells), and testes (Leydig cells). Leptin indirectly stimulates GnRH release, acting in concert with other molecules involved in appetite control (NPY, melanocortin). It also directly stimulates LH and FSH release from anterior pituitary cells. Leptin gene expression is promoted by estrogen and insulin. Anorexia and other states of hypoinsulinemia are characterized by reduced leptin concentrations, which correlate with body fat. Weight restoration in anorexia is associated with increases in leptin, changes that correlate with increases in serum LH and FSH. Thus, leptin may serve as a peripheral signal of adequate adipose stores that is capable of reactivating the hypothalamic–pituitary–gonadal axis and restoring menses during recovery from AN.[244] The changes in leptin in AN are also accompanied by changes in ghrelin, another peptide intimately involved in control of feeding behavior. Ghrelin is increased in anorexic females compared with age-matched control subjects and constitutionally thin women without AN. Ghrelin correlates negatively with body mass index (BMI) and leptin,[245,246] and partial weight recovery results in a 25% decrease in ghrelin levels in patients with anorexia.[245]

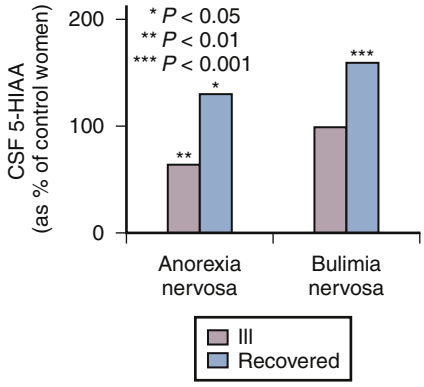


FIGURE 21.10 Cerebrospinal fluid 5-hydroxyindolacetic acid (5-HIAA) concentrations when ill and after recovery in people with anorexia and bulimia nervosa. Values are compared with those of healthy control women, which are set at 100%. *(Modified from Kaye W, Gendall K, Strober M. Serotonin neuronal function and selective serotonin reuptake inhibitor treatment in anorexia and bulimia nervosa.* Biol Psychiatry *44:825, 1998.)*

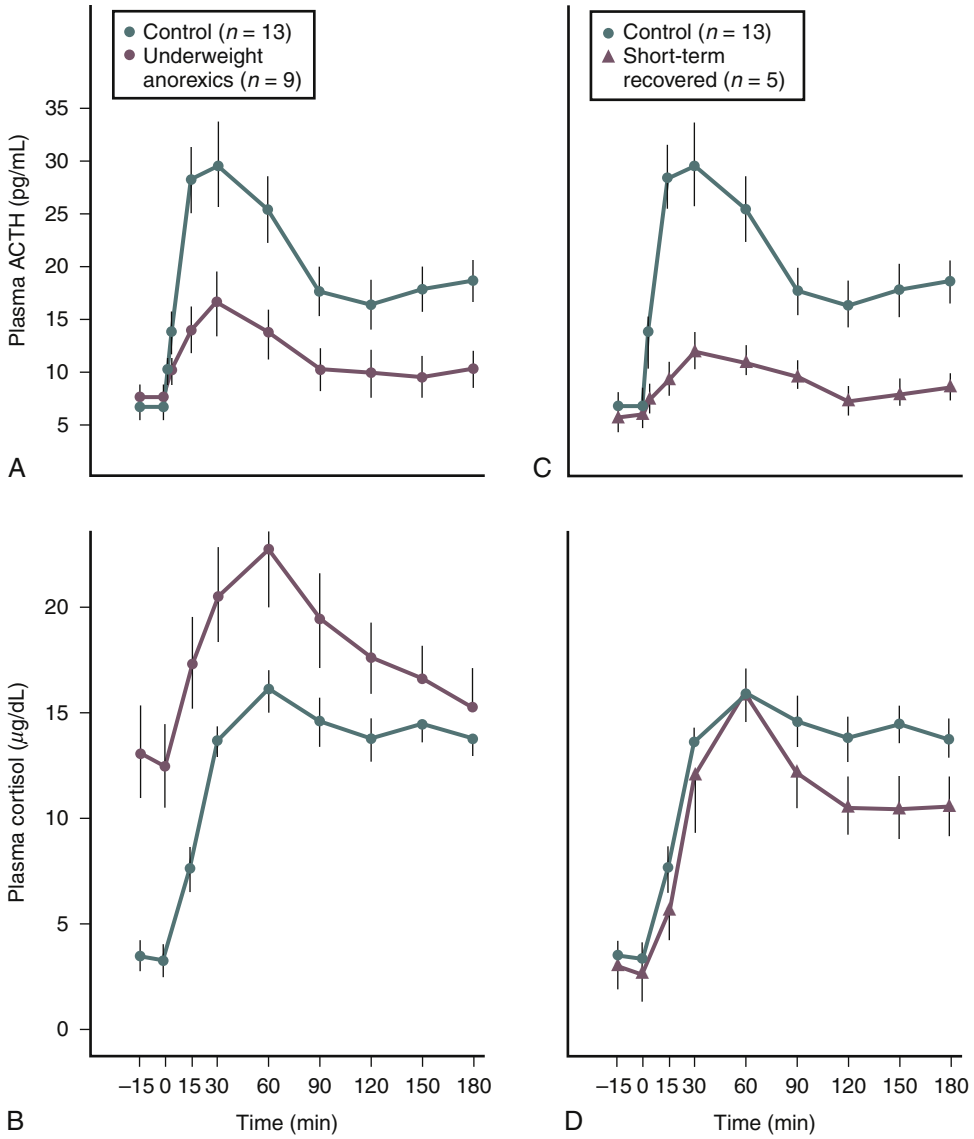


FIGURE 21.11 Response of plasma (ACTH) and cortisol to corticotrophin-releasing hormone (CRH) in control subjects and patients with anorexia nervosa while chronically underweight (**A** and **B**) and while in short-term recovery (**C** and **D**). *(Modified from Gold PW, Gwirtsman H, Avgerinos PC, Nieman LK, Gallucci WT, Kaye W, Jimerson D, Ebert M, Rittmaster R, Loriaux DL. Abnormal hypothalamic-pituitary-adrenal function in anorexia nervosa. Pathophysiologic mechanisms in underweight and weight-corrected patients.* N Engl J Med *314:1335, 1986. Copyright © 1986 Massachusetts Medical Society. All rights reserved.)*

Dehydroepiandrosterone sulfate and androstenedione are reduced in women with anorexia, but serum and urine cortisol levels are higher than normal. This preferential synthesis of cortisol over adrenal androgens is similar to that seen normally in prepubertal children and reflects relative 17, 20-desmolase deficiency.[247] The resulting hypercortisolemia is augmented by the prolonged plasma half-life of cortisol in AN.[248] In spite of elevated cortisol, ACTH levels are not elevated at baseline or in response to CRH, as illustrated in Figure 21.11.

These findings show that the negative feedback effects of this hypercortisolemia remain intact at the level of the pituitary, and that excess secretion of CRH and decreased clearance of CRH contribute to this hypercortisolemia.[249] The lack of clinical signs of hypercortisolemia may result from diminished tissue expression of glucocorticoid receptors in patients with anorexia.[250] Importantly, these alterations of the hypothalamic–pituitary–adrenal (HPA) axis resolve with long-term weight recovery.[230,249] The hypothalamic–pituitary–gonadal axis also recovers with restoration of ideal body weight, as shown in Figure 21.12.[231]

Although no absolute BMI or percentage of body fat has been found to correlate with the resumption of menses, more than 85% of patients who achieve a weight that is 90% of their ideal body weight note return of menses within 6 months.[251] However, amenorrhea persists in a minority of women (10% to 30%), despite weight gain. Of the latter, many admit to restricting their fat intake and score higher on questionnaires designed to detect anorexic behaviors.[229] Hypoestrogenemia in such women correlates with the degree of ovarian dysfunction, and ovarian ultrasound shows small, amorphous ovaries. However, small multifollicular ovarian cysts appear once weight gain starts, with the return of a dominant ovarian follicle once premorbid weight is achieved.[252] Those who recover from anorexia, resume regular menses, and have normal eating behaviors can successfully conceive and bear healthy children.[229]

Recovery from AN and restoration of ideal body weight requires both emotional and nutritional rehabilitation, and remains quite challenging, despite improvements that have been made in understanding the pathophysiology of AN during the last century. Standardized mortality rates in patients

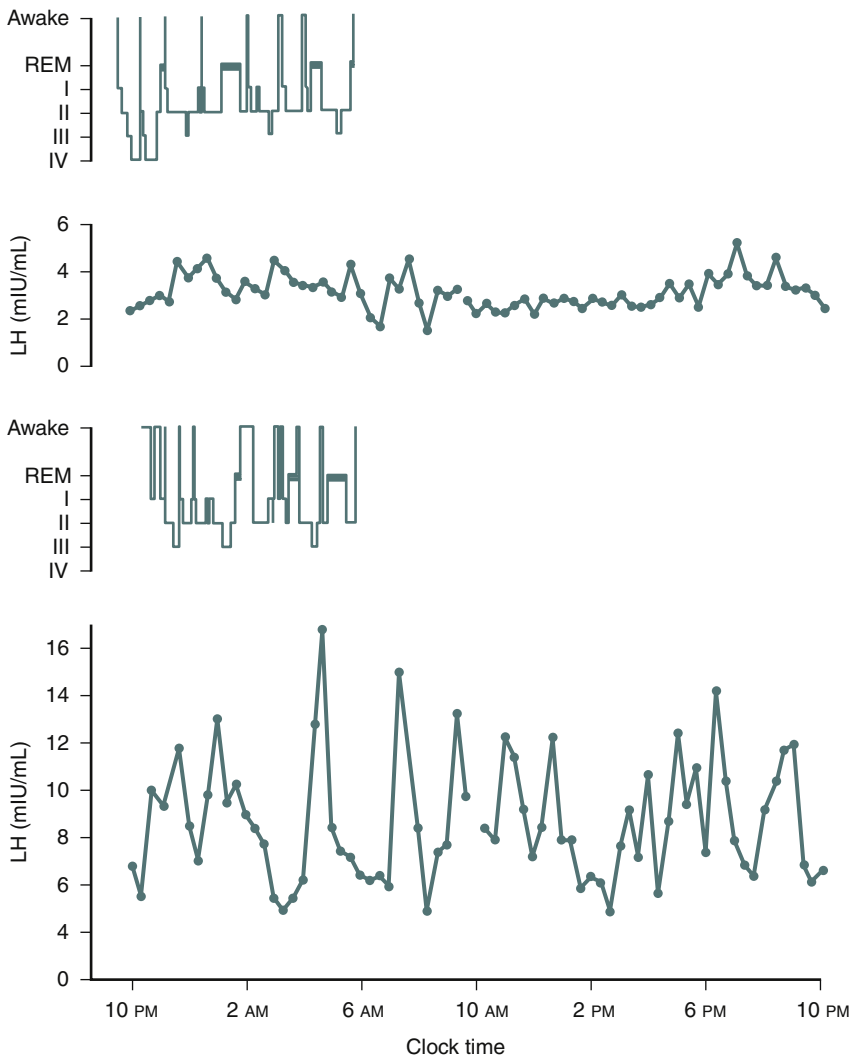


FIGURE 21.12 Plasma luteinizing hormone (LH) concentrations every 20 minutes for 24 hours during acute exacerbation of anorexia nervosa (*top*) and after clinical remission with return of body weight to normal (*bottom*). REM, rapid eye movement. *(Adapted from Boyar RM, Katz J, Finkelstein JW, Kapen S, Weiner H, Weitzman ED, Hellman L. Anorexia nervosa. Immaturity of the 24-hour luteinizing hormone secretory pattern.* N Engl J Med *291:861, 1974. Copyright © 1974 Massachusetts Medical Society. All rights reserved.)*

with AN are increased tenfold to thirteenfold when patients are followed for ten years.[253] The crude death rate in large meta-analyses, including more than 3000 patients with AN, was 5.9%.[254] The percentage of patients who recover entirely is less than 50%, whereas a significant proportion of patients have binge-eating behaviors at some point during their illness.[253] At least 10% still meet the criteria for AN after long-term follow-up. Factors associated with poorer outcomes included longer duration of illness before the first inpatient treatment and a lower BMI, illustrating the importance of early diagnosis and intervention.[255]

Unfortunately, the effects of severe undernutrition on other body systems can persist, even when anorexic patients restore their body weight. Osteopenia and osteoporosis are the most serious long-term consequences of AN, and they result from both decreased bone formation (related to energy deprivation) and increased bone resorption (related to hypoestrogenism). The majority of patients presenting with amenorrhea and anorexia have some degree of low bone density, and bone mass can decline by as much as 2% to 6% per year during periods of undernutrition.[229] Unfortunately, BMD does not always normalize after restoration of a healthy body weight and menstrual cyclicity, often because peak bone mass was not achieved during the adolescent and young adult years. The declines in bone density have been shown to correlate with the duration of anorexia and the age of onset of the disease.[228,229] Treatment of anorexia-associated osteopenia appears to require more than hormone replacement, likely because the osteopenia results from various nutritional and hormonal factors, not just estrogen deficiency.[256] For example, patients who received recombinant human insulin-like growth factor-1 (IGF-1) in combination with oral contraceptives had higher BMD than those who received oral contraceptives alone.[257]

Males account for only 5% of the anorexic population, but the diagnosis of AN in males is often delayed because the signs of reproductive dysfunction are harder to identify. Anorexia in men is strongly associated with depression and other underlying psychiatric disturbances, although the rate of comorbid substance abuse is higher than it is in women. Other features more prevalent in males with anorexia include a history of obesity, sexual identity concerns, and excessive exercising.[228] Boys are at higher risk for malnutrition-induced delays in growth and pubertal development, because they enter puberty later than girls. Studies in males with anorexia have shown abnormalities in the hypothalamic–pituitary–gonadal axis similar to those in women. Serum testosterone concentrations are often below

normal, as are mean 24-hour LH concentrations.[214,234] GnRH-stimulated gonadotropin levels are also lower than those of normal control male subjects. Unlike gonadotropin levels in anorexic females, basal LH and FSH do not correlate with BMI (and degree of weight loss) in males with anorexia.[234] This hypogonadotropic hypogonadism is likely the result of severe caloric restriction itself because caloric restriction in obese men had similar effects on sex steroid and gonadotropin levels.[258]

States of Undernutrition: Bulimia Nervosa

Bulimia nervosa (BN) is another eating disorder characterized by irregular feeding patterns, specifically binge eating, in individuals of normal body weight, many of whom aspire to body weight far below normal. These episodes of binge eating are followed by periods of self-induced vomiting, laxative abuse, or extreme exercise, all driven by an abnormal body image. BN affects approximately 2% of the general female population, and approximately one third of patients with BN who present for treatment have a history of anorexia. Both anorexia and bulimia are more common in patients with coexisting low self-esteem, depression, and anxiety.[226] Disturbances in the reproductive axis can be seen in patients with bulimia, but they do not define the condition, as in anorexia. Amenorrhea occurs in 30%.[259] Approximately half of women with bulimia show hypogonadotropic hypogonadism and no evidence of follicular development associated with decreased LH pulse frequency,[260,261] whereas others have normal gonadotropin secretion and normal follicular development, but impaired luteal-phase progesterone levels.[260] Patients with purging behaviors showed more severe reductions in LH responses to GnRH infusion than nonpurging patients with bulimia and control subjects.[262] As in women with anorexia, serum cortisol is higher in bulimic women than in control subjects,[261,263] possibly related to increased CRH stimulation.

States of Overnutrition

Overnutrition and *obesity* pose a much greater threat to human health in most parts of the world than does food scarcity. Associated morbidities, including diabetes, obstructive sleep apnea, cardiovascular disease, and osteoarthritis, often remain undiagnosed until later in adulthood, although their foundations are established in young adulthood. However, men and women of reproductive age can have obesity-associated reproductive dysfunction that brings them to medical attention much sooner than these other conditions. For example, the most common endocrinopathy in reproductive-age women is PCOS, which affects 5% to 10% of women in this age group and is associated with reproductive as well as cardiovascular and cosmetic complications.[264,265] Many patients with PCOS are obese, and adiposity correlates linearly with testosterone levels in these women.[266] Weight reduction can restore menstrual regularity and, therefore, fertility in many patients with PCOS.[265] Both lean and obese women with PCOS show rapid LH pulse frequencies, but increasing body weight is associated with decreases in LH pulse amplitude in women with PCOS.[267] This neuroendocrine dysfunction is a product of and contributes to hyperandrogenemia, thus creating a vicious cycle in women with PCOS.

Obesity itself in the absence of PCOS-associated hyperandrogenemia also affects the hypothalamic–pituitary axis. Historical analysis of menstrual cycle data shows a higher percentage of menstrual abnormalities or hirsutism in women who were heavier.[268] The number of menstrual cycles with evidence of luteal activity was lower in overweight or obese women than in normal-weight participants in the Study of Women's Health Across the Nation (SWAN) study. Women with BMI of 25 kg/m^2 or greater also had statistically longer follicular phases and shorter luteal phases.[269] Consistent with these population studies, detailed hormonal sampling studies have shown that excess body weight suppresses gonadotropins. LH amplitude was reduced by 50% in morbidly obese women (mean BMI, 48.6 kg/m^2) with regular cycles studied before gastric bypass surgery, although pulse frequencies did not differ. Nevertheless, the marked attenuation of pulse amplitude resulted in a lower mean LH compared with normal-weight control subjects.[270] This reduction in LH drive also results in deficient luteal stimulation and corpus luteum progesterone production, as reflected by lower urinary excretion of the progesterone metabolite pregnanediol glucuronide, illustrated in Figure 21.13.[269,270] Plasma E_2 and androstenedione concentrations are lower in obese women with regular cycles,[271] although E_1 does not change with body weight in most studies.[269,270]

Multiple factors are likely responsible for these obesity-associated neuroendocrine changes. The response to exogenous physiological-dosed GnRH was inversely related to body weight in women with PCOS, but not in normally cycling women, suggesting that obesity may blunt the pituitary response to GnRH.[272] Insulin may also interfere with pituitary gonadotropin secretion, although no clear relationship between LH and insulin has been identified. Hyperinsulinemia clearly suppresses hepatic secretion, however, thereby increasing free E_2 levels. Free E_2 may exert more negative feedback on the pituitary because it is more biologically active, thus decreasing gonadotropin production.[269] Leptin, an adipose-derived hormone, increases hypothalamic GnRH pulsatility and pituitary gonadotropin release,[244] although obesity is often associated with leptin resistance. Leptin also inhibits granulosa cell responses to hCG and may interfere with oocyte maturation. Thus, the exact relationship of leptin to the neuroendocrine changes described earlier is not fully understood. Similarly, the role of other adipose-derived substances, including TNF-α and interleukin-1β, is an area of active investigation because both have the ability to reduce pituitary response to GnRH as well as to impair corpus luteum function.[270]

Obesity in men is also associated with reductions in circulating gonadotropin and sex steroid concentrations. The hyperinsulinemia-associated decrease in hepatic SHBG decreases binding affinity for testosterone and results in greater aromatization of testosterone to E_2. The subsequent increase in free E_2 and the reduction in the testosterone-to-E_2 ratio have important implications for male sexual function.[273-275] Total testosterone is lower in obese men compared with normal-weight men, but free testosterone does not change.[274,276,277] Mean LH, LH pulse frequency, and LH pulse amplitude are suppressed in severely obese men (BMI > 35), again reflecting the negative feedback effects of elevated free E_2. More moderate degrees of adiposity (BMI 30 to 35) do not affect gonadotropin secretion.[277,278] These alterations in the hypothalamic–pituitary–gonadal axis can

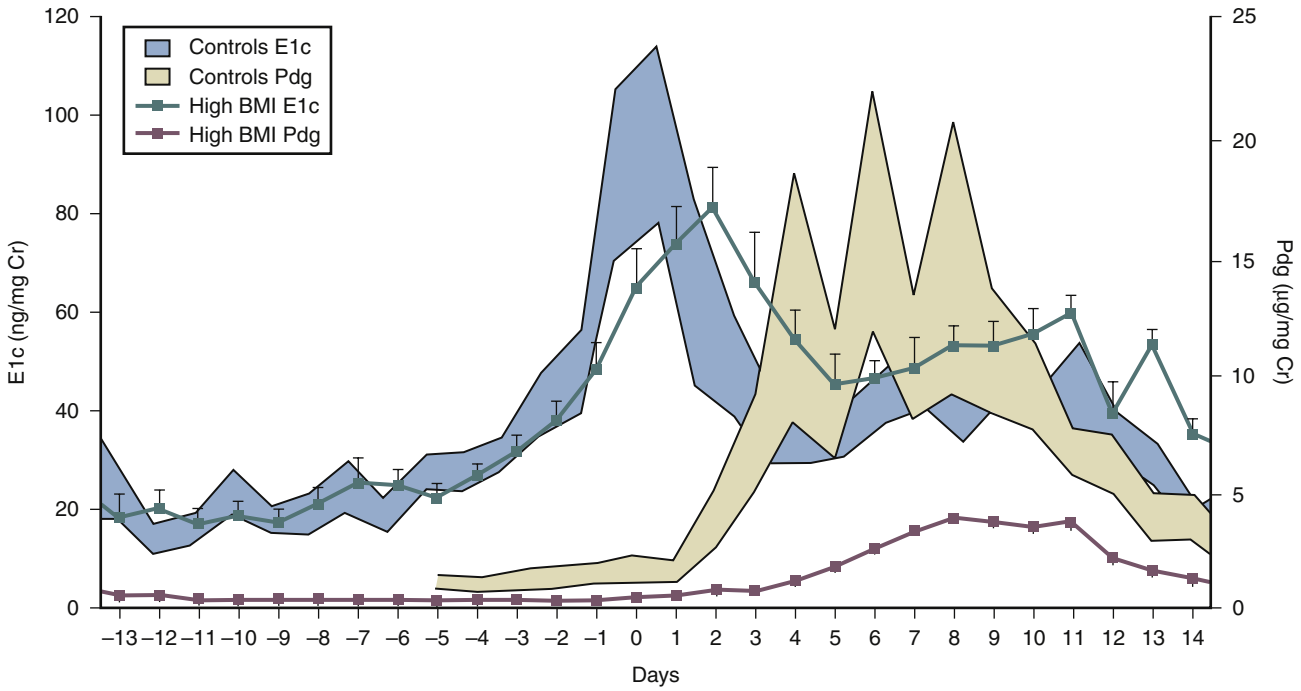


FIGURE 21.13 Daily urinary estrone conjugate (E1c) and pregnanediol glucuronide (Pdg) in women with a high body mass (BMI) versus mean values of control women ±1 standard deviation. Data are standardized to day 0, 13 days before ovulation. *(Modified from Jain A, Polotsky AJ, Rochester D, Berga SL, Loucks T, Zeitlian G, Gibbs K, Polotsky HN, Feng S, Isaac B, Santoro N. Pulsatile luteinizing hormone amplitude and progesterone metabolite excretion are reduced in obese women.* J Clin Endocrinol Metab *92:2468, 2007. With permission from The Endocrine Society.)*

decrease sperm concentrations.[273] Importantly, reducing hyperinsulinemia through weight loss allows for restoration of normal testosterone and LH levels.[278]

Effects of Exercise on the Neuroendocrine Components of the Reproductive Axis

Excessive exercise in the absence of eating disorders can result in hypogonadotropic hypogonadism due to hypothalamic dysfunction ("athletic amenorrhea"). The clinical manifestations of the reduced GnRH activity are varied and may include amenorrhea, oligomenorrhea, LPD, or anovulation. Menstrual cycle length is not a good marker of ovarian function in athletic women because it may be normal during cycles characterized by anovulation or LPD. In addition, one woman's cycle will differ over time, depending on the volume of her exercise and other environmental factors.[279] Exercise-associated menstrual dysfunction has been reported in women engaged in a variety of athletic activities, including long-distance running, rowing, skiing, tennis, gymnastics, ballet, fencing, and volleyball.[280] Dancers and long-distance runners have especially high rates of menstrual dysfunction (60% to 70%), but divers, cheerleaders, and gymnasts also have rates of amenorrhea (22%) that far exceed those in the general population.[264] Even recreational athletes are susceptible to exercise-associated menstrual dysfunction; 78% of such women had LPD or anovulation in at least one of three menstrual cycles.[279]

Risk factors for exercise-associated menstrual irregularities include the volume of training, eating behaviors, and gynecological age. Studies of distance runners have illustrated the effect of exercise volume on menstrual function, with the prevalence of amenorrhea increasing from 3% to 60% as weekly training mileage increased.[281] Surveys of eating behaviors among female collegiate athletes show relatively low rates of diagnosed eating disorders among this population (3.3% with AN; 2.3% with bulimia nervosa). Importantly, however, almost one third may be "at risk" for eating disorders, based on assessments of their attitudes toward eating, and these athletes are the ones most likely to report menstrual irregularities or bone injuries.[282] Although all menstruating females are susceptible to the disruptive effects of exercise on the reproductive axis, those who are younger and closer to the time of menarche (i.e., lower gynecological age) are more sensitive, regardless of body size, body composition, training mileage, or years of training.[280,283,284] This sensitivity of the hypothalamic–pituitary–gonadal axis appears to be maximal in the first 15 years after the onset of menses and wanes thereafter.[284]

Exercise-induced menstrual dysfunction is characterized by low gonadotropin and E_2 concentrations.[285] Whereas athletic women with normal cycles had 20% to 30% reductions in LH pulse frequency compared with regularly cycling sedentary women, amenorrheic athletes experienced an additional 20% reduction in LH pulse frequency,[286-288] as shown in Figure 21.14.[286]

Reductions in LH pulse amplitude and loss of sleep-associated changes in LH pulsatility also occur in the amenorrheic group.[286,289] FSH secretion is impaired during the follicular–luteal phase transition. These gonadotropin changes may be subtle enough that menses remain regular, but they likely contribute to the high rates of anovulation (12%) seen in recreational athletes. Ultimately, ovarian steroid production is reduced in the absence of appropriate gonadotropin stimulation and corpus luteum function is impaired. The former is reflected in Figure 21.15 *(top)*[286] by a reduction in urinary E_1 (E_1G) excretion in amenorrheic

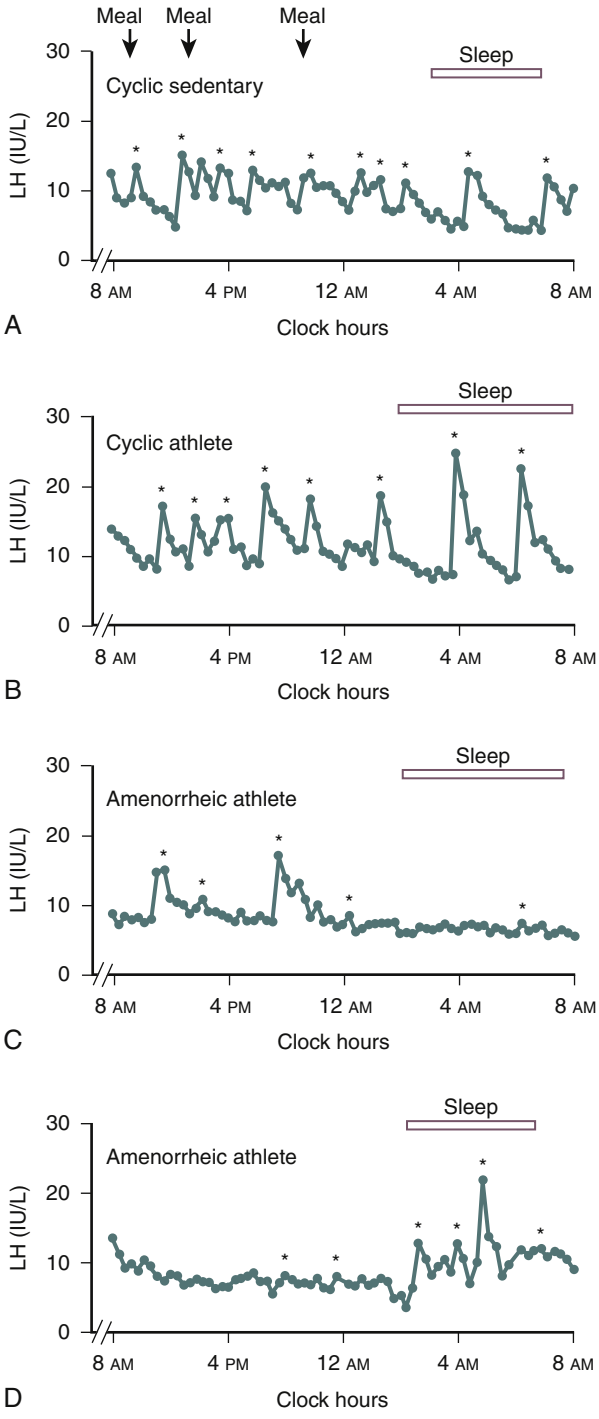


FIGURE 21.14 Serum luteinizing hormone (LH) levels at 20-minute intervals for 24 hours in a cycling sedentary women **(A),** a cycling athletic woman **(B),** and two amenorrheic athletic women (**C** and **D**). *Asterisks* indicate pulses, as identified by the Cluster pulse analysis program using a 2 × 1 cluster size and balanced T criteria of 2.1. *(Modified from Loucks AB, Mortola JF, Girton L, Yen SSC. Alterations in the hypothalamic-pituitary-ovarian and the hypothalamic-pituitary-adrenal axes in athletic women.* J Clin Endocrinol Metab *68:402, 1989. With permission from The Endocrine Society.)*

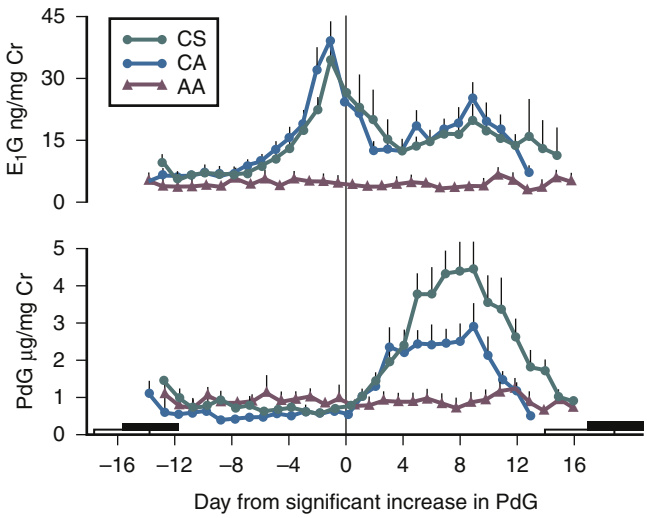


FIGURE 21.15 Mean (± standard error) daily urinary excretion of estrone glucuronide (E_1G) *(top)* and pregnanediol glucuronide (PdG) *(bottom)* in cycling sedentary (CS), cycling athletic (CA), and amenorrheic athletic (AA) women. Days are oriented from a significant increase in urinary PdG excretion, with day 1 being the day of the first significant increase. For urinary E_1G, 1 ng/mg creatinine (Cr) = 2.134 pmol/mg Cr; for PdG, 1 µg/mg Cr = 2.014 nmol/mg Cr. *(Modified from Loucks AB, Mortola JF, Girton L, Yen SSC. Alterations in the hypothalamic-pituitary-ovarian and the hypothalamic-pituitary-adrenal axes in athletic women.* J Clin Endocrinol Metab *68:402, 1989. With permission from The Endocrine Society.)*

athletes, whereas Figure 21.15 *(bottom)* shows urinary excretion of the progesterone metabolite prenanediol glucuronide (PdG) in these women. Reductions in PdG define luteal phase deficiency (LPD) and characterize 43% of the menstrual cycles in regularly cycling athletic women and can contribute to infertility.[286]

Provision of exogenous GnRH to women with exercise-induced amenorrhea illustrated that the etiology of this disorder is reduced hypothalamic GnRH secretion, rather than impaired pituitary or ovarian responsiveness. Submaximal graded doses of GnRH (2.5 µg, 5 µg, 10 µg, 25 µg) elicited exaggerated LH responses in athletic women compared with sedentary control women, whereas supraphysiological doses of GnRH (100 µg) in amenorrheic athletes resulted in greater LH secretion compared with regularly menstruating athletic women.[290]

Exercise also causes hypogonadotropic hypogonadism in men, with reductions in both gonadotropins and total and free testosterone levels that correlate with the intensity of endurance exercise.[291,292] High-mileage endurance exercise can also affect sperm count, density, motility, and morphology on semen analysis.[292-294] Unlike endurance exercise, resistance exercise is associated with transient increases in androgens and dehydroepiandrosterone sulfate in men.[295] Because hypogonadism in males is less often recognized by both medical providers and patients themselves, literature on the effects of exercise on the male reproductive axis is more limited.

Physical activity is associated with hypogonadotropic hypogonadism in some, but not all, female athletes. A prospective study of previously eumenorrheic women who engaged in distance running (65 miles/week) at or above the lactate threshold for 12 months or longer detected no

significant changes in menstrual cycle length, follicular and luteal phase length, or integrated serum progesterone and E_2 concentrations compared with control women who were not exercise-trained. Body weight, percentage of body fat, lean body mass, and caloric intake did not change during the 12-month training period, and none of the women had oligomenorrhea or amenorrhea. Only luteal phase length was shorter in the group exercising above the lactate threshold. This stability of the reproductive axis occurred despite significant increases in oxygen consumption in the training groups.[280] Importantly, the women in this study had few risk factors for exercise-induced reproductive dysfunction because their mean gynecological age was 17 years and they were carefully screened for coexisting psychiatric disease or eating disorders. Nevertheless, critics of this study note that the trained women may have been exercising enough at baseline to alter reproductive hormone secretion,[296] although there was little evidence that intensifying their exercise adversely affected their reproductive function.

The mechanisms whereby some women lose menstrual cyclicity when they initiate an exercise regimen are complex. Loss of body fat with intense exercise, with or without an underlying eating disorder, has long been thought to explain exercise-induced amenorrhea. Maintenance of a minimal percentage of body fat and a minimal weight for height was believed to be necessary to prevent menstrual dysfunction or restore menses in athletic women experiencing amenorrhea.[297] However, this theory has been disproved in multiple studies, none of which has been able to differentiate normally cycling athletes from oligomenorrheic or amenorrheic athletes by anthropometric features alone.[283,288,298,299] In addition, the changes in LH pulsatility are evident within 5 days,[300] much sooner than changes in adiposity appear.[301]

An imbalance between caloric intake and the amount of energy expended with exercise more likely explains the reduced hypothalamic GnRH drive observed in women with exercise-associated menstrual dysfunction.[287,302] Energy availability can be defined by the difference between energy intake and energy expenditure, with exercise being one component of energy expenditure. When energy availability is low, reproductive function suffers, as do thermoregulation, growth, and cellular maintenance.[303] Detailed nutritional assessments of athletic women who were expending 900 to 1000 kcal/day exercising showed that their daily caloric intake was similar to that of sedentary women. Thus, energy availability was significantly lower in the former, although none of these athletes had been formally diagnosed with an eating disorder. Fat and protein accounted for a smaller percentage of calories in the athletic women, and the athletic women with amenorrhea consumed 50% less fat than their regularly menstruating counterparts. This negative energy balance was reflected in insulin–glucose dynamics, with amenorrheic athletes showing marked hypoinsulinemia throughout the 24-hour observation period and glucose levels that were 10% lower. Women with more significant hypoinsulinemia had the lowest dietary fat intake and experienced greater reductions in LH pulse frequency.[287]

These effects of energy availability on hypothalamic GnRH secretion occur relatively quickly. When a negative energy balance is created experimentally, by restricting calories,[304] increasing expenditure,[305] or combining the two,[302] declines in LH pulse frequency occur within 1 week. Detailed studies in women whose energy availabilities were gradually decreased while they maintained a constant energy expenditure of 15 kcal/kg lean body mass (LBM)/day (kcal/kg LBM × day) illustrated that 30 kcal/kg LBM × day is the threshold of energy availability required to maintain normal reproductive function.[300] When energy availability falls below this threshold, LH pulse frequency declines and amplitude increases, as illustrated in Figure 21.16.[300]

The sensitivity of GnRH neurons to energy availability appears to decline with increasing gynecological age, suggesting that hypothalamic centers become desensitized to these metabolical signals over time. Caloric restriction combined with vigorous daily aerobic exercise altered LH pulse frequency in adolescents, but not adults, changes that appeared within 5 days of energy deprivation, as shown in Figure 21.17.[284] Correction of this negative energy balance in amenorrheic exercising monkeys who continued vigorous training was achieved with overnutrition, and the rapidity of recovery was directly related to energy consumption.[306,307]

These experimental data suggest a role for a peripheral marker of adequate energy stores that can also act centrally to regulate the reproductive axis. Derived principally from adipose tissue, leptin is one such hormone whose receptors have been identified in the hypothalamus, pituitary, ovary, and testes. Its effects are stimulatory in the CNS and inhibitory at the gonads.[244] Leptin concentrations in amenorrheic athletic women are significantly reduced compared with those in eumenorrheic athletic women,[308] and the normal diurnal pattern of leptin secretion is lost in the former.[309] Opposing the actions of leptin, ghrelin is an orexigenic hormone that stimulates the release of NPY and agouti-related protein from the arcuate nucleus of the hypothalamus. Exercise does not appear to alter ghrelin levels in the short term. Chronic exercise over 1 year in overweight sedentary women was associated with increases in ghrelin when the exercise was accompanied by weight loss. Not surprisingly, amenorrheic athletes had 85% higher levels of ghrelin than did women who were ovulatory and sedentary, ovulating and exercising, or exercising with an LPD.[310] Thus, both leptin and ghrelin are likely involved in the metabolical signaling cascade that mediates the reproductive effects of exercise.[311]

Exercise may also impair the hypothalamic–pituitary–gonadal axis in some women by acting as a chronic stressor and activating the HPA axis. This activation mobilizes fuel stores in an attempt to maintain metabolical homeostasis. Ultimately, however, this HPA activation impairs other hypothalamic–pituitary axes, including the hypothalamic–pituitary–gonadal axis, through changes in CRH, proopiomelanocortin, ACTH, endogenous opiates, adrenal corticosteroids, or neurotransmitters. Both regularly menstruating and amenorrheic female athletes demonstrated higher plasma cortisol levels compared with regularly menstruating nonathletes with preservation of a diurnal pattern.[286,287] The degree of hypercortisolism correlated negatively with the magnitude of reduction in LH pulse frequency.[287] Administration of carbohydrates during prolonged exercise was shown to attenuate these increases in cortisol in both rodents[312] and humans,[313] suggesting that

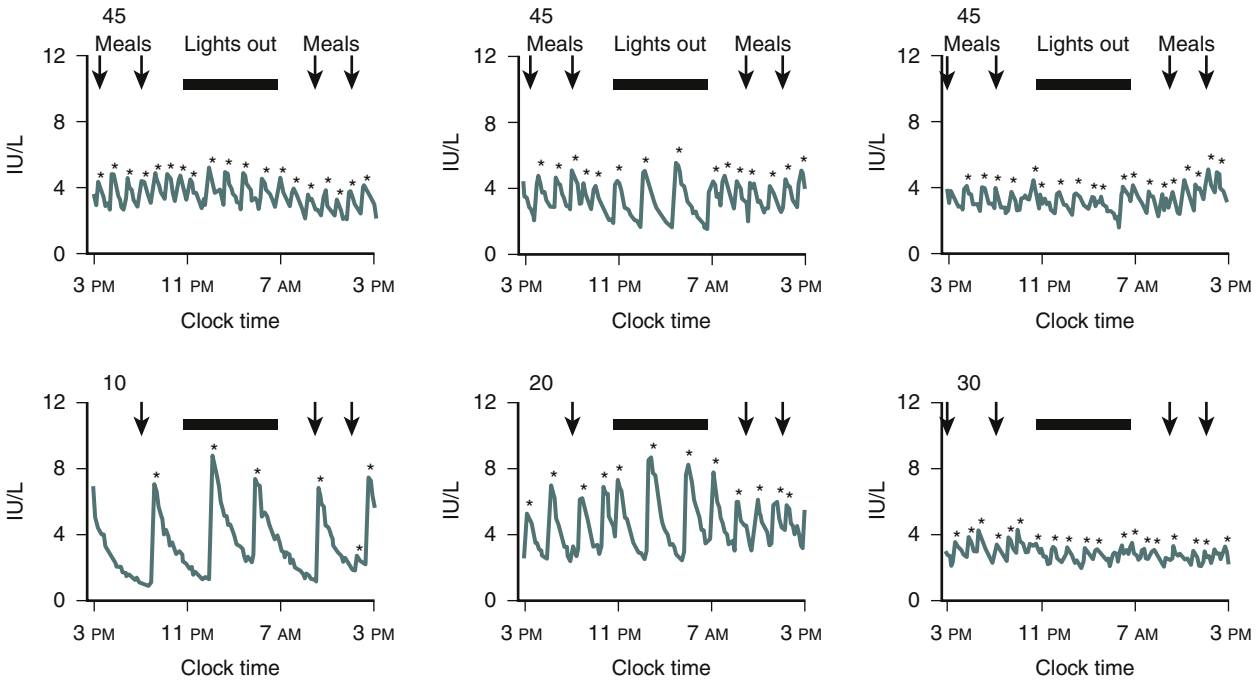


FIGURE 21.16 Twenty-four–hour luteinizing hormone (LH) pulse profiles for representative women after balanced energy availability treatments of 45 kcal/kg lean body mass (LBM) × day *(top)* and the paired restricted energy availability treatments of 10, 20, and 30 kcal/kg LBM × day, left to right, respectively *(bottom)*. *Asterisks* indicate LH pulses. The *black bar* indicates when lights were turned off. *Arrows* indicate meals. *(Modified from Loucks AB, Thuma JR. Luteinizing hormone pulsatility is disrupted at a threshold of energy availability in regularly menstruating women.* J Clin Endocrinol Metab *88:297, 2003. With permission from The Endocrine Society.)*

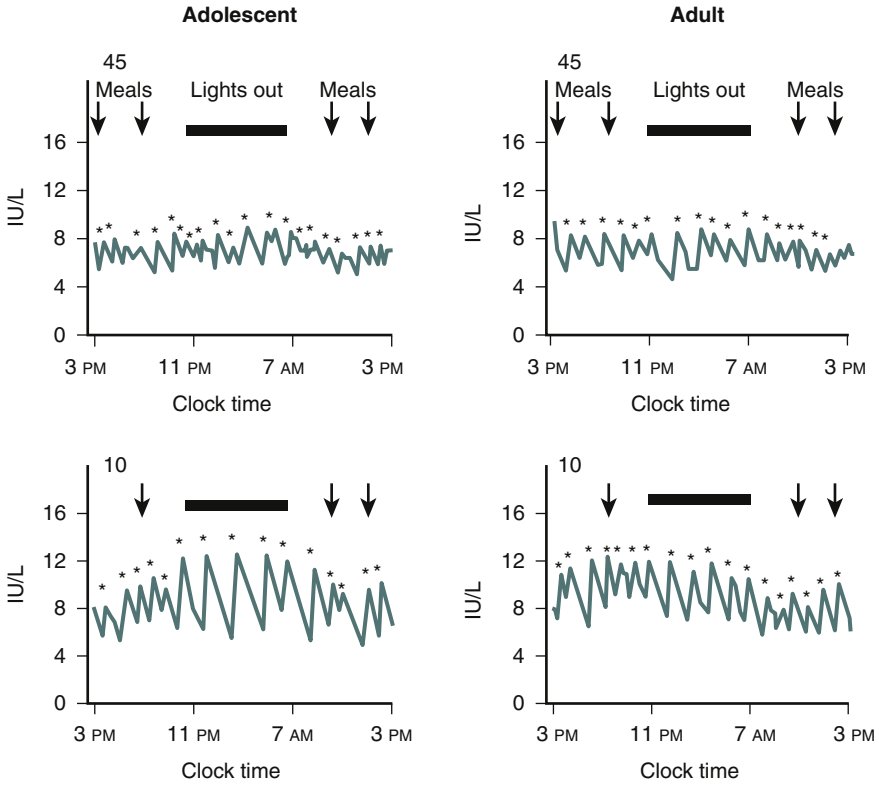


FIGURE 21.17 Twenty-four–hour luteinizing (LH) pulse profiles of a representative adolescent and adult after energy availability treatments of 45 and 10 kcal/kg fat-free mass/day. *Asterisks* indicate LH pulses. The *black bar* indicates when lights were turned off. *Arrows* indicate meals. *(Modified from Loucks AB. The response of luteinizing hormone pulsatility to 5 days of low energy availability disappears by 14 years of gynecologic age.* J Clin Endocrinol Metab *91:3158, 2006. With permission from The Endocrine Society.)*

this HPA axis activation may occur in response to inadequate energy availability and that dietary energy restriction is more disruptive to the reproductive axis than energy expenditure through exercise.[289]

Alterations in the somatotroph axis also occur as a result of exercise-associated metabolical changes. Both GH pulse frequency and mean 24-hour mean GH are augmented in amenorrheic athletes,[287,314] changes also observed in states of nutritional deficiency, such as AN.[315] These increases in GH levels do not effect a rise in circulating serum IGF-1, which directly stimulates LH release in rodent studies.[316] This lack of increase in IGF-1 likely reflects the increases in its binding protein (IGF binding protein-1 [IGFBP-1]), which is the most metabolically response IGF-binding protein. In the prevailing hormonal milieu associated with exercise (low endogenous insulin, elevated cortisol), IGFBP-1 increases and serves as another peripheral signal of fuel shortage, slowing hypothalamic GnRH release. Unlike IGFBP-1, the concentrations of other GH-binding proteins (GHBPs) fall 35% in amenorrheic athletes and are unchanged in regularly cycling athletes, demonstrating the role that nutrient availability plays in the regulation of hepatic GHBP synthesis. Exercise-associated variations in circulating catecholamine and free fatty acid levels may also contribute to this complex regulation of hypothalamic–pituitary GH secretion.[287]

Given its role in metabolism, it is not surprising that thyroid hormone is affected by the hypothalamic dysfunction that occurs with exercise of moderate intensity. Energy imbalance as a result of carbohydrate restriction causes triiodothyronine (T_3) to decline, the result of reduced 5′-diodinase activity. In men, increased energy expenditure through moderate exercise results in a rise in reverse T_3 and a fall in serum free T_3, changes similar to those seen in the "low T_3 syndrome."[317] Exercise in women is associated with significant reductions in T_4 and T_3, although only amenorrheic athletic women have alterations in free T_3 and reverse T_3 levels, both of which are significantly decreased compared with regularly menstruating athletes or sedentary women. Mean 24-hour TSH levels are not affected by this reduction in thyroid hormone negative feedback. Thyrotropin-releasing hormone (TRH) deficiency with exercise could explain these differences in women.[318] Alternatively, the nocturnal hypermelatoninemia observed in amenorrheic athletes[319] could modulate thyroid hormone levels because melatonin secretion from the pineal gland reduces serum thyroid hormone levels in animals and humans.[318,320,321] Finally, some studies suggest a genetic predisposition which contributes to the susceptibility to inhibition of the reproductive axis by stressors such as exercise, nutritional deficits, and psychological stressor.[322]

In summary, multiple metabolical signals are clearly involved with signaling to the GnRH pulse generator. Exercise can temporarily alter these metabolical signals if it creates an imbalance in energy availability. Such an imbalance can be prevented by supplementing dietary intake. Although most female athletes do not intentionally restrict their intake, few consume the additional calories needed to maintain a healthy energy balance. The exact nature of these interactions is complex and continues to be the subject of multiple investigations, but appears to be independent of body weight.

Long-Term Complications of the Hypoestrogenism Associated with Exercise

The complications associated with hypoestrogenism and exercise include infertility, lipid abnormalities, and premature bone loss.[303] Infertility results from poor or absent ovarian follicular development, impaired ovulation, or insufficient luteal-phase progesterone concentrations to support implantation. Amenorrheic athletes frequently have elevated low-density lipoprotein cholesterol levels[323] and endothelial dysfunction, despite their lack of body fat and low dietary fat intake.[324] Although the long-term effects of these cardiovascular risk factors in this population are not known, the adverse effects of amenorrhea on the skeletal system in young female athletes are well established. Estimates of the prevalence of osteopenia among female athletes range from 22% to 50%, whereas the prevalence of osteoporosis is much lower (0% to 13%).[325] Reductions in BMD are the result of increased bone resorption secondary to estrogen deficiency and decreased bone formation resulting from reduced energy availability.[303] Exercise itself may have differential effects on BMD, depending on the site examined. BMD at weight-bearing sites containing predominantly cortical bone, such as the femoral neck, was normal or increased in adolescent ballet dancers, whereas BMD was reduced at sites with predominantly trabecular bone, such as the spine and ribs. Sedentary amenorrheic girls with anorexia whose percentage of body fat was similar to the ballet dancers had lower BMD at all sites compared with regularly menstruating girls. Thus, weight-bearing exercise may offset the adverse effects of exercise-associated hypogonadism on cortical bone.[326] The skeletal effects of milder degrees of reproductive dysfunction, such as LPD, are unknown, and increasing evidence suggests that the declines in BMD associated with exercise-induced hypogonadotropic hypogonadism may not be reversible.[327]

The American College of Sports Medicine uses the term "the female athlete triad" to refer to the association of menstrual dysfunction, disordered eating, and osteoporosis in female athletes. Figure 21.18 illustrates the importance of

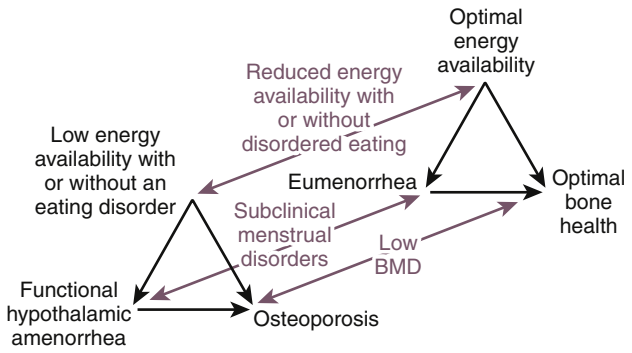


FIGURE 21.18 Female athletic triad. The spectrums of energy availability, menstrual function, and bone mineral density (BMD) along which female athletes are distributed *(purple arrows)*. An athlete's condition moves along each spectrum at a different rate according to diet and exercise habits. Energy availability, defined as dietary energy intake minus exercise energy expenditure, affects bone mineral density both directly via metabolical hormones and indirectly via effects on menstrual function and thereby estrogen *(black arrows)*. *(From Nattiv A, Loucks AB, Manore MM, Sanborn CF, Sundgot-Borgen J, Warren MP. The female athlete triad.* Med Sci Sports Exer *38:1867, 2007.)*

optimal energy availability for both reproductive and skeletal health.[303]

Caloric intake that is adjusted for the energy expended during exercise maintains normal hypothalamic–pituitary–gonadal function (eumenorrhea), while also providing nutrients for normal bone formation. Athletes who maintain such homeostasis often have BMD 5% to 15% above those of age-matched individuals. However, even mild but sustained reductions in energy availability can induce subtle degrees of menstrual dysfunction. Eventually, such dysfunction impairs estrogen-mediated bone resorption and BMD begins to fall below that expected. Not surprisingly, eumenorrheic athletes have higher BMD than amenorrheic athletes, and eumenorrheic athletes with disordered eating but no formal eating disorder have lower BMD.

Identifying adolescents and women with the female athlete triad can be difficult, especially when overt amenorrhea has not developed. Even in the absence of a clinical eating disorder, restrictive eating behaviors should raise concern and should be investigated further, potentially with the help of a mental health professional. A history of multiple stress fractures or fractures with minimal trauma might also be the first clinical manifestations of nutritional deficits.[326] Healthcare providers should be especially mindful of menstrual dysfunction in adolescent females engaged in sports where leanness is emphasized (i.e., gymnastics, cheerleading, diving). Such athletes should be encouraged to optimize their nutrition and vitamin D status to prevent exercise-induced menstrual dysfunction.[303] Exercise-associated menstrual dysfunction is a diagnosis of exclusion, however, and women should still be evaluated for pregnancy, hyperprolactinemia, thyroid dysfunction, premature ovarian failure, and anatomical abnormalities of the uterus, even if the history is consistent with exercise as the cause of oligomenorrhea or amenorrhea.

Treatment

Treatment of adolescents and adults with exercise-associated reproductive dysfunction requires a modification in diet or exercise behavior to increase energy availability, an intervention that is often difficult to implement.[328,329] Such nonpharmacological approaches are also more effective at increasing bone density than is hormone replacement therapy (HRT).[327,324] In the absence of weight gain, HRT has not been consistently effective at increasing BMD in patients with exercise-induced hypogonadotropic hypogonadism,[330] likely because it does not correct the abnormal metabolical milieu that may be contributing to premature bone loss. In addition, many athletes refuse to take estrogen because of the associated weight gain. HRT may be indicated in a woman with continued loss of BMD on serial bone densitometry scans, despite adequate nutritional intake and body weight. Bisphosphonates are not used for the bone loss associated with exercise-induced amenorrhea because of their long residence time in bone and the harm they may cause to future developing fetuses. Recombinant IGF-1 used in combination with HRT in the form of oral contraceptive pills may be better than oral contraceptives alone at increasing BMD, but is not currently used clinically.[257]

Infertility related to exercise-induced alterations of the hypothalamus can be treated with exogenous gonadotropins, but more conservative interventions, such as restoration of body weight, are much preferred. Women with low body weight and borderline nutritional status before conception may not augment their intake enough during pregnancy to sustain normal fetal growth, thus placing them at higher risk for spontaneous fetal loss. Progesterone can be prescribed for those women with LPD that does not correct, despite increases in energy intake or decreases in exercise.[303]

Stress and Functional Hypothalamic Amenorrhea

Undernutrition, overnutrition, and exercise are well-recognized causes of functional hypothalamic amenorrhea (FHA), which is defined by hypothalamic hypogonadism in the absence of any structural hypothalamic or pituitary abnormality. The mediators of this hypothalamic dysfunction are multiple and likely include insulin, leptin, ghrelin, CRH, and cortisol. FHA accounts for 34% of cases of secondary amenorrhea in adults, making it the most common cause of this disorder.[331] FHA also exists in women who do not meet the criteria for an eating disorder and do not exercise; such cases are often attributed to stress or are termed *idiopathic*. Life-threatening stress (i.e., experiencing refugee or concentration camps, wars, air raids) has long been known to cause amenorrhea in the majority of women; it resolves once environmental conditions improve.[332] Less severe stressors also contribute to menstrual dysfunction in some women. Characterization of women with such stress-associated menstrual dysfunction is complicated by the heterogeneity of the condition.

Stress is difficult to define objectively,[333] but Selye noted in 1939 that it was accompanied by activation of the HPA axis and that it reduced reproductive capacity.[334] This physiological response makes sense teleologically because it allows for preservation of adrenocortical function in the case of emergency at the expense of gonadal activity. Acute stress results in a brief increase in LH and testosterone, possibly as a result of a decrease in the metabolical clearance rate rather than a change in the secretory rate. However, this initial stimulatory phase is short-lived and is not nearly as clinically relevant as the response to prolonged stress, which has consistently been shown to inhibit hypothalamic GnRH secretion, to interfere with GnRH-induced LH production, and to impair gonadal response to LH and FSH. Stress-related increases in CRH, pro-opiomelanocortin, ACTH, endogenous opiates, adrenal corticosteroids, or neurotransmitters may modulate these central and peripheral effects on the reproductive axis. The interactions of each of these factors with each other and with the hypothalamus, pituitary, and gonads are extremely complex, as shown in Figure 21.19 and, therefore, difficult to distinguish experimentally.[335]

The decreased GnRH activity characteristic of FHA reflects CNS and hypothalamic responses to perceived or actual stressors. Different stressors elicit different neuromodulatory responses in different people, and the magnitude of these responses may be determined early in life by events that permanently influence the sensitivity of the HPA axis.[336] The severity of patients' self-reported psychomatic disturbance has been shown to correlate with the degree of gonadotropin suppression.[337] Most women are able to maintain normal menstrual cycles throughout stressful life events, periods of weight loss, and periods of intense exercise. In those who have amenorrhea, some degree of

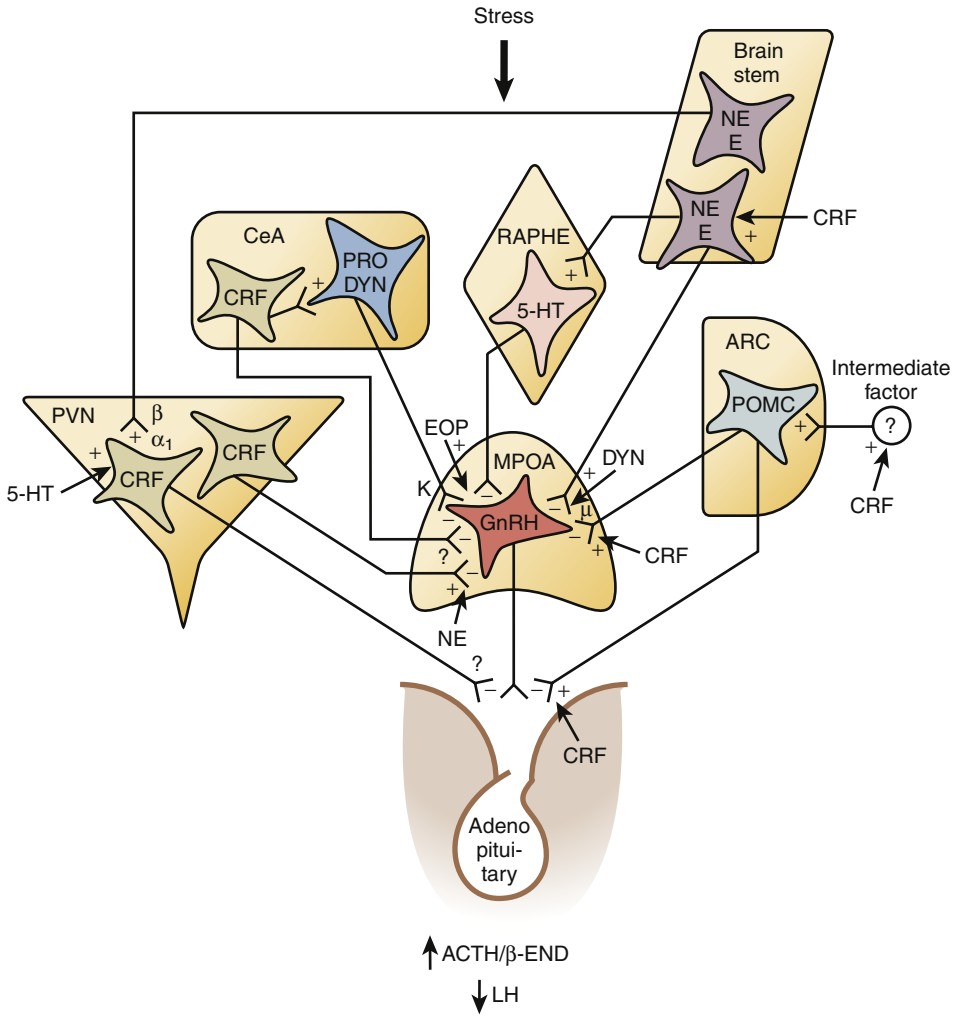


FIGURE 21.19 The main effects of physical stress on different brain areas, and peptidergic or aminergic pathways directly or indirectly involved in alteration of the activity of gonadotropin-releasing hormone (GnRH) neurons. ACTH, adrenocorticotropic hormone; ARC, arcuate nucleus; β-END, β endorphin; CeA, central amygdaloid nucleus; CRF, corticotropin-releasing factor; DYN, dynorphin; E, epinephrine; EOP, endogenous opioid peptides; NE, norepinpehrine; 5-HT, 5-hydroxytryptophan; LH, luteinizing hormone; MPOA, medial preoptic area; POMC, proopiomelanocortin; PRODYN, prodynorphin; +, stimulation; PVN, paraventricular nucleus; RAPHE, –, inhibition; ?, mechanisms unclear and remain to be fully investigated. *(Modified from Rivier C, Rivest S. Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms.* Biol Reprod *45:523, 1991.)*

menstrual irregularity often precedes the complete cessation of menses, whereas women with amenorrhea related to Cushing syndrome, hypothyroidism, Sheehan syndrome, or pituitary tumors usually have no history of menstrual dysfunction.[331] Similarly, athletes who have amenorrhea during periods of intense exercise often have a history of menstrual irregularity.[338] Clearly, in some individuals, the hypothalamic–pituitary–gonadal axis is more sensitive to disturbances in homeostasis than it is in others. Men are also susceptible to stress-associated reproductive compromise, but identifying functional declines in GnRH pulsatility is quite difficult in males. Asthenospermia (decline in sperm motility) may be the mildest manifestation of altered GnRH drive, but is usually only diagnosed in the workup of infertility.[339]

Gonadotropin secretion in women with FHA is inappropriately low, but the pattern of secretion varies during the course of illness. Studies in women with FHA who were of normal weight for height, who were not being treated for an eating disorder, and who were not exercising excessively at the time of sampling showed low-frequency LH secretion in 43%, low-frequency and low-amplitude secretion in 27%, low-amplitude secretion in 8%, apulsatile secretion in 8%, and an unclassified pattern in 14%, as shown in Figure 21.20.[340]

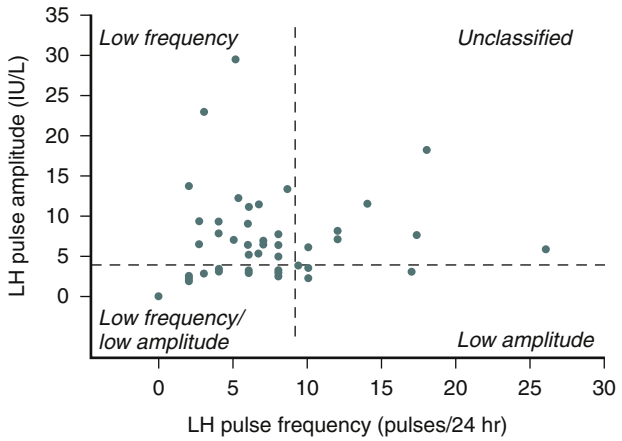


FIGURE 21.20 Classification of subjects with hypothalamic amenorrhea by luteinizing (LH) pulse frequency and amplitude, defined by mean –1 standard deviation in normally cycling women *(hatched lines)*. Low pulse amplitude was defined as less than 4 IU/L. Low pulse frequency was defined as fewer than 9 pulses/24 hr. *(Adapted from Perkins RB, Hall JE, Martin KA. Neuroendocrine abnormalities in hypothalamic amenorrhea: spectrum, stability, and response to neurotransmitter modulation.* J Clin Endocrinol Metab *84:1905, 1999. With permission from The Endocrine Society.)*

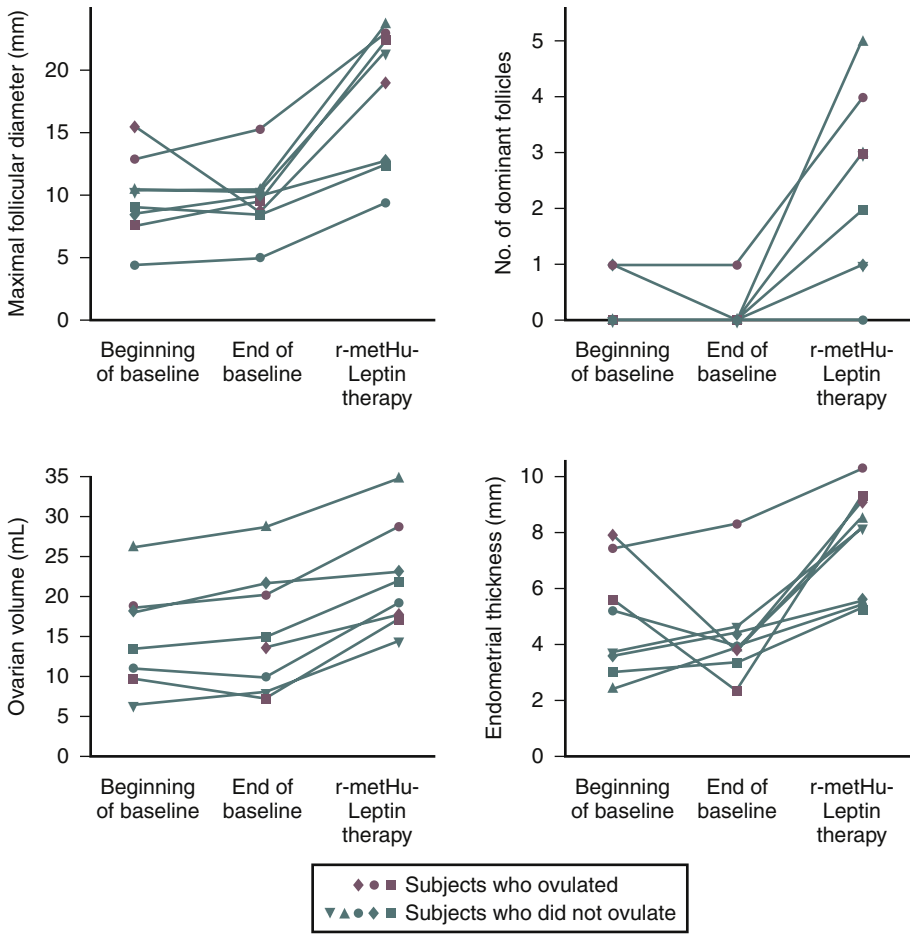


FIGURE 21.21 Follicular, ovarian, and endometrial ultrasonographic measurements at the beginning and end of the 1-month baseline period and at their maximum during recombinant methionyl human leptin (r-met-Hu-leptin) treatment. Each symbol represents one subject. *(Modified from Welt CK, Chan JL, Bullen J, Murphy R, Smith P, DePaoli AM, Karalis A, Mantzoros CS. Recombinant human leptin treatment in women with hypothalamic amenorrhea.* N Engl J Med *351:987, 2004. Copyright © 2004 Massachusetts Medical Society. All rights reserved.)*

Twelve patients participated in two to eight overnight sampling studies each, and LH secretory patterns changed in 75% of these women on repeated study. The majority of these women responded to naloxone infusion with increases in LH pulse frequency, implicating enhanced opioid tone as a cause of hypothalamic amenorrhea in a subset of patients. This naloxone response occurred in women regardless of their baseline neuroendocrine patterns, and has been supported by previous studies.[341] Thus, women with FHA do not appear to have a static defect of GnRH secretion. Rather, their hypothalamic sensitivity to endogenous opioids and other stress-associated factors is heightened, resulting in GnRH concentrations that rise and fall with changes in endogenous opioid levels. Such disordered GnRH pulsatility is unable to support follicular development and ovulation.[340]

Metabolical signals may also be responsible for inhibiting GnRH activity in idiopathic and stress-induced FHA. Specifically, both leptin and ghrelin have been implicated as markers of inadequate nutrition stores that are capable of signaling to the hypothalamus and suppressing the reproductive axis. In nonathletic women of normal weight with FHA, adipose-derived leptin levels are reduced compared with age-, weight-, and body fat–matched eumonorrheic control subjects, suggesting that energy deprivation may underlie menstrual dysfunction in these patients, as it does in patients with eating disorders and excessive exercise.[342,343] Administration of exogenous leptin to a small group of women with FHA increased maximal follicular diameter, ovarian volume, and endometrial thickness, as well as the number of dominant follicles, as shown in Figure 21.21.[344] LH pulse patterns normalized, and E_2, thyroid hormone, and IGF-1 levels increased in the majority of these women, despite the fact that they lost weight and experienced a decline in their percentage of body fat.[344]

Treatment with leptin over 36 weeks in women with FHA resulted in recovery of menstruation and also changes in markers of bone metabolism.[345]

Unlike the anorexigenic leptin, ghrelin is an orexigenic molecule that stimulates food intake. Ghrelin acts on the same neurohormonal pathways as leptin and is secreted by gastric oxyntic cells. Ghrelin levels correlate well with feelings of hunger and are higher in women with eating disorders and in exercising women who lose weight.[246] In addition, in animal studies, ghrelin has been shown to suppress LH pulse frequency in monkeys,[346] which has been confirmed for LH and FSH in human studies.[347] It may not be surprising that ghrelin levels are significantly higher in women with FHA, whose scores on tests of eating disordered behaviors were significantly higher than those of control women. Ghrelin elevation may be another mechanism that the body uses to stimulate appetite and suppress reproduction in the face of a negative energy balance.[348] Studies of male Army Rangers engaged in an elite combat leadership course also support the hypothesis that low energy availability is an important mediator of FHA. In these men, alterations in metabolical

and reproductive hormones during periods of semistarvation resolved after *ad libitum* refeeding, despite continued exposure to other stressors (heat, cold, sleep deprivation, extreme exercise, illness, injuries).[349] Thus, whether a reduction in energy availability occurs as the primary homeostatic insult, as it does in AN, or whether it is a secondary response to stress, it may be the proximate causes of the majority of reproductive disorders.[333] The hypogonadotropic hypogonadism associated with FHA may be the final common pathway by which the body serves to protect itself from additional stressors, such as childbearing.

Detailed overnight hormonal sampling studies in women with FHA showed the expected changes in gonadotropins. Mean serum LH concentrations, LH pulse frequency, and FSH were reduced by 30%, 53%, and 19%, respectively. Importantly, however, abnormalities in other hypothalamic–pituitary axes were also apparent. In addition, 24-hour cortisol secretion was 17% higher in women with FHA compared with eumenorrheic women,[350] but this hypercortisolemia did not suppress CSF CRH levels, suggesting hypothalamic resistance to cortisol negative feedback.[351] In addition, 24-hour PRL was lower, and nocturnal GH secretion was higher and more disordered. TSH was unchanged, but serum T_3 and T_4 were lower in the women with FHA. Administration of hypothalamic-releasing factors (GnRH, CRH, GH-releasing hormone [GHRH], TRH) showed normal pituitary responses in both groups, evidence that global hypothalamic dysfunction occurs in women with FHA.[350]

Treatment of Stress-Associated FHA

Restoring reproductive function in cases of stress-associated FHA is not as straightforward as the treatment of exercise-induced amenorrhea or anorexia-associated amenorrhea, in which case the pathological behaviors are obvious and potentially reversible. Women with all three types of amenorrhea tend to be perfectionists who desire social approval and who possess abnormal attitudes toward eating.[352,353] Detailed questioning is often required to uncover these unhealthy eating behaviors, which tend to be of the bulimic type in adult women and of the restrictive type in adolescents.[337,343] Women and adolescents with FHA tend to have lower self-esteem and a higher prevalence of psychological disturbances compared with eumenorrheic control subjects and women with amenorrhea due to other causes.[337,354] Cognitive behavioral therapy (CBT) can help to identify stressors and maladaptive attitudes toward eating and provide patients with problem-solving techniques to better cope with stress-inducing circumstances. CBT has also been shown to restore ovulation in up to 75% of women who were previously anovulatory, whereas observation alone was effective in only one of eight women during the 20-week study period. Responses to CBT occurred relatively quickly after CBT initiation (mean, 11.2 ± 3.3 weeks)[355] and were preceded by normalization of the hypercortisolemia characteristic of FHA.[356] Such psychological intervention is also more likely to allow for recovery of all endocrine and metabolical disturbances than is targeted treatment of the hypothalamic–pituitary–gonadal axis alone with sex steroid replacement.[357] Sex steroids can be considered after amenorrhea has persisted for 6 months or more despite lifestyle changes; lumbar spine BMD may improve in such patients.[358] Recombinant leptin may also have a potential role in preventing FHA-associated bone loss in the future, either alone or in combination with E_2, although leptin's suppressive effects on appetite are concerning in this population, which is already predisposed to altered eating behaviors.[359] Exogenous gonadotropins can be used to induce ovulation and achieve pregnancy in FHA, but they will not reverse the hypothalamic hypothyroidism and hypercortisolism that often accompany the hypogonadism. If pregnancy is achieved, obstetric and fetal outcomes may be adversely affected by failure to correct these other hormonal disturbances.[357,360]

Establishing recovery rates in cases of FHA unrelated to weight loss or exercise is complicated by the difficulty in defining this subset of patients. FHA of idiopathic etiology confers a poorer prognosis, with recovery rates in small longitudinal studies of 6 to 10 years' duration ranging from 29% to 61%. FHA related to stress almost always resolves once the causal factor is reversed, especially if there is associated weight loss that improves concurrently.[361,362] No neuroendocrine pattern was predictive of recovery.[361] The fact that few women with FHA of defined etiology do not experience recovery of menses, even after correction of the inciting factor, shows that both physiological and environmental insults are important.[361]

Medication-Associated Hypogonadotropic Hypogonadism

A variety of medications can interfere with the hypothalamic–pituitary axis as a result of their tendency to increase PRL.[363-385] Given that PRL release is under tonic inhibition by hypothalamic dopamine secretion, medications that interfere with hypothalamic release or transport of dopamine or that block pituitary dopamine receptors can raise serum PRL. The degree of PRL elevation in such cases is usually less than 100 ng/mL, a range that overlaps that seen with PRL-secreting microadenomas and with stalk deviation caused by other pituitary lesions.[385] More detailed description on the effects of medications on prolactin levels is described in Part 1, Chapter 3 of this edition.

Pituitary Dysfunction

Pituitary Tumors

Development of Pituitary Tumors: Etiological Considerations

Currently available information suggests that the majority of pituitary tumors develop from monoclonal tumor cell expansion of a mutated somatic cell.[386] Such mutations can occur in a familial setting within the germ cell line or may be sporadic. The development of a pituitary adenoma within a familial setting is known to occur in multiple endocrine neoplasia type 1 (MEN 1) and Carney complex (CNC)[387,388] as well as in familial acromegaly.[389] As discussed later, the MEN 1 syndrome is caused by mutations in the tumor suppressor gene *MEN1*, which is located on chromosome 11q13 and encodes a 610–amino acid nuclear protein. In patients with the *MEN1* gene, pituitary adenomas occur in approximately 40% of cases. Other mutations, such as that in the *CDKN1B* gene (which encodes for the cyclin-dependent kinase inhibitor $p27^{Kip1}$), are also considered to be possibly involved in the MEN 1 syndrome. CNC is associated with mutations in the protein kinase A1 alpha regulatory subunit gene on chromosome 2p16 in 60% of cases.[390,391]

Isolated familial somatotropinoma (IFS) is defined as more than two cases of acromegaly in a family in the absence of MEN 1 or CNC. The genetic pathophysiology is still unclear, even though a mutation in the gene encoding the aryl-hydrocarbon receptor–interacting protein has been found in some cases, including four Northern Irish families.[392-394] Somatic mutations in the $G_s\alpha$ gene *(GNAS1)* are found in a subset of GH-secreting tumors, with a prevalence ranging from 40% in white populations to 10% in Japanese populations. The oncogenic gsp mutation correlates with constitutively increased cyclic AMP response element–binding protein phosphorylation and activity, which then leads to increased Pit-1 transcription and GH synthesis.[391] An example of a sporadic mutation associated with pituitary cell tumors is the activating mutation in *GNAS1*, which is seen in McCune-Albright syndrome, a condition with multiple endocrine manifestations, including hyperplastic pituitary lesions.[395] Several *in vivo* and *in vitro* lines of evidence suggest that the clonal expansion of the mutated pituitary cell requires a secondary event for tumor progression.

This secondary event may be an additional somatic mutation or overexpression of genes in pituitary adenomas. Somatic mutations of the Ras oncogene have been associated with increased aggressiveness of prolactinomas and have been detected in metastasis of pituitary carcinomas.[396] Increased expression of cyclin E is associated with ACTH-producing pituitary adenomas. Overexpression of the pituitary tumor–transforming gene *(PTTG)* has been thought to be involved in pituitary tumorigenesis as all pituitary adenoma subtypes, and particularly invasive adenomas express *PTTG*. *PTTG* is a member of the securin family that regulates the separation of chromatids during mitosis.[397] Overexpression of *PTTG* is thought to be at least partly responsible for the aneuploidism often observed in pituitary tumors. Promotor methylation of the retinoblastoma gene and p16INK4a and low expression of the p27Kip1 protein all cause loss of tumor suppressor gene function and have been associated with the development and the aggressiveness of pituitary adenomas.[398,399]

Several growth factors have been shown to induce pituitary hyperplasia, with or without adenoma development. FGF-β, which is expressed in the pituitary and is a potent mitogen for neuroectoderm cells and their synthesis, is induced in NIH3T3 cells overexpressing *PTTG*. The heparin-binding secretory transforming gene *hst* seems to be associated with the aggressiveness of the tumor,[400,401] and pituitary TGF-α has been associated with estrogen-induced lactotrophic hyperplasia.

Few data concerning the effect of environmental factors on the development of pituitary tumors are available. However, the presence of the aryl hydrocarbon receptor on pituitary tumors, which is believed to mediate cell responses to toxins, such as dioxin,[402] suggests that such mechanisms may play a role in tumor development.

From both a morphological and a mechanistic perspective, pituitary hyperplasia must be distinguished from pituitary adenomas. Mechanisms subserving hyperplasia certainly include overstimulation of particular pituitary cell types by hypothalamic factors, such as GHRH and CRH, which may be secreted ectopically and lead to somatotrophic and corticotrophic hyperplasia. Moreover, situations in which negative feedback on the hypothalamic–pituitary system is absent as well may result in hyperplasia. For example, and as discussed later, untreated hypothyroidism may result in thyrotrophic hyperplasia, and inadequate glucocorticoid replacement after adrenalectomy is thought to be involved in the development of corticotrophic hyperplasia (Nelson syndrome).[391]

Pituitary Adenomas

Nonfunctioning Adenomas and Gonadotroph Adenomas

Nonfunctioning tumors of the pituitary either do not secrete hormones or do so at such a low level that clinical syndromes of hormone excess do not develop. The majority of these tumors are microadenomas (<1 cm) and clinically lead to no significant symptoms. Most of the nonfunctioning adenomas that do come to medical attention are macroadenomas that compress both pituitary and nonpituitary tissues and result in hypopituitarism.

In 70% of patients with a nonfunctioning macroadenoma, visual field defects are present.[403] More than 90% of patients have at least one pituitary hormone deficiency before surgery, most commonly, GH deficiency followed by hypogonadism. In 80% of these patients, secondary hypothyroidism occurs, and in 60%, secondary adrenal insufficiency is present before surgery.[404] In the rare case in which an adenoma secretes biologically active FSH, amenorrhea, high E_2 concentrations, and multiple ovarian follicles can occur.[405] In pubertal girls, these events may result in premature breast development and vaginal bleeding.[406]

Typically, the clinical diagnosis of a *gonadotroph adenoma* is made by an absence of symptoms that would suggest a different type of pituitary adenoma (e.g., of somatotrophic, corticotrophic, lactotrophic, or thyrotrophic origin) and by basal and stimulated secretion of gonadotrophins and their subunits. Approximately 80% of clinically nonfunctioning pituitary tumors are gonadotroph adenomas, and hypersecretion of more than one monomeric subunit, with or without LH or FSH secretion, is common. β-FSH is elevated in approximately 30% of patients, whereas the α subunit is elevated in approximately 20% of patients. Intact FSH hypersecretion occurs less frequently. In rare cases, LH hypersecretion is present, but seldom is sufficient to result in abnormally elevated testosterone levels.

In the setting of primary gonadal failure in general and in postmenopausal women in particular, serum concentrations of the gonadotropins and free subunits are also increased in the absence of an adenoma. Under those conditions, intravenous administration of TRH has been reported to help distinguish a normal hypothalamic–pituitary response to gonadal failure from a pituitary tumor. In approximately 40% of male patients and 69% of postmenopausal women with nonfunctioning pituitary adenomas, intravenous TRH results in an increase in serum concentrations of the gonadotropins or the free α/β subunit. In a study by Daneshdoost and colleagues, none of the healthy postmenopausal women had LH-β, LH, or FSH[407] responses to TRH. Unfortunately, TRH is not currently available for clinical use in the United States. Few data exist about the utility of measuring pituitary glycoprotein hormones or their free subunits, although some literature suggests that a postsurgical decrease in

circulating FSH concentration correlates with tumor size reduction. The final determination of the cellular basis of nonfunctioning pituitary tumors is based on immunohistochemistry. In most nonfunctioning tumors, immunostaining detects either intact pituitary glycoprotein hormones (FSH, LH, TSH) or the free subunits of these glycoproteins (α subunit, β-FSH, β-LH, β-TSH). In rare cases, PRL, ACTH, or GH is found. In fewer than 30% of nonfunctioning adenomas, no immunoreactive hormone or hormone subunit is seen (*null cell* pituitary tumors).[408]

Treatment. With regard to therapeutic strategies, nonfunctioning pituitary adenomas may express receptors for TRH, GnRH, and dopamine, but treatment with somatostatin analogs, GnRH agonists, GnRH antagonists, and dopamine agonists have shown inconsistent results.[409] In a small number of patients, 6-month combination therapy with cabergoline and octreotide resulted in a 30% tumor size reduction, but only in those patients who had detectable baseline or stimulated LH, FSH, and α subunit levels.[410] Temozolamide (an alkylating agent) has been reported to lead to a reduction in pituitary size in some cases in nonfunctioning pituitary adenomas.[411]

Nonfunctioning pituitary adenomas often are found as unsuspected pituitary lesions, discovered on an imaging study which was performed for unrelated reasons, so called incidentalomas. Based on the 2011 Endocrine Society Clinical Practice Guideline, surgical treatment is recommended when there is a visual deficit due to the pituitary lesion, other visual abnormalities, such as opthalmoplegia due to compression by the lesion exist, the lesion abuts or compresses the optic nerves or chiasm on MRI or in case of pituitary apoplexy with visual disturbance. The guideline suggests that surgery should be considered if there is significant growth of the pituitary lesion, in case of loss of endocrinological function, unremitting headache and if the lesion is close to the optic chiasm and the patient plans to become pregnant. Patients who do not meet criteria for surgical removal need to be followed with regular clinical assessments with imaging, laboratory tests, and visual field testing if necessary.[412]

Prolactin-Secreting Adenomas

Under physiological conditions, the regulation of PRL is inhibitory, mediated by dopamine through the type 2 isoform (D2) of the dopamine receptor. Dopaminergic neurons are primarily located in the arcuate nucleus (tuberoinfundibular system), but also in the rostral caudate and paraventricular nuclei (tuberohypophysial system).[413] Pituitary stalk injury or stalk compression interrupts these inhibitory circuits and results in increased PRL levels, which usually do not exceed 250 ng/mL.

Prolactin-secreting pituitary tumors include both prolactinomas and GH-producing pituitary tumors, which in 25% of cases cosecrete PRL. The latter observation reflects the fact that lactotroph and somatotroph cells derive from a common progenitor GH-expressing stem cell. Prolactinomas are classified as microadenomas (<10 mm) or macroadenomas (≥10 mm), with the risk of progression from microadenoma to macroadenoma estimated at 4% to 7% over a period of 8 years.[414] High PRL levels, larger tumor size, cavernous sinus invasion, and resistance to dopamine agonists are associated with increased aggressiveness. A serum PRL cutoff level of 3300 ng/mL predicts an invasive tumor with a specificity of 91%.[415] Specific clinical symptoms of prolactinomas in premenopausal women include hypogonadism, which may present as infertility, oligomenorrhea, or amenorrhea,[416,417] and galactorrhea. However, it cannot be overemphasized that galactorrhea may also occur in normoprolactinemic women. Kleinberg and colleagues reported that 86% of women with idiopathic galactorrhea without amenorrhea had normal PRL levels.[418] Regardless of its cause, the degree of PRL elevation correlates with the severity of hypogonadism. Serum PRL levels greater than 100 ng/mL are associated with hypogonadism and low E_2, which result in amenorrhea, hot flashes, and vaginal dryness. PRL levels of 50 to 100 ng/mL can be associated with both oligomenorrhea and amenorrhea, and levels of 20 to 50 ng/mL can result in a shortening of the luteal phase and resultant infertility.[186] Women with hyperprolactinemia and amenorrhea exhibit lower spine and forearm BMD compared with women with hyperprolactinemia and normal menses.[419] In men, sexual dysfunction is the main clinical manifestation of prolactinomas. Galactorrhea occurs in approximately 15% to 25% of men with microprolactinomas or macroprolactinomas and is less frequent compared with women. Osteoporosis can also occur in men with increased PRL levels.[420-422]

Besides PRL-producing pituitary adenomas, other causes of hyperprolactinemia include medications as well as hypothyroidism, chest wall trauma,[423] estrogens, chest wall tumors, herpes zoster of the chest, chronic renal failure, alcoholic cirrhosis, pregnancy, nipple stimulation, and the postictal state.[424] Idiopathic hyperprolactinemia is the diagnosis when no cause can be found. In a study by Sluijmer and Lappohn,[425] only 1 of 59 patients with idiopathic hyperprolactinemia who were followed for an average of 6.5 years had a pituitary adenoma.

In approximately 10% of patients with hyperprolactinemia, *macroprolactinemia* may be diagnosed. This condition is characterized by a predominance of high–molecular-mass circulating prolactin, which represents PRL complexed with anti-PRL immunoglobulins.[426] The cause of immunoglobulin production (mainly immunoglobulin G) is unclear. In a study that included more than 100 cases, dopaminergic treatment did not always result in normalization of serum PRL concentrations. Clinical symptoms associated with macroprolactinemia are sometimes absent because of the weak *in vivo* biological activity of macroprolactin. In patients with increased serum PRL levels and few or no symptoms related to PRL excess, this condition should be considered.[426] While gel-filtration chromatography (GFC) is currently considered the gold standard for macroprolactin measurement, this method is labour intensive and for routine use polyethylene glycol (PEG) precipitation is preferred.[427]

Falsely low PRL measurements can be encountered in the presence of very high PRL levels, reflecting the hook effect. In this case, the antigen (PRL) is present in the serum in excess and there is not enough antibody to bind both sides of the antigen. The assay value is significantly lower than the real value. Dilution of the samples will resolve the problem. The hook effect should be considered in the presence of a large pituitary tumor and low levels of circulating PRL.[428]

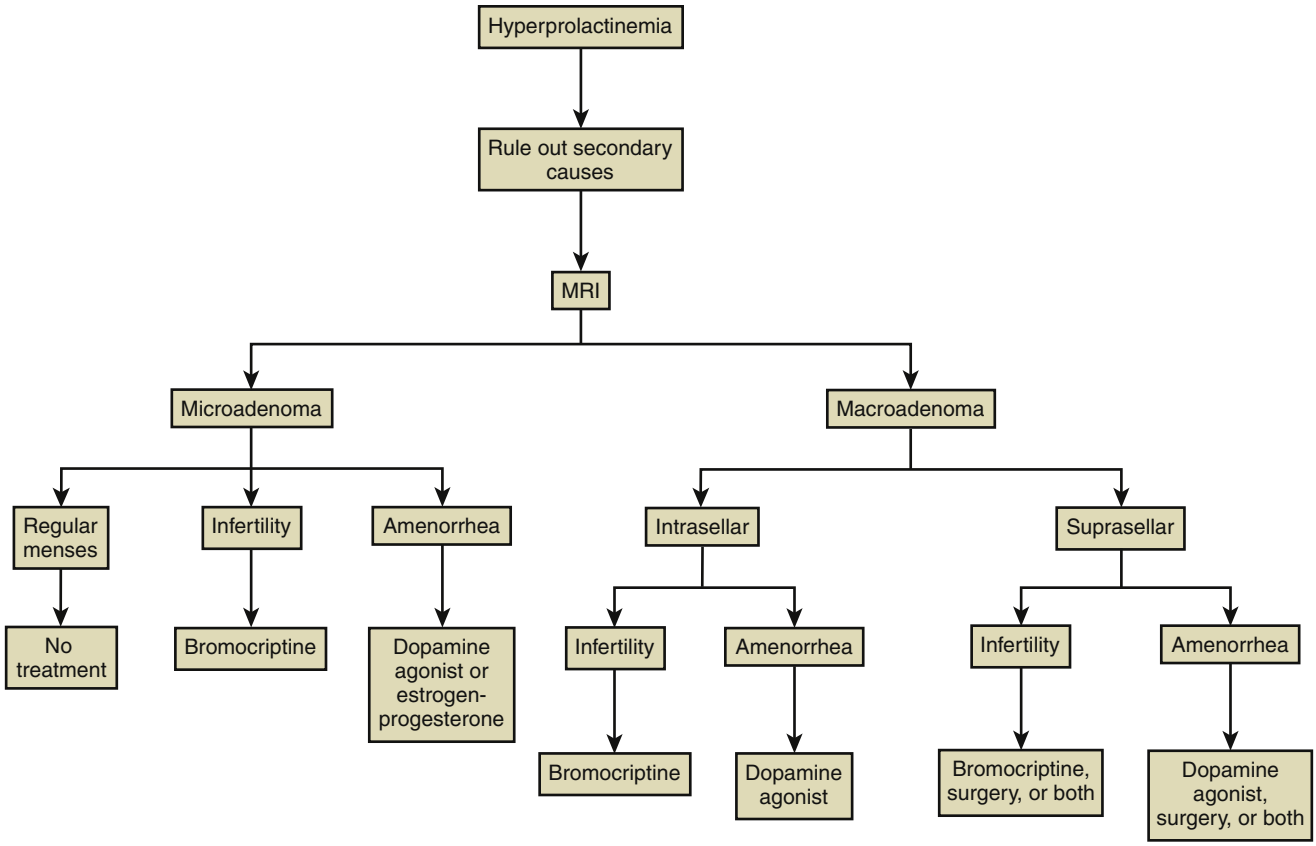


FIGURE 21.22 Algorithm for the management of prolactinoma in women. *(Reproduced from Schlechte JA. Clinical practice. Prolactinoma.* N Engl J Med *349:2035, 2003, with permission.)*

Treatment. The primary treatment objectives in patients with PRL-secreting pituitary adenomas include normalization of PRL levels, restoration of gonadal function, reduction of tumor size, and recovery of pituitary function. Both surgical and medical approaches to achieve these goals are available (Fig. 21.22).[429] For a detailed description of treatment options the reader is referred to Part 1, Chapter 3 of this edition.

Growth Hormone–Secreting Adenomas

Growth hormone is secreted in a pulsatile fashion by somatotrophic cells within the anterior pituitary gland. GH pulses can be detected every 3 to 4 hours and are primarily regulated by the interaction between the hypothalamic peptides somatostatin and GHRH. Most GH pulses occur at night. Somatostatin determines the trough levels of GH, whereas GHRH stimulates the release of GH from the pituitary as well as the increase in GH gene transcription.[430] Approximately 75% of pituitary GH circulates in the 22-kDA form, with 5% to 10% in the 20-kDA form.[431]

Growth hormone–secreting pituitary tumors represent approximately 2.8% of all pituitary tumors,[432] and are monoclonal in origin, suggesting intrinsic genetic alterations as the cause for their development. In a subset of GH-secreting tumors, a mutation of the *gsp* gene has been found, with the prevalence ranging from 40% in whites to 10% in Japanese. Other candidate genes associated with GH adenomas include *PTTG*, *H-ras*, *MEN1*, and *P16INK4a*. *PTTG* overexpression in pituitary tumors has been shown to correlate with tumor size. In 25% of GH-secreting pituitary adenomas, PRL is cosecreted.[433,434] Adenomas with plurihormonal secretion often have no apparent clinical symptoms and are called *silent somatotroph adenomas*, with increased IGF-1 and PRL levels.

In very rare cases, *ectopic GHRH secretion* is the cause of increased GH secretion. In such cases, MRI shows a normal or mildly enlarged pituitary because GHRH leads to hyperplasia of the somatotrophs.[435] Sources of increased GHRH secretion include hypothalamic tumors, bronchial carcinoids, and tumors of the pancreas, gastrointestinal tract, thyroid, and adrenals. Ectopic GH secretion by nonendocrine tumors has also been reported.[436]

Growth hormone–producing adenomas are phenotypically diverse, with two predominant histological forms, *densely granulated* and *sparsely granulated*. The two forms differ in their morphological features and respective growth patterns. *Densely granulated* GH cell adenomas show slow, expansive growth without invasion, resulting in the gradual development of acromegalic features. Such adenomas exert pressure on the surrounding nontumorous gland, resulting in the formation of a pseudocapsule, which helps with surgical removal. The *sparsely granulated* GH cell adenoma grows more rapidly and diffusely and is often invasive. Mixed adenomas expressing both GH and PRL are also more likely to invade surrounding tissues.[437]

Table 21.2 compares the most frequent symptoms found in acromegalic patients with symptoms found in patients with nonfunctioning pituitary tumors and prolactinomas.

Table 21.2 Comparison of Symptoms Between Acromegalic Patients and Patients with Nonfunctioning Pituitary Tumors and Prolactinomas

	Total Percentage		
Feature	Nonfunctioning (*n* = 99)	Acromegaly (*n* = 176)	Prolactinoma (*n* = 96)
Hypogonadal symptoms*	**35**	38	**70**
Headache	**48**	40	**46**
Galactorrhea	19	9	**49**
Visual deficit	**48**	26	19
Acral enlargement	—	**86**	—
Maxillofacial changes	—	**74**	—
Excessive perspiration	—	**48**	—
Arthralgia	—	**46**	—
Weight gain	13	18	13
Fatigue	20	26	17

*Hypogonadal symptoms include decreased libido, oligo- or amenorrhea, infertility, and erectile dysfunction.
The most frequent features observed for each tumor type are shown in **bold** print.
From Drange MR, Fram NR, Herman-Bonert V, Melmed S. Pituitary tumor registry: a novel clinical resource. *J Clin Endocrinol Metab* 85:168, 2000.

Many of the signs and symptoms of acromegaly develop slowly and insidiously, and patients are often treated for seemingly nonendocrine conditions long before the diagnosis of acromegaly is made. The average time between the onset of recognized symptoms and diagnosis is approximately 10 years.[432] Consequently, more than 70% of GH-secreting adenomas are macroadenomas at the time of diagnosis.[438] Besides the compressive symptoms of pituitary tumors, such as headache, visual field defects, and cranial nerve palsies, clinical symptoms of GH hypersecretion include hyperhidrosis, acral enlargement, thickness of the soft tissues of the hands and feet, prognathism, jaw malocclusion, macroglossia, arthralgias and arthritis, bilateral carpal tunnel syndrome, proximal myopathy, frontal bossing, skin tags, colon polyps, cardiomyopathy, hypertension, diabetes mellitus, sleep apnea, and visceromegaly. Cardiac disease is the primary cause of death in untreated acromegaly, with 50% of patients dying before the age of 50 years. Hypertension occurs in approximately 40% of patients, and left ventricular hypertrophy is present in more than 50% of normotensive acromegalic patients.

The serum IGF-I level is the best biochemical marker of clinical disease activity in acromegaly. Both GH and IGF-I correlate with mortality and when GH and IGF-I levels are controlled, mortality is close to the level expected in the general population.[439] Before the availability of IGF-I assays, dynamic tests were used and the oral glucose tolerance test ([OGTT] 75-g oral glucose load with serum GH sampling every 30 minutes over 2 hours) was the recommended test for the diagnosis and assessment of therapeutic outcome postoperatively. The OGTT may help diagnostically when there is a discrepancy between GH and IGF-I data, but it does not add additional diagnostic value when IGF-I levels are clearly elevated. A GH nadir during OGTT of less than 1 μg/L theoretically excludes acromegaly and indicates postoperative remission. However, the results of the OGTT must be interpreted with caution in conditions such as puberty, pregnancy, hepatic and renal disease, AN, and diabetes mellitus, all of which may cause inadequate GH suppression. A random GH level of 0.4 μg/L or less and an IGF-I level in the age-matched and sex-matched reference range excludes the diagnosis of acromegaly.[440]

Treatment. Treatment options for GH-secreting adenomas include surgery (with or without presurgical medical treatment), medical treatment, and RT. Data on postsurgical cure rates and recurrence rates must be interpreted by carefully reviewing the specific cutoff levels used for defining cure or recurrence and the assays used to measure IGF-I and GH.[441] Surgical cure rates also depend on the experience and workload of the surgeon.[442] Frank and colleagues[443] defined remission by a basal GH level of less than 2.5 ng/mL, a normal sex- and age-adjusted IGF-I level, and a GH nadir of less than 1 ng/mL after OGTT, and reported a cure rate of 83% in microadenomas and 64.5% in macroadenomas. Intraoperative invasiveness of the tumor decreased the cure rate to 44%, which was similar to the findings of other studies that reported that GH is controlled in approximately 80% of microadenomas. In macroadenomas, on the other hand, IGF-I and GH are controlled in fewer than 50% of cases. Using these criteria, long-term surgical remission rates range from 42% to 62% and multimodality (surgery followed by RT or medical treatment) remission rates vary from 52% to 97%.[438] When comparing the microscopic surgery with the endoscope approach, the latter provides the advantage of enhanced visualization of the suprasellar compartment and cavernous sinus walls.[444] In a series of 60 acromegalic patients who underwent endoscopic transsphenoidal surgery, Jane and colleagues[445] report that remission was achieved in 100% of microadenomas and 61% of macroadenomas with endoscopic transsphenoidal surgery in acromegalic patients. Interestingly, prior to transsphenoidal surgery, age and gender were not predictive of remission. Remission was defined as a normalized IGF-I, a post OGTT GH level below 0.4 ng/mL and a random GH of less than 1 ng/mL. Independent of which cutoff levels are used, both GH and IGF-I should be measured 3 months after surgery, although stabilization of IGF-1 levels can take up to 12 months. In addition, preoperative treatment with long-acting somatostatin analogs can have a prolonged suppressive effect on GH of up to 3 months postoperatively.[435] The postoperative GH level is the most important determinant of mortality outcome, with published data suggesting that a random GH level of less than 2 μg/L measured by radioimmunoassay is associated with the reversal of increased mortality and continued suppression of GH. Based on the

analysis of Jane and colleagues[445] a postoperative day 1 or day 2 morning GH level of less than 2.5 ng/mL is a strong predictor of remission. The risk for all-cause mortality in patients with acromegaly is increased by 32%[439] and persistence of disease (using the less stringent criteria of 5 μg/L) is associated with a 2.4- to 4.8-fold enhanced mortality.[446] If GH and IGF-I are normal 3 months postsurgery, annual follow-up measurements are recommended. The postsurgical serum GH level during the OGTT should be as low as 0.3 μg/L measured by a current two-site assay.[447] The IGF-I level should be within the age-adjusted reference interval according to the latest consensus statement of the Joint Consensus Conference of the Growth Hormone Research Society and the Pituitary Society, published in 2004.[440] Residual pituitary function should be assessed no later than 3 months postsurgery.[440]

Medical therapy includes *dopamine agonists* (e.g., cabergoline, bromocriptine), *somatostatin analogs* (e.g., octreotide, lanreotide), and the *GH receptor antagonist* pegvisomant. Dopamine agonists can provide adequate control in a minority of patients with acromegaly, and it may take 3 months to achieve maximal suppression of GH and IGF-I. Measurements should be performed annually after normalization. Vilar and colleagues[448] reported normalization of PRL and IGF-1 and 70% to 90% tumor shrinkage with cabergoline in two cases of somatotroph macroadenomas cosecreting PRL and GH. Cabergoline in combination with somatostatin receptor ligands may improve the responsiveness of patients with acromegaly that is otherwise resistant to maximal doses of somatostatin receptor ligands.[449] High-dose dopamine agonists used alone lead to a GH level of less than 5 ng/mL in only 15% of patients and normalize IGF-I levels in fewer than 10%. The advantage is the relatively low cost of the drugs, but disadvantages include the incidence of side effects in approximately 30% of patients.

Satisfactory GH and IGF-I levels are achieved in approximately 65% of patients, and IGF-I levels are normalized in 70% with somatostatin analogs. The effects are inversely related to pretreatment GH levels. Approximately 50% of patients show 30% average tumor volume shrinkage. Transient gastrointestinal symptoms, such as nausea, vomiting, and diarrhea, can occur during the first 2 weeks of therapy, and 25% of patients have gallbladder sludge and gallstones, which are usually diagnosed within the first 2 years after the start of treatment. Somatostatin analogs inhibit insulin secretion and may cause deterioration of glucose metabolism. Therefore, fasting blood glucose and hemoglobin A_{1C} should be measured annually. Although octreotide is given subcutaneously three times daily, there are also long-term depot somatostatin preparations (octreotide long-acting release) available that are given every 3 months.[450]

The GH receptor antagonist pegvisomant directly inhibits GH action in the periphery without inhibiting GH secretion. It normalizes circulating IGF-I levels in more than 95% of the patients treated for up to 36 months, whereas circulating GH values may rise during the first weeks, but then plateau. Pegvisomant is indicated after failure of other therapy options. Of 160 patients, 2 exhibited progression of tumor growth under pegvisomant therapy; therefore, patients should have an MRI every 6 months during the first year of pegvisomant therapy and annually thereafter. Therapy is monitored with IGF-I measurements every 6 months. In addition, liver enzymes should be monitored over the initial 6 months of therapy, given the potential transaminitis that can occur with GH antagonist therapy.[451] Observation of 1288 acromegalic patients over 2.1 years on pegvisomant showed that it is effective and safe. There was a low incidence of pituitary size increase (3.2%) and liver enzyme elevations (2.5%).[452]

Indications for the use of RT in acromegaly include conditions in which surgery or medical therapy does not lead to GH and IGF-I normalization. RT techniques include external radiation with a cyclotron or cobalt 60 source, proton-beam therapy, and stereotactic ablation by gamma knife radiosurgery. Whereas RT decreases GH levels to less than 5 ng/mL in 90% of patients within 18 years, fewer than 5% show a normalization of IGF-I.[453] An overall reduction in GH levels of 30% to 50% can be expected in the first 1 to 2 years, with a slower rate of decline after that. Pituitary deficiency develops in 50% of patients within 10 years of RT. GH and IGF-I should be measured 2 years after conventional multiple-dose RT, and pituitary function should be assessed annually. After gamma knife therapy, GH and IGF-I should be measured after 1 year and residual pituitary function measured at 6-month intervals.[440]

ACTH-Secreting Adenomas

In 70% of cases, hypercortisolism (Cushing syndrome) is caused by excessive ACTH secretion from the pituitary (Cushing disease).[454] Cushing disease is almost always caused by a solitary corticotroph adenoma[455] and presents more often in women than in men. The reported incidence is between 0.7 and 2.4 cases per million inhabitants per year, and patients have a fourfold higher mortality rate compared with age-matched and sex-matched peers as a result of cardiovascular complications.[456] Approximately 90% of the adenomas are microadenomas (<1 cm in diameter). The etiology of Cushing disease is unclear. Monoclonal tumor cell expansion is one possibility. Based on the findings in a patient with generalized glucocorticoid resistance due to a missense mutation of the glucocorticoid receptor preceding the development of Cushing disease,[457] others speculate that decreased negative feedback might also play a role in corticotroph adenoma formation. The cyclin-dependent kinase inhibitor p27 *(Kip1)* is believed to be involved in corticotroph tumorigenesis, and cytogenetic studies show loss of chromosomes 2, 15q, and 22. Brg 1 and histone deacetylase 2 (HDAC2) are considered candidates for glucocorticoid resistance and are also believed to be involved in the tumorigenic process of the adenoma.[458]

Symptoms of Cushing disease result from excessive cortisol production, with its widespread systemic effects. One of the earliest signs of Cushing disease is weight gain, often associated with increased abdominal, supraclavicular, facial, and dorsocervical fat.[459,460] However, proximal muscle weakness, with preservation of distal strength, plethora, and ecchymoses are considered the most discriminant indicators of hypercortisolism.[461,462] Such muscle weakness affects the upper arms and legs and results from cortisol-induced mobilization of amino acids from the muscle. If hypokalemia is present, muscle weakness is significantly more prominent. Cutaneous atrophy leads to wrinkling of the skin, which is especially obvious at the dorsum of the hands and elbows. The skin also can show striae around the hips, abdomen,

shoulders, and breasts. These striae are purple, in contrast to the white or pink striae found in generalized obesity, and the width of hypercortisolism-associated striae often is greater than 1 cm. The skin frequently shows increased bruisability as a result of increased fragility of the capillaries. Vellus hair, especially on the upper cheeks and forehead, can also be found. Other skin manifestations include those associated with fungal infections. Many patients have emotional and cognitive changes that correlate well with the degree of hypercortisolism and include depressed mood, irritability, tearfulness, insomnia, and anxiety.[463] Osteoporosis is present in 40% to 80% of patients, and a history of fractures of the feet, ribs, and vertebrae is not unusual. Hypertension occurs in approximately 80% of patients, and type 2 diabetes is present in 20% to 30%.[464,465] Menstrual and sexual dysfunction occurs in up to 75% of patients. A summary of the clinical manifestations of Cushing disease is shown in Table 21.3.

The diagnosis of Cushing disease does not rely on a single test, but often requires the combination of several biochemical tests. The procedures used to confirm the diagnosis are based on the fact that, in hypercortisolism (Cushing syndrome), the normal circadian rhythm of cortisol is no longer present and the normal feedback characteristics of glucocorticoids on the HPA axis are disturbed. To confirm hypercortisolism, the consensus statement by Arnaldi and colleagues stated that the 24-hour urinary free cortisol (UFC) measurement should be the initial screening test.[461] The 24-hour UFC is reported to have specificity of 100%, but sensitivity ranges from 45% to 71%. The authors suggest that up to three 24-hour UFC measurements should be obtained. When measuring urinary cortisol high-performance liquid chromatography, interfering substances, such as carbamazepine and digoxin, can produce false-positive results. Therefore, the preferred method for measurement of 24-hour UFC is tandem mass spectrometry, which is less likely to produce false-positive results. Of note, if the glomerular filtration rate is less than 30 mL/min, urinary cortisol excretion is decreased, resulting in a false-negative result.[466]

Another first-line diagnostic procedure is the low-dose (1 mg) overnight dexamethasone suppression test (DST). The recommended level for a normal plasma cortisol level after a low-dose overnight DST is less than 1.8 µg/dL (50 nmol/L), with a required cortisol assay sensitivity of 1 µg/dL.[461] Some centers suggest the 2-day low-dose DST as a

Table 21.3 Frequency of Clinical Signs and Symptoms of Cushing Syndrome in Five Series of Adults (1952-1982) and Two Series of Children (1994, 1995)

Sign/Symptom (%)	Plotz et al 1952 (*n* = 33)	Sprague et al 1956 (*n* = 100)	Soffer et al 1961 (*n* = 50)	Urbanic and George 1981 (*n* = 31)	Ross and Linch 1982 (*n* = 70)	Magiakou et al 1994 (*n* = 59)	Weber et al 1995 (*n* = 12)
Obesity or weight gain	97	84	86	79	97	90	93
Decreased linear growth	—	—	—	—	—	83	80
Hypertension	84	90	88	77	74	47	—
Plethora	89	81	78	—	94	—	—
Round face	89	92	92	—	88	—	—
Hirsutism	73	74	84	64	81	78	58
Thin skin	—	—	—	84	—	—	—
Abnormal glucose tolerance	94	—	84	39	50	—	—
Easy bruising	60	62	68	77	62	25	17
Weakness	83	—	58	90	56	45	50
Osteopenia or fracture	83	—	56	48	50	—	—
Electrocardiogram changes or atherosclerosis	66/89	—	34	—	55	—	—
Menstrual changes	86	35	72	69	84	78	20
Decreased libido (men/women)	86	—	100/33	55	100	—	—
Depression or emotional lability	67	—	40	48	62	—	25
Headache	58	—	—	—	47	—	50
Striae	60	64	50	51	56	61	—
Edema	60	—	66	48	50	—	—
Acne	82	64	—	35	21	47	58
Buffalo hump	—	67	34	—	54	—	—
Female balding	—	—	51	—	13	—	20
Lipid abnormalities	39	—	—	—	—	—	—
Decreased wound healing	42	—	—	—	—	—	—
Delayed bone age	—	—	—	—	—	11	—
Accelerated bone age	—	—	—	—	—	8	—
Pigmentation	—	—	—	—	—	14	8

From Newell-Price J. Clinical manifestations of CD. *Endocr Rev* 19:647, 1998.

first-line screening test[467] in which the patient takes 0.5 mg dexamethasone orally every 6 hours. Urine is collected for UFC on 2 baseline days and is measured on the second day of dexamethasone administration, or cortisol is measured at 9 AM and 48 hours after the first dose. A normal response consists of a decrease in UFC to less than 10 μg/24 hours on the second day after dexamethasone administration or a plasma cortisol level of less than 1.8 μg/dL on the morning of the last dose of dexamethasone.[461]

Findling and Raff[468] suggested that measurement of a late-night or bedtime salivary cortisol level is an excellent surrogate for documenting an increased midnight serum cortisol level and is the test of choice. The assay has sensitivity and specificity of greater than 90% to 95%. Although this approach may be the most useful for the practicing endocrinologist, false-positive findings may be encountered in certain conditions, such as hypertension and advanced age, and in the setting of psychiatric disease.

Exogenous hypercortisolism always must be considered in the evaluation of patients for Cushing syndrome. The use of all prescription and over-the-counter medications and herbal remedies must be assessed, including injections into joints. This diagnosis is suggested by findings of suppressed ACTH and cortisol with no response to CRH or ACTH. Screening for synthetic glucocorticoids in serum or urine may be useful. Factitious hypercortisolism due to exogenous ACTH injections shows similar laboratory data as Cushing disease, but attention to the patient's history and a search for injection sites may show the cause.[469]

Other factors to consider include the effects of *exogenous compounds*, such as *estrogens*, *antiepileptic drugs*, and *glycyrrhetinic acid*, on cortisol measurements. Estrogen administration increases corticosteroid-binding globulin levels, with a concomitant increase in total plasma cortisol levels.[470] However, free cortisol levels and 24-hour UFC levels are normal. Pharmacological agents, such as phenytoin, barbiturates, and rifampicin, have an effect on the metabolism of dexamethasone by increasing its hepatic conjugation and biliary excretion. Thus, measurement of urinary free cortisol rather than a DST is suggested in these patients.[471,472] Glycyrrhetinic acid, an extract from licorice, has been shown to increase UFC by inhibiting 11β-hydroxysteroid dehydrogenase, which converts cortisol to cortisone. As a result, there is a cortisol excess, especially in the kidney, with increased concentrations in the urine, but not in the plasma. Therefore, urinary cortisol measurements can be misleading in patients taking licorice.[473]

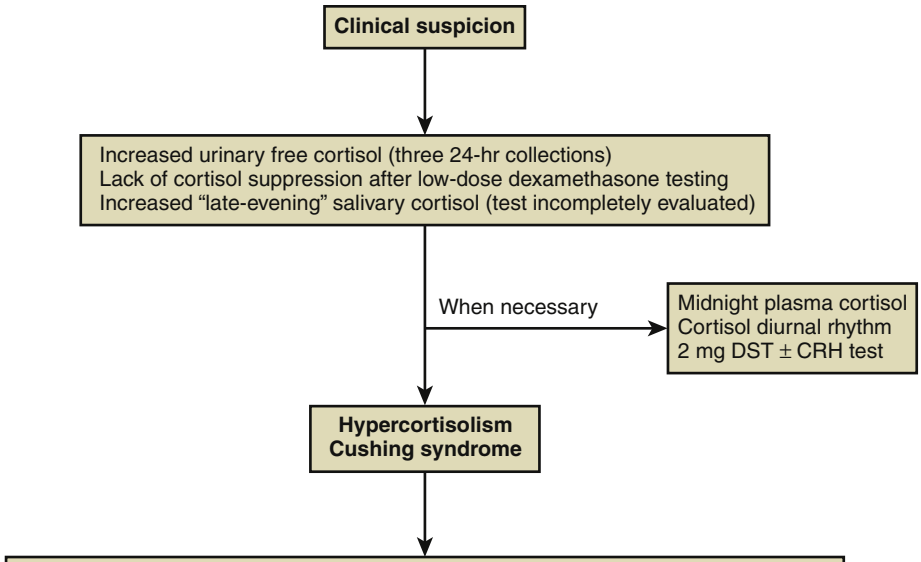
Once the diagnosis of Cushing syndrome is made, the next challenge is to determine whether the hypercortisolism is ACTH-dependent or ACTH-independent. The interpretation of circulating ACTH levels depends on proper preanalytical sampling and on the use of an assay that can reliably detect plasma ACTH values of less than 10 pg/mL. ACTH values of greater than 10 pg/mL suggest ACTH-dependent Cushing syndrome (Cushing disease), whereas values of less than 10 pg/mL are most often consistent with ACTH-independent Cushing syndrome. However, caution is warranted in the interpretation of ACTH levels, given that Cushing disease can occur with ACTH values of less than 10 pg/mL in rare cases,[474] and adrenal hypercortisolism can be present with values of greater than 20 pg/mL. Values of 10 to 20 pg/mL are considered indeterminate, and it is prudent to repeat measurements of ACTH or perform a CRH test. If the ACTH response to CRH is greater than 20 pg/mL, Cushing disease is very likely.

If the diagnosis of an ACTH-dependent pituitary adenoma seems probable, MRI may help to locate the adenoma. In 65% of cases, MRI shows a pituitary tumor. However, the diagnosis of Cushing disease cannot be based on MRI imaging alone, given that some ACTH-secreting adenomas may not be visible, even after gadolinium enhancement, and nonsecreting incidentalomas may be present and misinterpreted as the source of ACTH.

Cushing's disease accounts for 70% to 90% of cases of ACTH-dependent hypercortisolism. However, ectopic ACTH production must be excluded. Currently, the procedure of choice to exclude ectopic ACTH production is IPSS. In experienced centers, bilateral IPSS has very high sensitivity (95% to 99%) for diagnosing Cushing disease. The test is recommended in patients with ACTH-dependent Cushing syndrome who have clinical, biochemical, or radiological findings that are discordant or equivocal.[475] An IPPS-to-peripheral cortisol ratio (IPSS/P) greater than 2 in the basal state and 3 after CRH administration suggests the diagnosis of Cushing disease. False-negative results with IPSS can occur as a result of unsuccessful catheterization or anomalous venous drainage; the additional measurement of PRL can be helpful in such cases.[476] IPSS has limited utility in lateralization of ACTH-producing pituitary adenomas within the gland. In a literature review of 313 cases, the diagnostic accuracy ranged from 50% to 100%, and a gradient of 1.4 or greater across both sides of the pituitary predicted the tumor location in 78% of cases. In the case of asymmetrical drainage, IPSS predicted the lateralization of the adenoma in only 44% of patients. Cavernous sinus sampling has been proposed as an alternative to IPSS for lateralization because the cavernous sinuses are closer to the pituitary. Accurate localization was reported in 73.3% of cases without CRH and in 93.3% of cases with CRH stimulation.[477]

A less invasive test for distinguishing ectopic ACTH production from pituitary-dependent Cushing syndrome is the single-dose, 8 mg overnight DST, which has 92% sensitivity and 100% specificity for Cushing disease when morning plasma cortisol is suppressed by 50%, according to Tyrrell and colleagues.[478] Aron and colleagues reported sensitivity of 81% and specificity of 66.7%, with both the 2-day, high-dose DST (2 mg dexamethasone every 6 hours for 48 hours) and the 8 mg, single-dose overnight test using the 50% threshold.[479] Patients with ectopic ACTH secretion were significantly older (mean, 51 years versus 40.2 years), were more likely to be male (58.8% versus 27.4%), had a shorter duration of clinical findings (11.6 months versus 39.9 months), and were more likely to have hypokalemia (50% versus 8.6%). Such patients also had higher baseline 24-hour UFC levels (mean, 3015 μg/day versus 422 μg/day) and higher plasma ACTH levels (210 pg/mL versus 78 pg/mL)[479] compared with patients with Cushing disease. Figure 21.23 describes a possible diagnostic approach to determine the source of hypercortisolism.[461]

Special mention should be made of differentiating patients with Cushing syndrome from those with the much more common PCOS. Women of reproductive age who have hypercortisolism have symptoms similar to those of women with PCOS, including oligomenorrhea or amenorrhea,



	Adrenal	Pituitary	Ectopic
ACTH	Low	Normal/high	Normal/very high
CRH test	No response	Response	Rare response
DEX 8 mg	No suppression	Suppression	Rare suppression
CT/MRI adrenal	Mass(es)	Normal/hyperplasia*	Normal/hyperplasia*
MRI pituitary	Normal	Tumor (60%)	Normal
BIPSS	Not applicable	Gradient (pit/periph.)	No gradient (pit/periph.)

*Nodules

FIGURE 21.23 Cascade of tests to diagnose Cushing syndrome. ACTH, adrenocorticotropic hormone; BIPSS, bilateral inferior petrosal sinus sampling; CRH, corticotrophin-releasing hormone; CT, computed tomography; MRI, magnetic resonance imaging; pit/periph, pituitary/peripheral. *(Reproduced from Arnaldi G, Angeli A, Atkinson AB, et al. Diagnosis and complications of Cushing's syndrome: a consensus statement.* J Clin Endocrinol Metab *88:5593, 2003, with permission.)*

adrenal hyperandrogenism with acne or hirsutism, and metabolical syndrome.[461] Kaltsas and colleagues speculated that PCOS may be one manifestation of Cushing syndrome. In their small group of women, they found that 46% had ovaries with morphological features of PCOS.[480] Because the prevalence of PCOS is significantly higher than that of Cushing syndrome, the latter should always be considered in the differential diagnosis of PCOS. Especially in the case of mildly elevated cortisol levels, an endocrine profile suggestive of PCOS can be present (estrogen sufficiency, normal or increased androgen levels, enlarged ovaries). Kaltsas and colleagues also found that patients with proven Cushing syndrome had significantly higher blood pressure (both systolic and diastolic) than women with PCOS.[480]

Treatment. The treatment of choice of Cushing disease is selective transsphenoidal adenoma resection. The procedure has a mortality rate of less than 1%, and the most common postsurgical complication is transient DI, which may be present in up to 28% of patients.[481] The goal of surgery is selective adenomectomy and preservation of as much normal pituitary tissue as possible. If it is not possible to identify the tumor, hemihypophysectomy on the side of the gland with the ACTH gradient on IPSS is often performed. Recovery of the HPA axis after successful surgery rarely occurs before 3 to 6 months and commonly is not confirmed until 1 year after the procedure. Until recovery is confirmed, patients are treated with glucocorticoid replacement.

The definition of cure of Cushing disease is still under debate. Several reports suggest that serum morning cortisol levels of less than 5 μg/dL measured at day 1 or day 2 after surgery (first surgery) are associated with sustained remission in 93% of patients after 32 months.[482] Others suggest that postsurgical remission is likely if serum cortisol levels are 2 μg/dL or less within 72 hours of surgery.[483,484] When applying a serum cortisol threshold of 1.8 μg/dL or less measured 5 to 14 days after surgery and at least 24 hours after the last dose of hydrocortisone, the reported surgical remission rate was between 40% and 65%, and the 10-year incidence of recurrence was 25%.[482] Other studies reported postoperative remission rates of 60% to 90%.[485] Of note, postoperative detectable serum cortisol levels above those suggested earlier may still be compatible with cure and can decrease within 2 weeks. However, the absence of secondary adrenal insufficiency 4 to 6 weeks postoperatively suggests that the patient has persistent hypercortisolism or will have a recurrence.[468] When no histological confirmation of tumor removal can be made, remission is achieved in only 50% of patients.[486] Reasons for surgical failure include residual tumor within the pituitary gland, tumor located ectopically in the intrasellar or perisellar region, and residual invasive tumor within the dura mater of the sella turcica or the cavernous sinus[487,488] suggests that the lateral invasion of the tumor into the medial wall of the cavernous sinus may be caused by the anatomy of the lateral venous drainage of the pituitary gland. The confluent pituitary veins that penetrate the pituitary capsule and the dura of the cavernous sinus produce an anatomical route of least resistance. When surgery is unsuccessful, additional therapeutic options include repeated transsphenoidal surgery, medical treatment, RT, and bilateral adrenalectomy. A report by Locatelli and colleagues[485] suggests a favorable effect on remission in patients with initial surgical failure when the repeat surgery is done within 15 days. Figure 21.24 summarizes their suggested management of Cushing disease.[485]

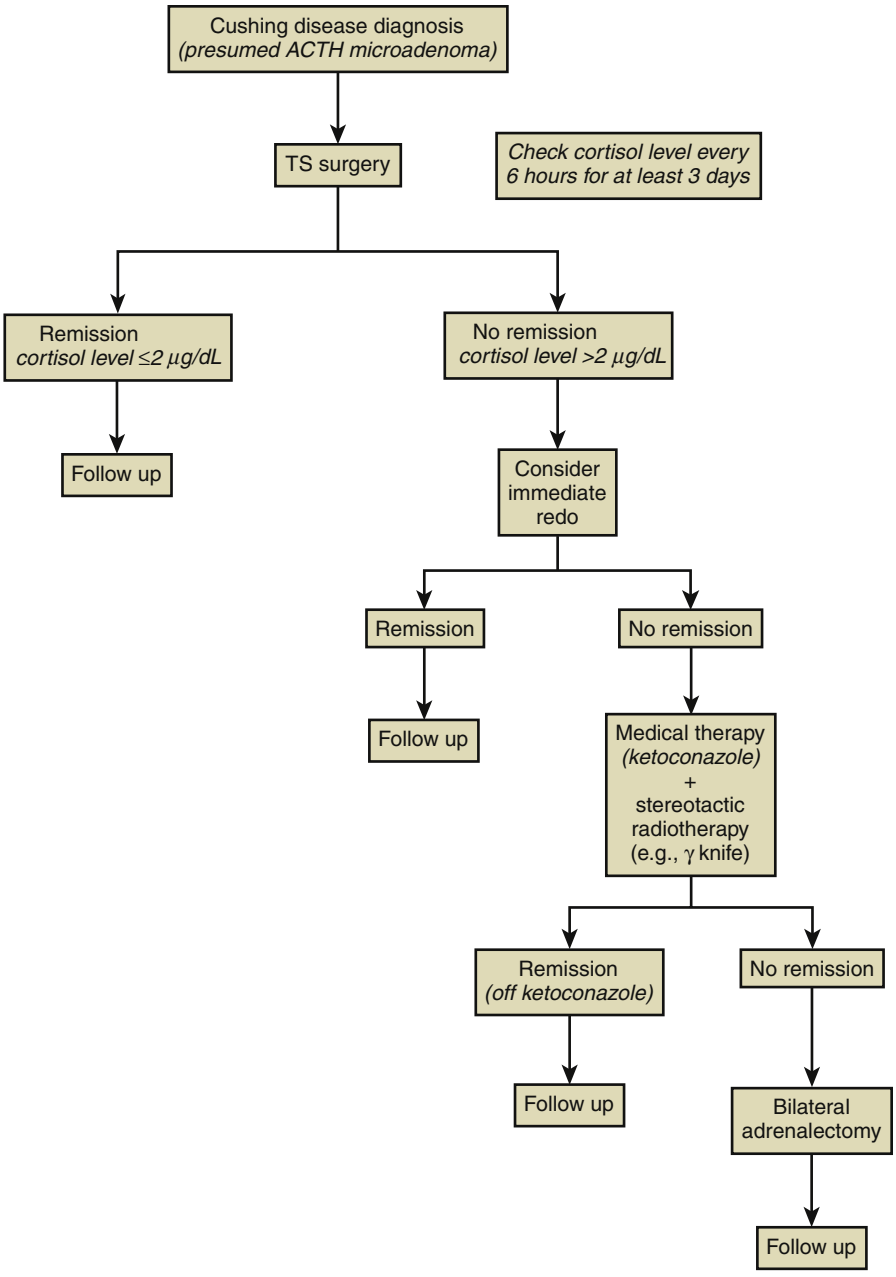


FIGURE 21.24 Therapeutic algorithm for the management of Cushing disease. ACTH, adrenocorticotropic hormone TS, transsphenoidal. *(Reproduced from Locatelli M, Vance ML, Laws ER. Clinical review: the strategy of immediate reoperation for transsphenoidal surgery for Cushing's disease.* J Clin Endocrinol Metab *90:5478, 2005, with permission.)*

When surgical intervention fails or is likely to be unsuccessful (e.g., in patients with cavernous sinus or dural invasion), pituitary radiation—either conventional or gamma knife—is recommended. Remission rates of 76% have been reported for gamma knife RT.[489,490] The expected normalization of cortisol levels occurs 12 to 36 months after RT; thus, adrenostatic therapy is required in the interim.[489,490]

Medical treatment targets adrenal steroid secretion and includes ketoconazole, metyrapone, aminoglutethimide, mitotane, and etomidate. *Ketoconazole* is an imidazole antifungal agent and an inhibitor of cytochrome P450 enzymes that blocks adrenal steroid synthesis at several points. The principal side effects of ketoconazole include hepatic toxicity, skin rash, gastrointestinal upset, and gynecomastia.[491,492] *Metyrapone* blocks the cortisol synthetic pathway by inhibiting the enzyme 11β-hydroxylase. The drug achieves cortisol trough levels 2 hours postdose and is given in three to four doses per day. However, the drug is not approved by the U.S. Food and Drug Administration for this use and has been withdrawn from the U.S. market. The main side effects include hirsutism, acne, edema, hypertension, and gastrointestinal upset.[201] *Aminoglutethimide* blocks the conversion of cholesterol to pregnenolone and causes a reduction in the synthesis of all hormonally active steroids, including corticosteroid production. Its side effects include a morbilliform rash, fever, and dizziness. *Mitotane* blocks adrenal cortisol synthesis and has direct cytotoxic effects on the adrenals. In high doses, it can achieve remission in up to 83% of patients. In lower doses and in combination with RT, remission can be achieved in up to 80% of patients.[493] The onset of effect can take up to 6 to 8 weeks, and effects can last for weeks and even months after discontinuation due to its lipophilic activity. Major side effects are related to gastrointestinal and neurological toxicity. Elevated liver enzyme levels, abnormal

platelet function, and hypercholesterolemia are other side effects. High doses are used for adrenocortical carcinoma and can cause anorexia and ataxia. *Etomidate* is an imidazole-derived anesthetic agent that inhibits adrenocortical 11β-hydroxylase. Given as a nonhypnotic low-dose infusion (0.1 mg/kg/hour or lower), it has been shown to control cortisol levels in severe hypercortisolemia.[494] It acts rapidly, but its use is limited because it must be given parenterally. *Mifepristone*,[495] a type 2 glucocorticoid receptor antagonist, which is currently under investigation has been shown to rapidly decrease circulating cortisol levels in malignant and benign hypercortisolism. Another agent, *Pasireotide*, has a unique, broad somatostatin-receptor-binding profile, with high binding affinity for somatostatin receptor subtype 5. In a double blind, phase 3 study, urinary free cortisol levels decreased by approximately 50% by month 2 and remained stable for 12 months. The combination of *pasireotide* with a dopamine agonist and low dose ketoconazole induced biochemical remission in 88% of patients with Cushing disease.[496,497]

In cases of persistent hypercortisolism or intolerable side effects related to the adrenostatic therapy, bilateral adrenalectomy is an option. It has no risk of hypopituitarism and may be chosen over RT in younger patients who worry about postradiation hypopituitarism and loss of reproductive function. Disadvantages of bilateral adrenalectomy include the lifelong requirement to take glucocorticoid and mineralocorticoid replacement therapy as well as a 50% risk of Nelson syndrome (discussed later in the chapter). Prophylactic pituitary radiation before adrenalectomy can reduce this risk to 25%.[498]

Nelson Syndrome

The first case of Nelson syndrome (NS) was reported in 1958.[499] Since that time, various criteria have been used to define this syndrome, including: (1) the presence of a pituitary macroadenoma, and (2) the presence of high ACTH levels after bilateral adrenalectomy. Nelson and colleagues suggested that the definition need not include the presence of a pituitary adenoma.[500] More recently, others have used the term *corticotroph tumor progression* for NS.[501]

The pathogenetic mechanisms underlying the development of a Nelson tumor are not fully understood. NS is believed to be in part the result of lack of cortisol feedback after adrenalectomy. Other findings support a role for mutations in regulatory genes.[457,502] Some studies suggest that ACTH stimulation after CRH infusion is greater and more prolonged in NS compared with Cushing's disease.[503] Studies of the ACTH secretion profile in NS show an altered secretion profile compared with patients with Cushing disease.[504] Immunohistochemically, tumors stain strongly positive for ACTH.

Published data suggest that the prevalence of NS after adrenalectomy ranges from 8% to 50%.[498,505] The interval between adrenalectomy and the diagnosis of NS ranges from 0.5 year to 24 years. According to Assie and colleagues,[506] a shorter duration of Cushing disease and a relatively high plasma ACTH concentration in the year after adrenalectomy are predictive of corticotrophic tumor progression. In their study, tumor progression was first diagnosed as early as 3 years after adrenalectomy. Others found a relationship between pretreatment urinary cortisol levels and the occurrence of NS.[507] Kelly and colleagues[508] reported that a shorter duration of the disease before adrenalectomy is also predictive of tumor progression.

The clinical features of NS are caused by the mass effects of the tumor on surrounding structures, secondary loss of other pituitary hormones, and the effects of the high serum alpha-melanocyte–stimulating hormone (α-MSH) concentration (a derivative of proopiomelanocortin) on the skin. Cutaneous hyperpigmentation of hand lines, scars, gingivae, and areolae is present.[509] The degree of pigmentation reflects the level of serum α-MSH.

Treatment. Treatment strategies for Nelson syndrome include surgical intervention and RT, which rarely cure the disease. There have also been therapeutic approaches with drug treatment. Because the peroxisome proliferative-activated receptor (PPARγ) is expressed in corticotroph adenomas, the effect of PPARγ agonists on tumor growth has been documented. Using a rodent model, some studies have suggested that PPARγ agonists have a favorable effect on retarding tumor growth and lowering ACTH and cortisol levels.[510,511] However, studies in humans that have focused on the effects of rosiglitazone (given over 8 weeks) have not shown a reduction in ACTH. Longer-duration studies and the evaluation of PPARγ agonists with stronger agonist activity warrant consideration.[512] Other neuropharmacologic approaches, such as treatment with valproate or dopamine agonists, are rarely effective. Studies investigating neurosurgical interventions show success rates of 10% to 70%.[513] RT of the pituitary after adrenalectomy has been shown to reduce the risk of NS by 50% in some studies.[498]

Thyrotropin-Secreting Tumors and Thyrotroph Hyperplasia

The term *thyrotropin-producing pituitary tumors* describes two distinct clinical conditions: true thyrotroph neoplasia, which results in secondary hyperthyroidism, and pituitary hyperplasia, resulting from long-standing hypothyroidism.[514] It is important to distinguish one from the other because the therapeutic approaches are different.

Thyrotrophic adenomas (TSHomas) account for 0.5% to 1% of all pituitary adenomas, with a prevalence of one to two cases per million. They have no sex preference. TSHomas are almost always benign but are, in approximately 90% of cases, macroadenomas, which are highly invasive.[515] Such tumors have been shown to be monoclonal in origin.[516] Previous thyroid ablation appears to have deleterious effects on the size of the pituitary adenoma. Approximately 72% of tumors secrete TSH alone. In 28% of cases, hypersecretion of TSH is associated with hypersecretion of the α subunit and can also be associated with hypersecretion of GH and PRL and, in rare cases, with gonadotropins. No association with ACTH is known.

Clinically, patients present with symptoms of hyperthyroidism (goiter and clinical thyrotoxicosis) and with mass effects of the pituitary adenoma (visual field defects, headache). However, the size of the tumor in the imaging study does not correlate with serum TSH levels.[514] Clinical characteristics of patients with TSHomas are shown in Table 21.4. More than 90% of patients present with a goiter. Approximately one third had an inappropriate thyroidectomy or radioiodine thyroid ablation before the

Table 21.4 Clinical Characteristics of Patients with Thyroid-Stimulating Hormone–Secreting Pituitary Adenoma Reported in the Literature (Updated to January 1996)

	No Thyroid Ablation % *(n)**	Previous Thyroid Ablation % *(n)*	All Patients % *(n)*
Age (yr)†	41 ± 15 (156)	42 ± 13 (80)	41 ± 14 (236)
Sex (female %)	52 (168)	62 (87)	55 (255)
Goiter	92 (114)	97 (63)	94 (177)
Thyroglobulin autoantibodies or thyroid peroxidase autoantibodies	11 (63)	2 (43)	8 (106)
Anti-thyrotropin receptor autoantibodies	5 (40)	3 (33)	4 (73)
Exophthalmos	8 (79)	4 (49)	6 (128)
Menstrual disorders‡	40 (30)	23 (40)	30 (70)
Galactorrhea‡	50 (12)	17 (18)	30 (30)
Visual field defects	40 (73)	45 (53)	42 (126)
Headache	23 (44)	13 (61)	17 (105)
Tumor size			
Microadenomas and intraseller macroadenomas	34 (155)§,‖	19 (88)§,‖	29 (243)
Macroadenoma with extrasellar extension	39 (155)¶	32 (88)¶	36 (243)
Invasive macroadenoma	27 (155)	49 (88)	35 (243)

**n* = number of patients for whom the information was available.
†Mean ± standard deviation.
‡Data include women with or without associated prolactin hypersecretion.
§*P* = not significant *versus* macroadenoma with extraseller extension (by Fisher's exact test).
‖*P* < 0.0006 *versus* invasive macroadenoma (by Fisher's exact test).
¶*P* < 0.006 *versus* invasive macroadenoma (by Fisher's exact test).
From Beck-Peccoz P, Brucker-Davis F, Persani L, et al. Thyrotropin-secreting pituitary tumors. *Endocr Rev* 17:610, 1996.

TSH-secreting pituitary adenoma was discovered. Typically, the concentration of thyroid hormone is elevated in the presence of detectable TSH. Some reports suggest that TSH molecules in patients with TSHomas have increased bioactivity.[517] It has been suggested that an α subunit or TSH ratio above 1 indicates a TSHoma.[518] The TSH inhibition test using T_3 (80 to 100 μg) given for 8 to 10 days can be used to confirm the presence of a TSHoma. The lack of suppression confirms the autonomy of the TSH secretion by the adenoma, but the test is contraindicated in the elderly and patients with coronary heart disease.[514] In the rare cases when no pituitary adenoma is present, resistance to thyroid hormone must be considered.

Treatment. The primary approach to treatment of TSHomas is surgical removal of the pituitary tumor. Approximately two thirds of TSHomas respond to surgery or irradiation.[514] An undetectable TSH level 1 week after surgery typically indicates successful surgery. A measurable TSH level after the T_3 suppression test suggests the need for additional treatment after surgery. If surgery is not an option, RT followed by somatostatin analog administration can be considered.

Pituitary Hyperplasia

Pituitary hyperplasia as a consequence of untreated hypothyroidism is rarely symptomatic, but may present with hypothyroidism, headache, visual defects, hyperprolactinemia, amenorrhea, or galactorrhea. A goiter is present in 16% of cases. In children, presenting signs and symptoms include abnormal puberty, delayed bone age, and short stature.

Treatment. Treatment is medical, with adequate thyroid hormone replacement resulting in total tumor regression in 62% and partial regression in 29% of cases,[514] occurring sometimes within 1 or 2 months. In children, initial therapy can lead to pseudotumor cerebri; such a complication should be considered when symptoms worsen after initiation of thyroid hormone replacement.

Adenomas of Multiple Endocrine Neoplasia Type 1

The combined occurrence of tumors of the parathyroid gland, pancreatic islet cells, and the anterior pituitary is characteristic of *multiple endocrine neoplasia type 1* (MEN 1; also called *Wermer syndrome*). The *MEN1* gene is located on chromosome 11q13 and encodes the protein MENIN. More than 90% of tumors from patients with MEN 1 have loss of heterozygosity, suggesting that the *MEN1* gene is a tumor-suppressor gene. The overall incidence of MEN 1 is 0.25%. It is 1% to 18% in patients with primary hyperparathyroidism and less than 3% in patients with pituitary tumors.[519,520] All forms of MEN 1 are either inherited as autosomal dominant syndromes or occur sporadically. A patient may be considered to have MEN 1 if two of the three principal related tumors (parathyroid, pancreatic islet [e.g., gastrinoma, insulinoma, glucagonoma, VIPoma], and anterior pituitary [prolactinoma, GH-producing, ACTH-producing, nonfunctioning]) are diagnosed.[521] Associated tumors include carcinoid tumors, adrenocortical tumors, lipomas, facial angiofibromas, and collagenomas, as well as thyroid tumors.[522] The incidence of pituitary tumors in patients with MEN 1 ranges from 15% to 90%. The majority of the pituitary tumors are microadenomas, and approximately 60% secrete PRL. Fewer than 25% secrete GH, and 5% secrete ACTH. The rest are nonfunctioning and secrete glycoprotein subunits.

Treatment. The treatment of these pituitary tumors is identical to that of other isolated pituitary tumors. Because of its autosomal dominant inheritance,[523] distinction between sporadic and familial forms is important for first-degree relatives who have a 50% risk of having the disease.

Carcinoma of the Pituitary

Pituitary carcinomas are rare neoplasms that can be distinguished from invasive adenomas by the presence of craniospinal or systemic metastasis. An unresolved question is whether pituitary carcinomas develop from pituitary adenomas or occur *de novo*. Studies addressing this question have been limited by the small number of patients and the lack of sufficient follow-up.[524] Pituitary carcinomas are defined by metastases. Whether extensive invasion of the brain by a pituitary tumor is a further criterion is a matter of controversy.[525] Adenomas that meet these criteria should be followed carefully.

Immunohistochemically, MIB-1 (Ki-67) and p53 staining correlate with the aggressiveness of pituitary tumors.[526] Studies of molecular markers suggest that the *Ras* oncogene may play a role in the anaplastic progression of GH-producing tumors. Expression of the *p53* gene has been shown to be increased during the transformation of a pituitary adenoma to a pituitary carcinoma.[527] Loss of heterozygosity on autosomes 1p, 3p, 10q26, 11q13, and 22q12 has also been shown to be present in ACTH-producing pituitary carcinomas.[528] Other factors, such as telomerase and its subunits, have been shown to be increased in PRL-producing pituitary carcinoma.[529] Animal studies have suggested a connection between the loss of the retinoblastoma gene *(Rb)* and the development of pituitary carcinomas, observations that could not be confirmed in benign ACTH-positive adenomas in humans.[530]

The prevalence of pituitary carcinoma is 0.2% of all adenohypophysial neoplasms,[531] with the majority of such tumors being endocrinologically active (88%). Available data suggest that 12% are null cell pituitary tumors, 33% are PRL-producing tumors, 42% are ACTH-producing tumors, 22% are associated with Nelson syndrome, 6% are GH-producing pituitary tumors, 5% are gonadotroph-producing tumors, and approximately 1% are TSH-producing tumors.[531,532] PRL-producing pituitary tumors (71%) and ACTH-producing tumors (57%) have the highest propensity to metastasize.[533] An average 15.3-year interval between adenoma diagnosis and the development of carcinoma has been reported in cases of Nelson syndrome.[531]

The prognosis of pituitary carcinoma is poor. In a study of 15 patients with pituitary carcinoma, 80% died of metastatic disease within 7 days to 8 years after diagnosis. Sixty-six percent of the patients died within 1 year.[531] Sironi and colleagues reported a mean survival time for patients with PRL-secreting carcinomas of 2.4 years.[534]

The majority of pituitary carcinomas are hormone-secreting, are invasive, and result in symptoms from local mass effect. Sites of pituitary tumor metastasis include the brain, spinal cord, bone, liver, lymph nodes, ovary, heart, and lung. In ACTH-secreting pituitary carcinomas, ACTH levels range from 145 to 280,000 pg/mL.[535] Serum PRL levels associated with PRL-secreting pituitary carcinomas range from 6 to 22,000 ng/mL, and increased levels are associated with tumor recurrence and metastasis.[531,536]

Treatment. Treatment options for pituitary carcinomas include medications specific for the pituitary tumor as well as RT and chemotherapy. Dopamine agonists have a role in the treatment of PRL-producing pituitary tumors, but control serum PRL levels for a limited time. Neither adjuvant RT to the sella and distant metastases nor stereotactic radiation has been shown to have an effect on long-term outcome. Similarly, different chemotherapy protocols with agents such as carmustine, hydroxyurea, and 5-fluorouracil have not improved outcomes.[532]

Temozolamide, a lipophilic imidazotetrazine derivative that is converted to a methylating alkylator agent, MITC (methy-triazene-1-yl)-imidazole-4-carboxide) which induces DNA damage, has shown to be effective in pituitary carcinoma and is generally well tolerated.[537-540]

Metastatic Disease Involving the Pituitary

Metastatic disease to the pituitary has been found in 1% to 3.6% patients at the time of pituitary resection.[541] If both the pituitary and the surrounding sella turcica are evaluated, metastases are found in 27% of patients with malignant tumors.[542] Of tumors known to metastasize to the pituitary, cancer of the breast is the most common (accounting for up to 33% to 40%), followed by cancer of the lung (33% to 36%). Others include prostate, colon, and other gastrointestinal tumors; lymphoma; leukemia; and thyroid tumors.[543] Most metastatic disease is found in the posterior pituitary gland (57%), followed by the anterior gland (13%), and both lobes (12%). This preference for the posterior pituitary is probably explained by its direct arterial blood supply.[544] Of interest, metastases from breast cancer seem to occur most often in the anterior as opposed to the posterior pituitary. Perhaps surprisingly, most metastases to the pituitary are clinically silent and only 7% have been found to have produced symptoms in an autopsy series. Clinical features include DI, followed by ophthalmoplegia, visual impairment, headache, and anterior hypopituitarism. Kimmel and O'Neill[545] suggested that 14% to 20% of patients presenting with DI may have pituitary metastases.

Although there are no imaging characteristics that are specific for pituitary metastases, Fassett and Couldwell[543] have suggested that thickening of the pituitary stalk, loss of the high-intensity signal of the posterior pituitary, isointensity of T1- and T2-weighted MRI scans, invasion of the cavernous sinus, and sclerotic changes around the sella turcica may make metastatic disease more likely.

Treatment. Treatment includes surgery, RT, and chemotherapy, which do not change the mean survival rate in most cases, which ranges from 6 to 22 months.[546]

Pituitary Apoplexy and Sheehan Syndrome

Pituitary Apoplexy

Pituitary apoplexy is a potentially life-threatening condition that results from acute expansion of an infarcted or hemorrhagic pituitary adenoma. Symptoms typically associated with pituitary apoplexy include sudden severe headache, visual field defects, decreased visual acuity, and ophthalmoplegia.[547] Other symptoms are nausea, vertigo, meningismus, facial pain, and in some cases, hemiparesis and Horner syndrome. Headache occurs in nearly 100% of cases and is believed to be a consequence of meningeal irritation from blood or dural stretching. In cases of extension to the lateral wall of the sinus, cranial nerve damage can occur in association with ophthalmoplegia. The accompanying

hypopituitarism and possible pressure on the brain stem can result in decreased consciousness. The symptoms are often similar to those of other disease entities, such as ruptured intracranial aneurysms and bacterial or viral meningitis.

All types of pituitary tumors have a similar risk of apoplexy, and the incidence of apoplexy within pituitary tumors has been estimated at 2% to 7%.[548,549]Approximately 50% to 80% of cases of apoplexy occur in patients who were unaware of the presence of pituitary tumors before the apoplectic event. The ratio of men to women presenting with apoplexy is 2:1, and most patients present in the fifth or sixth decade.[547] Precipitating factors described in the literature include hypertension, hypotension, bromocriptine therapy, anticoagulation, surgery, diabetic ketoacidosis, RT, and pregnancy.[550-555]

The most reliable method to diagnose pituitary apoplexy is MRI. Early MRI images show predominant hyperintensity on T1 images and predominant hypointensity on T2-weighted images.[556] Whereas pituitary apoplexy can be confirmed by MRI in essentially all cases, CT scans may not show this disorder in 54% to 79% of patients.[547] Because vascular emergencies are more common than pituitary apoplexy, head CT is often the first procedure performed to evaluate the symptoms that are common to both conditions. Thus, the low sensitivity of CT scanning for the diagnosis of pituitary apoplexy must not be overlooked when no aneurysm is identified.

Treatment. Usually timely surgical intervention to provide decompression is essential. Although the outcome of pituitary apoplexy is unpredictable, cranial nerves III, IV, and VI seem to recover more easily than do the chiasmal and optic nerves, especially if decompression is achieved within 7 days. DI occurs in only 2% to 3% of patients, whereas reports show that 88% of patients are GH-deficient, 76% do not secrete LH, and 66% have secondary adrenal insufficiency.[547] Verrees and colleagues reported that decompression within 3 days after the onset of symptoms resulted in full return of pituitary function in 73% of patients.[547]

Indications for conservative treatment of pituitary apoplexy should be made by a multidisciplinary team of experts in neurosurgery, endocrinology, and ophthalmology. This approach can be tried in patients, who are without neuroophthalmic signs or with mild and stable signs. Management includes steroid administration and careful monitoring, including repeated formal assessment of visual fields and acuity and neurological assessment, as well as evaluation of the pituitary function.[557]

Sheehan Syndrome

Sheehan syndrome is a form of pituitary apoplexy that results from hypotension secondary to severe postpartum uterine hemorrhage. In rare cases, it is seen without massive bleeding or after normal bleeding. It has been suggested that the enlarged pituitary during pregnancy is more prone to have ischemic necrosis after postpartum bleeding. The presence of a small sella has also been proposed as a potential risk factor for Sheehan syndrome.[558] It may be difficult to distinguish Sheehan syndrome from postpartum autoimmune hypophysitis (AH), given that pituitary antibodies have been described in both disorders.[559] In the early stage of Sheehan syndrome, MRI shows an enlarged pituitary, and in later stages, an empty sella can be present.[560] Clinical manifestations include failure of postpartum lactation and failure to resume regular menstrual cycles postpartum, together with the loss of pubic and axillary hair. Other symptoms of anterior hypopituitarism can be present. In the case of hypopituitarism, replacement therapy (especially glucocorticoids) must be initiated as soon as possible. Partial or complete spontaneous recovery has been reported.[561]

Empty Sella Syndrome

Primary Empty Sella Syndrome

Primary empty sella syndrome is a radiological finding. It refers to the anatomical state that occurs as a result of intrasellar herniation of the subarachnoid space through an opening in the diaphragma sellae, allowing spinal fluid to enter the sella. The result is a compressed and often posteriorly displaced pituitary gland. The term *primary empty sella syndrome* is used when no identifiable cause can be found. The majority of patients are asymptomatic. Of those who have symptoms, 80% are middle-aged women who are overweight and hypertensive.[562] Chronic headache is the most common symptom and often the only one. Elevated intracranial pressure is believed to be an important contributing factor, and 10% of patients with benign intracranial hypertension also have empty sella syndrome.[563] In 5% of patients, moderate hyperprolactinemia resulting from stalk compression is evident. In 10% of patients, CSF rhinorrhea is present and is the result of CSF pulsation-associated thinning of the sella floor.[564]

Secondary Empty Sella Syndrome

Secondary empty sella syndrome can be the consequence of previous surgery, radionecrosis, or pituitary apoplexy. In contrast to primary empty sella syndrome, there is no association with body habitus or sex. Visual symptoms are not uncommon and are usually the result of chiasmal prolapse. Panhypopituitarism or selective pituitary hormone deficiencies can occur. Having established the diagnosis, the treatment is primarily based on the complications (endocrinological, ophthalmological, CSF rhinorrhea).

Lymphocytic Hypophysitis

Autoimmune hypophysitis, also referred to as *lymphocytic hypophysitis*, is the most common form of chronic inflammation that primarily affects the pituitary gland. AH is a rare disease that should be part of any differential diagnosis of a nonsecreting pituitary mass during pregnancy or postpartum. Outcomes of this disease range from spontaneous recovery to death. Women are affected much more commonly than are men (9:1 occurrence rate, and symptoms include those of sellar compression, such as headache and visual disturbance, pituitary deficiency, and DI).[565] DI can be masked by coexisting glucocorticoid deficiency, given that glucocorticoids inhibit the secretion of ADH from the paraventricular nucleus and suppress the synthesis of aquaporin 2, which is an ADH-dependent water channel in the renal collecting tubules.[566-568] In AH, the anterior pituitary corticotrophin-secreting cells are most frequently impaired, whereas the least common finding is hyperprolactinemia. The clinical diagnosis of AH may be difficult.

Indeed, 40% of cases are misdiagnosed preoperatively as pituitary adenomas. AH should always be considered when there is a temporal relationship with the identification of pituitary pathology and pregnancy or the postpartum state. The strong association between pregnancy and AH might be explained by the presence of low-titer serum antibodies, which are directed against alpha-enolase, a 49-kDa cytosolic pituitary protein that is expressed in the placenta.[569,570] However, others have found a similar association between these antibodies and nonfunctioning pituitary adenomas, thus calling into question the specificity of these antibodies. Other autoantibodies have been associated with the disease as well. AH has been associated with other autoimmune diseases, such as Hashimoto thyroiditis, polyglandular syndrome type 2, Graves disease, and systemic lupus erythromatosis.[565] Imaging studies often show an enlarged pituitary, sometimes with suprasellar extension and occasionally with thickening of the infundibulum. In rare cases, an empty sella is present.[571] The histopathology of lymphocytic hypophysitis shows lymphoplasmacytic infiltrates.

Treatment. Visual loss related to AH can necessitate surgical intervention. If surgery is not urgently needed, medical treatment with corticosteroids and sequential MRI are recommended. There are also reports of successful treatment with azathioprine[572] and methotrexate[573] after unsuccessful glucocorticoid therapy. RT has been used successfully in patients who have failed medical treatment or had repeated recurrences.[574,575]

The differential diagnosis of hypophysitis should also include sarcoidosis, fungal infections, tuberculosis, Langerhans cell granulomatosis, IgG4-related hypophysitis, Wegener's granulomatosis, Tolosa-Hunt syndrome, CTL4 blockade-related hypophysitis.[576]

Pituitary Hemochromatosis

Primary hemochromatosis is the most common form of hemochromatosis. It is an autosomal recessive disorder caused by a single site mutation of the *HEFE* gene.[577] This gene is located near the human leukocyte antigen complex and produces the *HEFE* glycoprotein, which binds to the transferrin receptor and decreases the affinity of this receptor for iron-bound transferrin. *Secondary hemochromatosis* may result from disorders including thalassemia, aplastic anemia, blood transfusion, long-term kidney dialysis, and chronic liver disease. Patients with hemochromatosis of both types have increased iron deposition in different organs, including the liver, skin, heart, joints, pancreas, hypothalamus, and pituitary. The most common feature of hemochromatosis is fatigue; other symptoms include arthritis, heart failure, diabetes mellitus, hepatic cirrhosis, hyperpigmentation, hypothyroidism, and hypogonadism.[578]

Iron has been found as aggregates of ferritin and hemosiderin in the cytoplasm of all five anterior pituitary cell types, but with a propensity for the gonadotrophs. The cytotoxic effect seems to correlate with the quantity of iron stored. The most common consequences of deposition of iron in the pituitary gland and hypothalamus are hypogonadotropic hypogonadism and GH deficiency.[579] Thirty percent of men with hemochromatosis have low gonadotrophin and testosterone levels resulting from impaired LH and FSH response to GnRH by the damaged gonadotrophs.[580] Siminoski and colleagues[117] described a patient with idiopathic hemochromatosis with no LH or FSH response after clomiphene administration, suggesting that a defect in the hypothalamic GnRH response can also occur. In patients with iron overload, MRI shows low signal intensity,[581] and some authors suggested that the MRI T2 relaxation rate of the pituitary correlates with the extent of pituitary siderosis.[582]

Treatment. Treatment options include phlebotomy and desferrioxamine, which is a chelating agent.[583]

Iatrogenic Pituitary Dysfunction

Hypopituitarism has been described in patients after *radiation therapy* for nonpituitary disorders, such as primary brain tumors and nasopharyngeal carcinoma, as well as in children who undergo prophylactic cranial irradiation for leukemia. GH is usually the first pituitary hormone affected by cranial RT, followed by the gonadotropins ACTH and TSH.[133] The total dose of radiation administered determines the severity of the pituitary deficiencies.[133] Children and adults differ with regard to the threshold dose for the risk of hypopituitarism.[138] Agha and colleagues reported that 41% of adult patients who had irradiation of a nonpituitary brain tumor had hypopituitarism.[135] After RT, regular evaluation is mandatory to check for recurrence and pituitary function, even though the site of radiation may have been distant from the hypothalamic–pituitary region.[134]

Radiation of the pituitary, most often used to treat residual disease after pituitary surgery or recurrent pituitary tumors, typically uses either gamma knife or linear accelerator–based radiosurgical techniques. Gamma knife treatment delivers a high dose (25 Gy to the periphery of secretory tumors and 12 to 20 Gy to the periphery of nonsecretory tumors) in a single fraction. Hypopituitarism occurs in approximately 30% of patients postradiation, with thyroid and GH deficiencies being the most common.[584] Data on the outcome of treatment with linear accelerator–based treatments are comparable to those for gamma knife treatment, with 20% to 40% of patients requiring hormone replacement 10 to 20 years after linear accelerator radiation.[585] Additional longer-term studies are needed to evaluate the hormonal effects of both treatment options.

Chemotherapy, alone or in combination with RT, can affect the hypothalamic–pituitary axis. Patients treated with chemotherapy (e.g., vincristine, vinblastine) may have syndrome of inappropriate antidiuretic hormone secretion as a result of cytotoxic effects on the paraventricular and supraoptic neurons, leading to increased vasopressin release.[586] Autopsy findings have related cyclophosphamide therapy to vasopressin depletion of the posterior pituitary as well as to infundibular necrosis.[587]A rare case of pituitary apoplexy after treatment with vinblastine, cisplatin, and methotrexate for squamous cell carcinoma was reported by Davies and colleagues.[588] Prolonged high-dose exogenous glucocorticoid therapy in children with leukemia or lymphoma can lead to secondary adrenal insufficiency via suppression of the HPA axis. Children treated with chemotherapy often exhibit growth retardation after which "catch-up growth" occurs. When catch-up growth does not occur after 1.5 to 2 years, such children should be tested for GH deficiency.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 22

Polycystic Ovary Syndrome and Hyperandrogenic States

R. Jeffrey Chang

Understanding the clinical significance and pathophysiology of polycystic ovaries and polycystic ovary syndrome has evolved over more than 150 years. The first description of enlarged, polycystic ovaries surrounded by a smooth capsule was reported in 1844.[1] This was followed by similar observations, including a description on hyperthecosis in 1897.[2] In the early 1900s, there was a growing awareness that dysfunctional uterine bleeding was associated with multiple cystic follicles of the ovary, which in part, led to the therapeutic recommendation of bilateral ovarian wedge resection. In 1926, it was demonstrated in rodents that gonadotropic extract derived from the urine of pregnant women was capable of inducing multiple ovarian cyst formations.[3] This finding suggested that abnormal secretion of anterior pituitary hormones may be responsible for the morphological changes in the ovary. Subsequently, in 1935, the classic description of polycystic ovaries was reported by Stein and Leventhal, which codified the association with hyperandrogenism, amenorrhea, and infertility as well as established the syndrome named for the authors.[4] As investigators began to study the pathogenesis of this disorder and the number of relevant publications increased, there followed a gradual and distinct terminological conversion to what has become known as *polycystic ovary syndrome* (PCOS).

Currently, the term polycystic ovary syndrome refers to a multi-system reproductive-metabolical disorder that has evolved over decades and stands to be further defined in years to come. The principal clinical manifestations are hyperandrogenism and irregular menstruation, the latter of which leads to infertility. The ovaries of women with these symptoms are polycystic and conform to a specific anatomical appearance that may be detected on ultrasound imaging, although the ovarian morphology can exist in women without the overt clinical manifestations. The associated metabolical dysfunction includes insulin resistance, dyslipidemia, and, in the United States, an increasing prevalence of obesity. The intriguing nature of PCOS rests with the underlying mechanism(s) responsible for the juxtaposed abnormalities of hypothalamic-pituitary-ovarian-adrenal function with those of altered metabolical physiology. All of these factors may contribute to the clinical phenotype, pose increased long-term health risks, and serve as targets for therapeutic intervention in women with PCOS.

Epidemiology

Prevalence

It is estimated that the prevalence of PCOS is 4% to 12% of women in their reproductive years of life,[5-7] which designates this disorder as the most common reproductive endocrinopathy of women. Estimates may vary among reports based on the study population and whether ultrasound imaging of the ovary was included in the diagnostic criteria. In a study of 277 unselected women undergoing employee entrance physical examinations, 4.6% were found to have PCOS.[5] Of the 11 women with PCOS, all had oligomenorrhea and of 9 women in whom androgen levels were assessed, 8 exhibited hyperandrogenemia. These results confirmed earlier reports in which the diagnosis of PCOS was highly likely in women with hirsutism and oligomenorrhea.[8,9]

In the absence of hirsutism, it has been demonstrated that women with anovulation have a high incidence of PCOS. In 206 non-hirsute women, biochemical assessment revealed elevated androgens consistent with the diagnosis in 87% of those with oligomenorrhea and 32% with amenorrhea.[10] In contrast, women with hirsutism alone were reported to have ultrasound findings of polycystic ovaries regardless of whether the patient had oligomenorrhea, amenorrhea, or regular cycles.[9,11] In these women, increases in circulating androgen levels may have facilitated the development of polycystic ovaries. This notion is supported by studies in which female to male transsexuals treated with androgen acquire the typical appearance of polycystic ovaries.[12]

While the sonographic demonstration of polycystic ovaries in the presence of typical clinical features is regarded as confirmatory for the syndrome, a small portion of women with PCOS have been shown to have regular ovulation accompanied by normal follicle development as determined by serial ultrasound.[13] Moreover, it has been clearly demonstrated that polycystic ovaries can be found in normal ovulatory women without a history of hyperandrogenism.[14] In a study of 104 young Danish women with polycystic ovaries, only 43 were found to have other symptoms that fulfilled the criteria for PCOS.[15] Thus, 58% of women with polycystic ovaries were essentially normal without menstrual abnormalities or evidence of hyperandrogenism. Similarly,

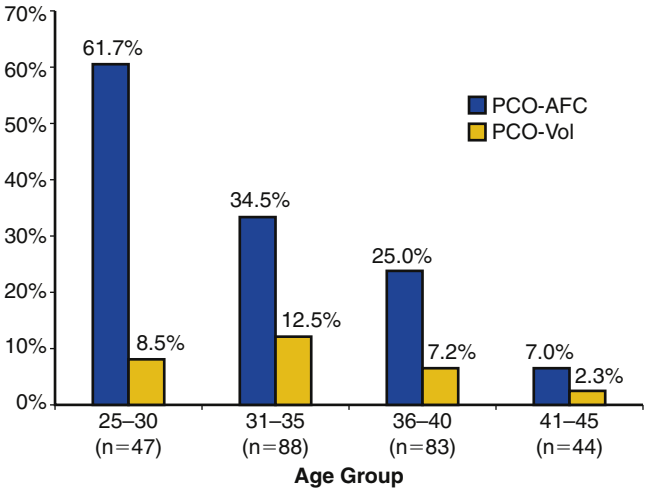


FIGURE 22.1 Percentage of subjects by age group meeting PCO antral follicle count (AFC) and volume (vol) according to Rotterdam criteria. *(Adapted from Johnstone EB, Rosen MP, Neril R, et al. The polycystic ovary post-Rotterdam: a common, age-dependent finding in ovulatory women without metabolic significance.* J Clin Endocrinol Metab *95:4965–4972, 2010.)*

polycystic ovaries were recently shown to be a relatively common finding in a large series of well characterized normal women from age 25 to 45 years old.[16] Of 257 women, polycystic ovaries were identified in 32% overall with the greatest percentage, 62%, occurring in women 25 to 30 years old followed by progressive decline in older groups (Fig. 22.1). The physiological relevance of polycystic ovaries in normal women is not clear, although a longitudinal study suggested that such individuals are not at increased risk for PCOS.[17] Thus, while the requisite identification of polycystic ovaries to establish the diagnosis appears to be clinically appropriate, the morphogenesis of the polycystic ovary may not necessarily be unique to the disorder.

Familial Occurrence

It has been well documented that PCOS tends to aggregate within families.[18] Moreover, several studies have addressed the prevalence of PCOS among first-degree relatives. Based on clinical evidence and laboratory confirmation of hyperandrogenism, anovulation, and polycystic ovaries, the likelihood of PCOS in sisters and mothers of affected women has been reported to be considerably higher than those of normal controls. In a study of 115 sisters of 80 probands, PCOS was demonstrated in 22% of reproductive-aged siblings whereas hyperandrogenemia was found in an additional 24%.[18] Additional studies of families with PCOS have revealed that 32% to 66% of sisters and 24% to 52% of mothers had the syndrome.[19,20] In contrast, in a study of sisters and mothers of Turkish women with PCOS revealed rates of 16% and 8%, respectively.[21] In most of these studies the prevalence of insulin resistance and impaired glucose tolerance was considerably greater in the relatives compared to normal controls.

A PCOS phenotype for men and male siblings of affected women with PCOS has not been established. However, circulating dehydroepiandrosterone sulfate (DHEA-S) levels in first-degree male relatives have been shown to be significantly higher than those of unrelated body mass index (BMI)-matched controls.[22] Serum testosterone (T), sex hormone binding globulin (SHBG), luteinizing hormone (LH), and follicle stimulating hormone (FSH) were similar between groups.

Collectively, these results indicate that first-degree relatives of PCOS women are at significant risk for having PCOS. The findings support a genetic basis for hyperandrogenemia, which may account, at least in part, for the familial clustering of this disorder.

Clinical Description

Hirsutism

The most distinctive and visible clinical feature of PCOS is hirsutism, the degree of which is variable from mild to severe. The rate of hair growth is important clinically as gradual and progressive growth indicates a functional etiology, whereas the rapid appearance of thick, pigmented hair often suggests a neoplastic source of androgen production. In PCOS, increased hair growth is commonly found on the side of the face, upper lip, and chin extending down to the neck region (Fig. 22.2). This pattern of hirsutism may be accompanied by extension of pubic hair growth towards the umbilicus and include excess hair on the extremities, abdominal flank, and back, although these areas are not considered specific sites of sexual hair growth. More severe cases include the appearance of hair on the chest. Progressive hyperandrogenism may be associated with temporal balding and male pattern baldness.

In PCOS, the amount of hirsutism has been correlated to serum androgen concentrations. However, individual variation in hair growth may reflect ethnic differences, which may account for slight variations of prevalence of PCOS in different parts of the world.[7,23-25] Coexisting conditions that alter the bioactivity of androgens, such as hypothyroidism and obesity may also give rise to excessive hair growth. These conditions are associated with lowered sex-hormone binding globulin (SHBG), which provides increased availability of free testosterone. Sequence variations within the coding region of separate SHBG alleles were identified in a heterozygous woman who presented with severe hyperandrogenism during pregnancy.[26] Serum SHBG levels were barely detectable and non-protein bound testosterone concentrations markedly higher than the normal reference range. A single nucleotide polymorphism within an allele encoded a missense mutation that permitted normal steroid hormone binding, but caused abnormal glycosylation and decreased serum SHBG.

Menstrual Irregularity

In PCOS, menstrual dysfunction is primarily characterized by irregular, infrequent, or absent menstrual bleeding. Typically, this pattern of bleeding is an extension of postmenarchal irregularity and monthly menstrual cyclicity is never established. In some women, the onset of chronic anovulation emerges beyond adolescence, but this is unusual. Prolonged heavy bleeding should raise consideration of abnormal endometrial hyperplasia and even endometrial adenocarcinoma. In approximately 20% of women, there is

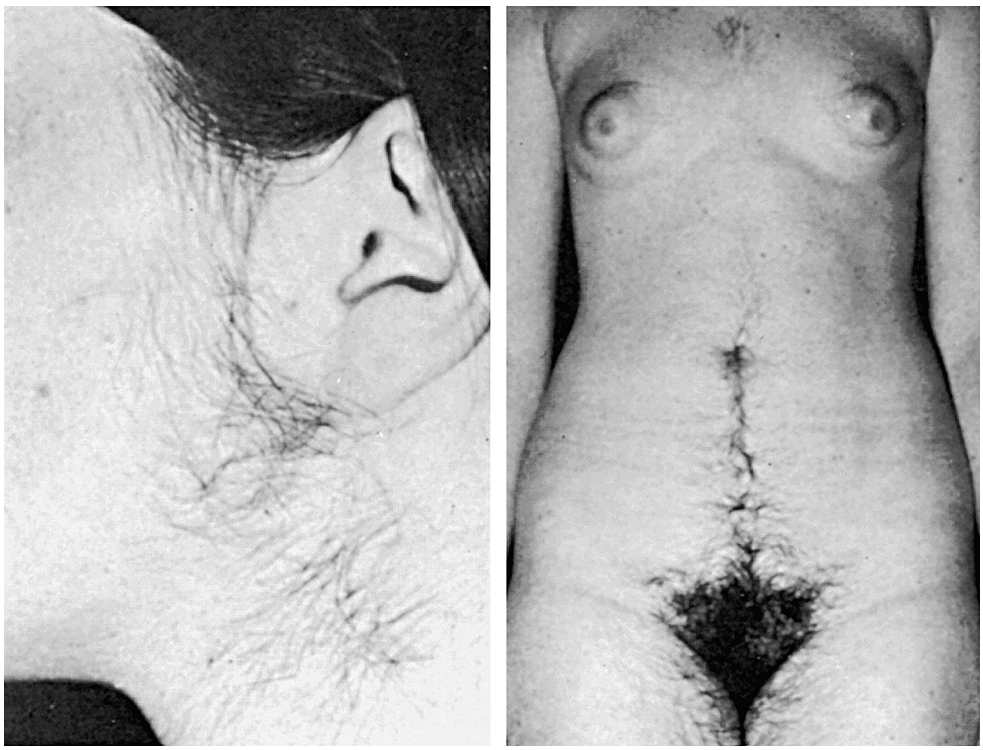


FIGURE 22.2 Moderately severe hirsutism in a young woman. Note the male pattern of hair distribution.

complete absence of menses, whereas 5% to 10% of cases demonstrate regular ovulatory function. Recognition of normal ovulation in PCOS is significant in that a history of regular menstrual cycles does not exclude the diagnosis. It has been reported that in late reproductive life, PCOS women begin to experience regular ovulation for unknown reasons.[27] Aging PCOS women with regular menstrual cycles appear to have a smaller follicle cohort, higher serum FSH levels and lower androgen levels compared to age-matched PCOS with persistent anovulation.[28] Whether changes in the follicle population or alterations in the ovarian endocrine milieu may be responsible for resumption of ovulation in late-age PCOS women remains to be determined.

Ovarian Morphology

Classically, women with PCOS have enlarged ovaries with numerous peripheral small antral follicles and increased central stroma (Fig. 22.3). While the process that leads to this morphological appearance is not completely understood, it has been proposed that normal follicular growth appears to occur up to the midantral stage, after which maturation ceases and progressively, the majority of follicles undergo atresia.[29] As the follicle reaches the midantral stage, the granulosa cell layer becomes progressively degenerative and the entire structure may have the appearance of a thin-walled cyst. In contrast, the theca cell layer that surrounds the follicle becomes substantially hyperplastic and thickened compared to that of normal follicles and is responsible for increased androgen production (Fig. 22.4). However, that follicle development becomes arrested at the midantral stage does not necessarily signal the immediate onset of atresia. In granulosa cells from unstimulated ovaries of women with PCOS, viability measures have indicated robust cell survival with substantial steroidogenic potential compared to cells obtained from ovaries of normal women.[30] It was concluded that the majority of follicles in PCOS are not apoptotic, but retain ample functional capacity. These findings were consistent with a recent study in which PCOS granulosa cells exhibited decreased apoptotic nuclei and proteins and increased anti-apoptotic protease gene expression compared to that of normal cells.[31] After midantral arrest, there appears to be a progressive accumulation of follicular fluid that results in expansion of the antrum which gives rise to the classical appearance of cystic follicles. As the follicle enlarges, the granulosa cell layer becomes progressively degenerative and thin.

The ovarian follicle population in PCOS also is distinctive in that histomorphometric studies have revealed a twofold to threefold increase in the numbers of primary, secondary, and tertiary follicles compared to those of the normal ovary.[32] Whether the ovaries are endowed with a greater number of follicles or whether the rate of programmed cell death is decelerated compared to the normal ovary has not been systematically studied. In the classical description of the polycystic ovary, the central ovarian stroma appears abnormally increased occupying at least 25% of the medullary portion of the ovary. This hyperplastic stroma may be responsible for displacing the cystic follicles towards the periphery.

A mechanism for the morphogenesis of the polycystic ovary has not been established, although a role for androgen excess on follicle growth and development has been suggested from ovarian morphology in hyperandrogenic women with congenital adrenal hyperplasia and androgen producing ovarian tumors.[33-35] In particular, polycystic ovaries have been demonstrated in male to female transsexuals receiving long-term androgen treatment.[12,36] Nonhuman primates treated with subcutaneous silastic capsules containing testosterone were observed to develop enlarged

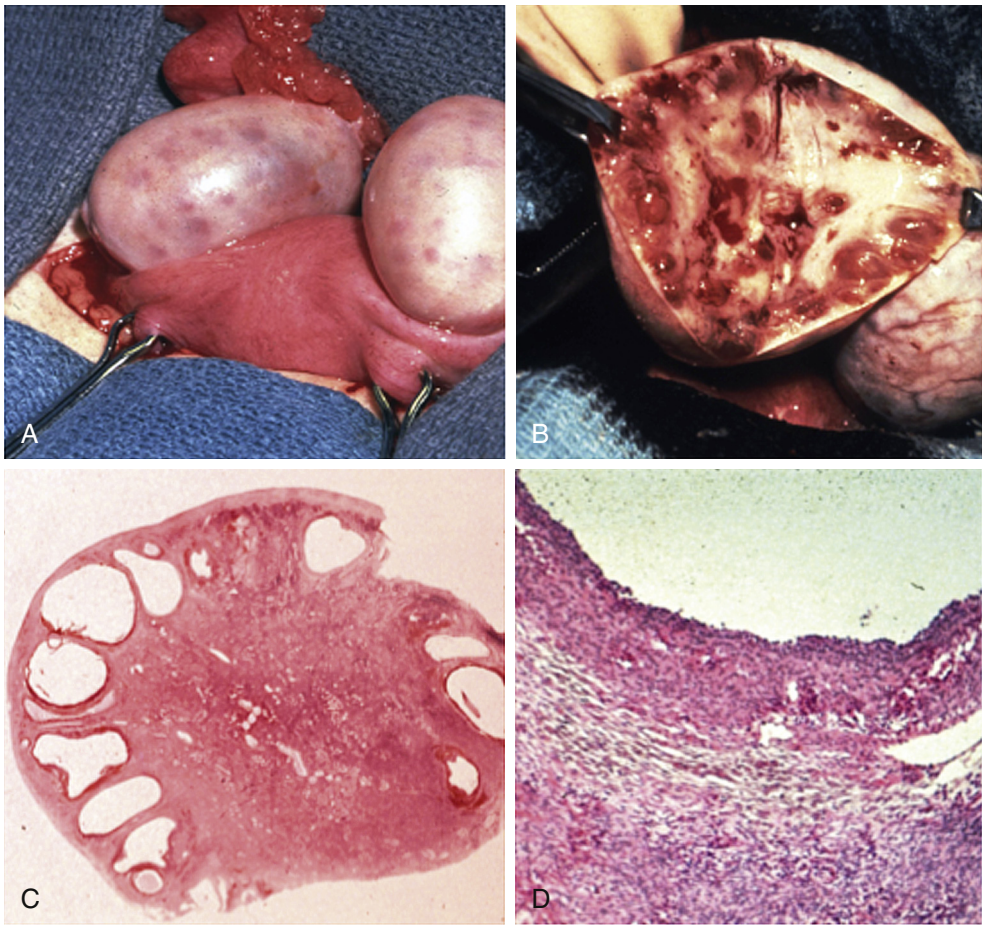


FIGURE 22.3 The gross and microscopic characteristics of the polycystic ovaries. **A**, Bilateral enlarged ovaries with a smooth and thickened capsule. **B**, On cut section, multiple follicular cysts surrounded by abundant ovarian stroma are found throughout the cortex of the ovary. **C**, Subcapsular antral follicles arrested at midstage of development. **D**, Hyperplasia of theca cell layer; note relative thin granulosa cell layer.

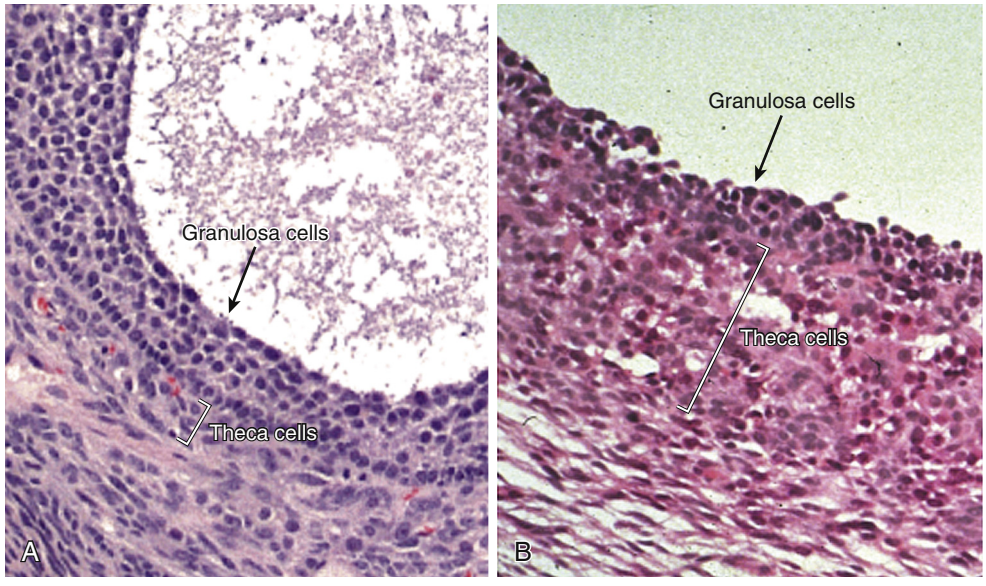


FIGURE 22.4 Histology of midantral follicle wall of a normal and polycystic ovary. **A**, Normal follicle with healthy appearing granulosa and theca cells. **B**, PCO follicle with thickened theca cell layer with disrupted and degenerative granulosa cells; note difference in width of theca and granulosa cell layers.

ovaries and increased follicle number.[37] *In situ* hybridization studies revealed that androgen receptor (AR) messenger ribonucleic acid (mRNA) was expressed primarily in healthy small- and medium-sized antral follicles compared to large preovulatory follicles.[38] Moreover, AR mRNA was found to colocalize with that of the FSH receptor and testosterone treatment increased the expression of FSH receptor mRNA.[39]

Alternatively, there is evidence to suggest that antimüllerian hormone (AMH) may be involved with the morphogenesis of the polycystic ovary. AMH is a product of the granulosa cell and mRNA expression is initially detected in primary follicles. With subsequent growth, the concentration of AMH intensifies and the greatest expression is observed in large preantral and small antral follicles.[40-42] Thereafter, the expression declines and is not observed in

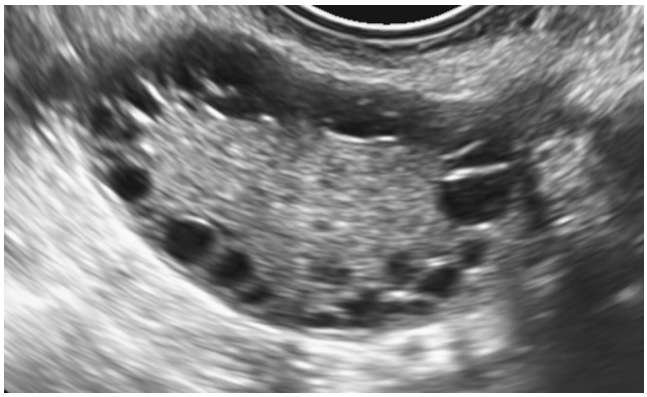


FIGURE 22.5 Polycystic ovary on ultrasound. *(Courtesy of Dr. G. Garzo.)*

late stage antral follicles, preovulatory follicles, or the corpus luteum. It has been proposed that AMH has two potential functions. First, AMH may serve to inhibit recruitment of primordial follicles into the pool of growing follicles to prevent early depletion.[42,43] Second, AMH may decrease follicle sensitivity to gonadotropin stimulation to control the number of large preantral and small antral follicles that reach the preovulatory stage.[43,44] These proposed actions of AMH are consistent with the report of decreased AMH expression in preantral and early antral follicles of women with PCOS compared to values observed in normal women.[45] In addition, the number of small antral follicles in PCOS women and normal women has been positively correlated to circulating levels of AMH.[46,47] With two functions so critical to folliculogenesis, the regulation of AMH is paramount in understanding its role. However, the regulation of AMH production by granulosa cells is not well understood.

The polycystic ovary has been characterized by ultrasound examination (Fig. 22.5). At a consensus conference in Rotterdam, Netherlands in 2003, a polycystic ovary was described as having 12 or more follicles per ovary or an ovarian volume of greater than 10 cm^3.[48] This description should not be confused with the ultrasound appearance of an ovary in women recovering from hypogonadotropic hypogonadism. In these individuals, multiple small antral follicle growth is common and may appear similar to a polycystic ovary. In women undergoing ovulation induction, multiple follicles formation may occur as result of ovarian stimulation. Thus, implication of a multi-follicular ovary or polycystic ovary should be considered within the realm of the clinical setting.

Obesity

Early studies of the clinical features of PCOS demonstrated that obesity was present in a little more than 50% of cases.[49] However, there is epidemiological evidence that the incidence may be greater, at least in the United States, than that previously described. Commonly, an increase in the upper body or central distribution of fat gives rise to an increased waist to hip ratio as compared to obese women without PCOS.[50] This fat distribution pattern has been termed android obesity and can be found in other hyperandrogenic states, diabetes, and hyperlipidemia. Notably, there is a preponderance of visceral fat compared to peripheral fat not unlike the distribution of adipose tissue in individuals with insulin resistance.[51] In contrast, women with gynecoid obesity generally have an enhanced accumulation of normal fat in the hips, buttocks, and thighs. As to whether PCOS patients are predisposed to obesity has not been clarified. It has been shown that postprandial thermogenesis may be decreased in PCOS, thereby contributing, at least in part, to weight gain.[52] However, resting energy expenditure in PCOS appears to be equivalent to that of normal weight-matched control, which suggests a relative disparity of increased caloric intake and decreased total energy expenditure.[53]

Insulin Resistance

It has been well-documented that women with PCOS are insulin resistant and have compensatory hyperinsulinemia as a result of their disorder.[50,54,55] The prevalence of insulin resistance in PCOS has been reported to range between 20% to 40%.[56,57] That insulin resistance is common in obesity may account, in part, for the rather wide prevalence. Nevertheless, independent of obesity, the presence of a defect in insulin action in PCOS has been clearly established.[50] Generally, the degree of insulin resistance is mild, although the prevalence of glucose intolerance and subsequent diabetes has been reported to be as high as 31% and 7.5% respectfully.[57] Moreover, in these individuals the progression to diabetes appears to be relatively rapid.[58,59] Commonly, fasting hyperglycemia is not evident; instead there is a primary dysfunction of postprandial glucose uptake that results in severe peripheral insulin resistance.[60,61]

Not only are women with PCOS predisposed to insulin resistance and risk for diabetes, but evidence exists that first-degree relatives also exhibit disordered glucose metabolism and insulin secretion. Importantly, this predisposition includes both sisters and brothers of affected individuals.[62,63] Aside from the increased risk for diabetes, there is indirect evidence to indicate that insulin resistance may worsen the clinical manifestations of PCOS. Administration of insulin lowering drugs has been shown to improve insulin sensitivity, reduce androgen levels, and restore ovulation in some, but not all patients with this disorder.[64-67] Insulin resistance may also contribute to metabolical dysfunction in PCOS, including an increased likelihood of lipid abnormalities.[68,69] In addition, the association of insulin resistance with visceral fat distribution is underscored by the displacement of central fat to the peripheral compartment with improvement of insulin sensitivity following administration of insulin lowering drugs or weight reduction.[66,70]

The discovery and documentation of insulin resistance in PCOS has been achieved by time-consuming and complex procedures conducted in the course of clinical investigation. By comparison, identification of insulin resistance in the clinical setting has been difficult as most patients exhibit normal fasting blood glucose levels and increased circulating insulin levels are not common. As a result, effective and convenient screening tests to determine evidence-based therapeutic modalities have been limited.

Acne and Acanthosis Nigricans

Women with PCOS may experience increased skin oiliness secondary to excessive stimulation of the pilosebaceous



FIGURE 22.6 Acanthosis nigricans. Note the darkened, velvety plaque along the nape of the neck giving the appearance of hyperpigmentation in a patient with severe insulin resistance, hirsutism, and polycystic ovaries.

unit by increased androgen production. However, increased sebaceous gland activity in PCOS is not associated with acne nor is acne correlated with increased ovarian androgen production. Therefore, as an isolated symptom, acne should not be considered a sign of PCOS.

Perhaps, more common is the finding of acanthosis nigricans, which has been observed in 5% to 50% of hyperandrogenic women and is related to the presence and severity of hyperinsulinemia.[71] This dermatological condition features symmetrical, darkened, velvety plaques that may appear most commonly appear on the nape of the neck, in the intertriginous areas of the body such as skin folds, and on pressure bearing surfaces such as knuckles and elbows (Fig. 22.6). Acanthosis nigricans originates from epidermal hyperkeratosis and dermal fibroblast proliferation. There is no evidence of an increased number of melanocytes or melanin deposition despite apparent increased pigmentation. Acanthosis nigricans is considered a potential marker for insulin resistance and diabetes in adults. Reduction of hyperinsulinemia is associated with improvement in the darkened skin areas.

Infertility

A significant number of patients will offer infertility as a presenting feature of PCOS.[49] Clearly, anovulation would appear to be the primary defect responsible for the failure to achieve pregnancy in this disorder. However, other potential considerations may exist which may preclude fertility. There is evidence that women with PCOS have a higher incidence of spontaneous pregnancy loss, the mechanism of which remains unclear.[72] It has been reported in a small series of women that the prevalence of polycystic ovaries in women with recurrent miscarriage was 56%.[72] Subsequently, in a much larger study, it was observed that polycystic ovarian morphology was not predictive of pregnancy loss among women with recurrent pregnancy loss.[73] Clearly, additional research is necessary to determine the prevalence of recurrent pregnancy loss in PCOS as well as the underlying mechanism.

PCOS in Adolescence

Ovarian Morphology in Adolescence

An early attempt to estimate the prevalence of polycystic ovaries in a general adolescent population revealed that 28% of 119 girls had 10 or more follicles per ovary.[74] More recently, in a large cohort from Australia it was demonstrated in 230 adolescents that the median antral follicle count was 11 with an interquartile range of 7 to 14.[75] From this cohort it was shown that 35% had polycystic ovaries using Rotterdam criteria.[76]

In normal adolescent girls with regular cycles and no hirsutism, it was reported that 9% exhibited polycystic ovaries using older criteria.[74] Later, using Rotterdam criteria, ultrasonography in 74 nonobese girls with regular menstrual cycles and no evidence of hyperandrogenism revealed a 34% prevalence of polycystic ovaries.[77] A similar rate, 40%, was reported in 20 postmenarchal girls with regular menstruation function and in whom hyperandrogenism had been excluded.[78] Collectively, the data suggest that polycystic ovaries are relatively common in the normal adolescent population. The precise rate of polycystic ovaries in this group is unclear due to the limited numbers of subjects studied to date. Nevertheless, an increased prevalence in adolescence is not dissimilar to that reported for normal young adults.[15,16]

There is little information about ovarian morphology in adolescent girls with PCOS. Ibanez and colleagues identified polycystic ovaries in only 38% of adolescent and young women who presented with evidence of androgen excess or irregular bleeding.[79] Similar findings were reported in adolescent PCOS girls who had been referred for oligomenorrhea and hirsutism.[80] Polycystic ovaries were identified in 35% of these individuals.

Recently, Brown and coworkers studied ovarian morphology in normal high school students and adolescents with PCOS based on the presence of hirsutism and oligomenorrhea.[81] Employing magnetic resonance imaging, 17 of 21 normal individuals (81%) had 12 or more follicles per ovary (Fig. 22.7). In 5 of 21 normal adolescents, ovarian volume was larger than 10 mL. Of 19 girls with PCOS, each had polycystic ovaries by follicle count and ovarian volume. While the number of subjects in this study was small, the difference between these findings and those of others may have been due to magnetic resonance technique and improved visualization of small antral follicles. A direct comparison between magnetic resonance and ultrasound of the ovary has not been conducted in this population.

In adolescent girls with irregular menstrual cycles, the prevalence of polycystic ovaries is significant. In a study of nonobese adolescent girls with oligomenorrhea, polycystic ovaries were demonstrated in 45%.[74] Similarly, it was reported in healthy girls with menstrual irregularities that the ultrasound appearance of the ovary was polycystic in

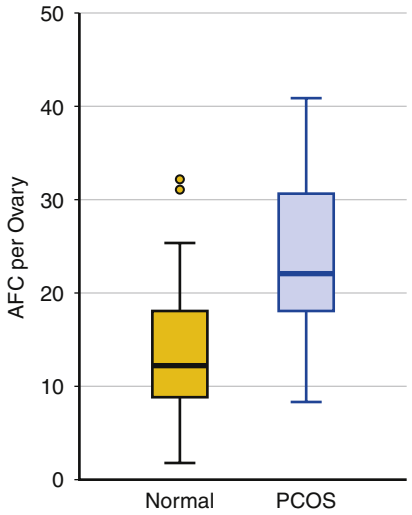


FIGURE 22.7 Box-and-whisker plot showing antral follicle count (AFC) per ovary in normal adolescent girls and adolescent girls with polycystic ovary syndrome (PCOS) assessed by magnetic resonance imaging. Horizontal small bars represent the 5th to 95th percentile range and the boxes indicate the 25th to 75th percentile range. The horizontal line within each box corresponds to the median. O, values beyond the 95th percentile. *(From Brown M, Park A, Shayya R, et al. Detailed morphometric analysis of the ovary in adolescent girls with polycystic ovary syndrome and age-matched controls.* J Magn Reson Imaging. *Jan 4. doi: 10.1002/jmri.23992. [Epub ahead of print] 2013.)*

41% of cases.[82] Subsequent examinations performed 2 to 7 years later showed that the percentage of individuals with polycystic ovaries increased despite no change in the rate of anovulation. In contrast, 40% of oligomenorrheic girls with elevated LH levels and enlarged ovaries were shown to spontaneously normalize ovarian function and size during longitudinal follow-up.[83] These results point out that the polycystic ovary is a common finding in adolescent girls with abnormal menstrual cycles and suggest that the likelihood of spontaneous restoration to normal ovarian morphology is relatively small as long as menstrual irregularity persists.

Clinical Presentation

Characteristically, the symptoms of PCOS emerge insidiously coincident with changes that accompany normal pubertal development (see Chapter 18). That the emergence of PCOS can commonly be traced to the events of puberty suggests that this disorder may be related to an abnormal expression of those factors that initiate and regulate the process of puberty. Because the duration of menstrual irregularity that accompanies normal puberty may be variable, it is difficult to rely solely on this historical feature as a basis for diagnosis. Moreover, with the recognition that some PCOS women may exhibit normal ovulatory function, evidence of regular cyclic bleeding does not preclude the disorder in adolescence. Rather, early detection of PCOS in adolescent girls is predicated primarily on hyperandrogenic symptoms such as hirsutism and acne. In the obese individual, associated metabolical-reproductive abnormalities may create uncertainty as to the mechanism of hyperandrogenism. Reduction in SHBG is directly correlated to obesity, thus giving rise to increased free testosterone levels. In addition, obese adolescents, particularly those with evidence of acanthosis nigricans, are highly likely to have insulin resistance with compensatory hyperinsulinemia, which is known to suppress SHBG and probably contributes to excess ovarian androgen production. An ultrasound image of a polycystic ovary virtually confirms the diagnosis. However, the utility of ultrasonography is often limited by the necessity of an abdominal versus vaginal approach and the difficulty securing adequate imaging in obese girls.

It has been well-documented that adolescent girls with PCOS have increased levels of circulating androgens as well as elevated LH levels.[74,83] Previous studies have shown that hyperandrogenic girls with likely PCOS exhibited changes in gonadotropin secretion patterns that were similar to those found in adult PCOS.[74,82-84] Increased concentrations of serum LH are accompanied by an increase in pulse frequency and amplitude. In addition, mean serum levels of testosterone and androstenedione were elevated. Twenty-four hour pulsatile LH secretion studies have revealed that premenarchal hyperandrogenic girls demonstrated higher LH levels while awake whereas the sleep entrained increases were minimal compared to those of developmentally matched control subjects.[84] In post-menarche, there was greater LH pulse activity during waking hours whereas pulse frequency was slowed with sleep (Fig. 22.8). By comparison, in postmenarchal normal girls, LH levels were lower and the pulse frequency reduced while awake whereas during sleep LH pulse frequency was significantly less than that of hyperandrogenic girls. Thus, the pattern of gonadotropin secretion in the postmenarchal normal control resembled that of the younger premenarchal hyperandrogenic girl. These findings suggest that the transition through neuroendocrine puberty may be accelerated in hyperandrogenic girls compared to normal puberty. Moreover, the data pose the question as to whether there is a chronological difference in the onset of increased LH levels and pulse frequency between hyperandrogenic and normal pubertal girls.

Role of Obesity

The effect of obesity on androgen levels and gonadotropin secretion in adolescents has recently been examined. In a cross-sectional study, McCartney and colleagues showed that during early puberty obese girls at Tanner stage 1-3 had significantly increased serum testosterone levels compared to nonobese controls accompanied by corresponding decreases of circulating LH concentrations.[85,86] In addition, obese girls exhibited hyperinsulinemia and increased HOMA values compared to that of normal weight controls which may have accounted, in part, for hyperandrogenemia in the presence of lowered serum LH. In early pubertal girls, diurnal variation was observed for both testosterone and progesterone with levels lower at night and rising in the early morning hours. The incremental change in both hormones was significant in nonobese girls, but failed to achieve statistical significance in obese girls likely because of too few numbers. Nevertheless, the heightened level of progesterone in obese girls may have contributed to reduced LH levels in early puberty.

This concept relates to responsiveness of the GnRH pulse generator to progesterone negative feedback. In normal adult women, progesterone administration inhibited LH pulse frequency whereas in women with PCOS, LH secretion was unaltered.[87] However, after androgen blockade by anti-androgen treatment, rapid LH release was

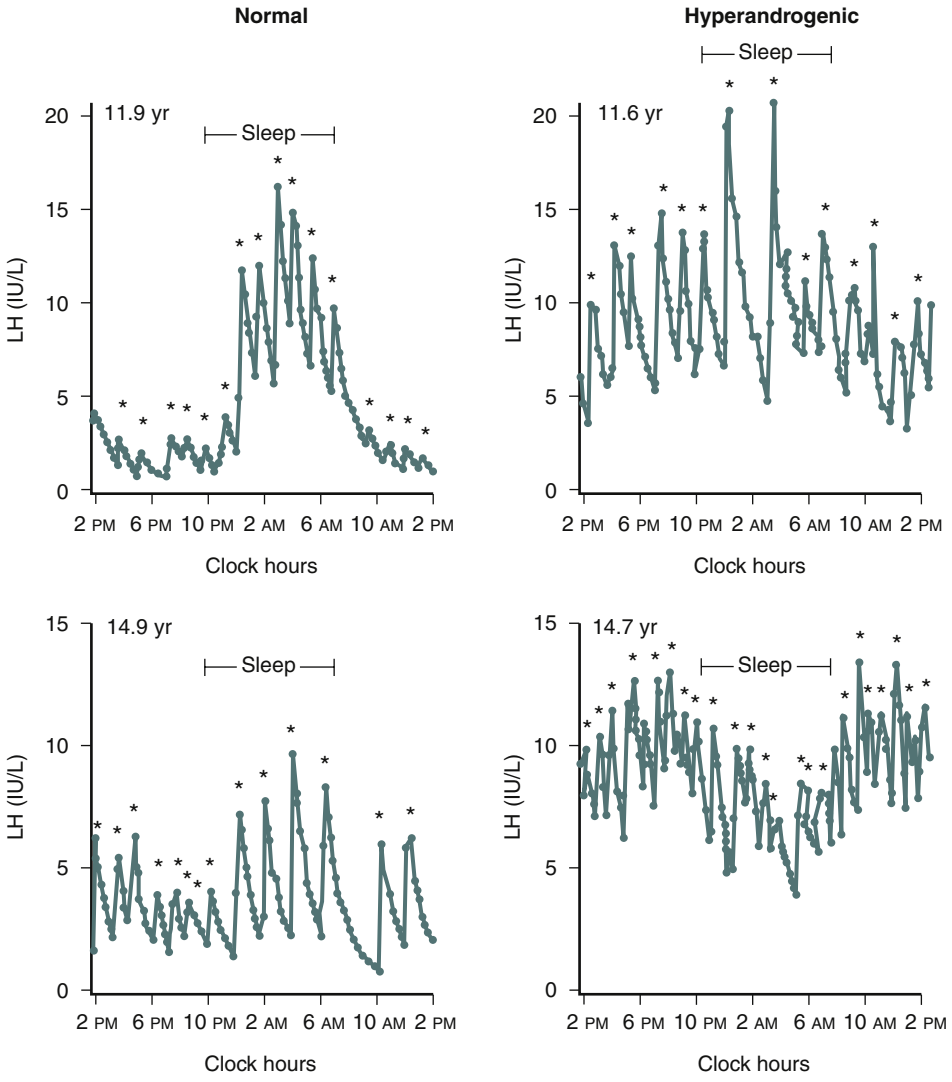


FIGURE 22.8 Twenty-four-hour pulsatile luteinizing hormone patterns in premenarchal and postmenarchal hyperandrogenic girls *(right)* and two developmentally matched healthy girls *(left)*. Asterisks indicate significant pulses. Open bars represent sleep periods. *(Adapted from Apter DA, Butzow J, Laughlin GA, et al. Accelerated 24-hour luteinizing hormone pulsatile activity in adolescent girls with ovarian hyperandrogenism: relevance to developmental phase of polycystic ovarian syndrome.* J Clin Endocrinol Metab *79:119–125, 1994.)*

reduced by progesterone administration which implied that hyperandrogenemia may have contributed to increased LH secretion.[88] This proposed mechanism is suggested in young adolescents as some girls with PCOS treated with progesterone failed to exhibit a reduced rate of LH release.[89]

Evidence for this was provided by an intensive and vigorous examination of 24-hour LH secretion in nonobese and obese girls through puberty.[90] The 24-hour pattern of LH secretion in nonobese girls during early adolescence (Tanner stage 1-3) was characterized by greater release during sleep than that observed while awake. The nighttime rise of LH declined in the very early morning hours and was correlated to a corresponding increase of serum progesterone. In contrast, in obese girls, LH secretion was unaltered during the sleep-wake cycle and there was no relationship to circulating progesterone levels. With advanced development (Tanner stage 3-5) obese adolescents exhibited increasing day and night time LH secretion that was greater than that observed in nonobese individuals, although pulse amplitude remained significantly higher in nonobese girls. The pattern of LH secretion in the obese group was essentially equivalent to those with PCOS. Similar findings were reported in a study of LH secretion during early puberty.[91] Normal weight girls at Tanner stage 3 demonstrated a sleep related rise of mean circulating LH that was significantly greater than that observed in an obese group of adolescents.

Thus, in the absence of hyperandrogenemia, diminished LH secretion in early puberty may be a consequence of obesity during this stage of development. However, in general, obesity confers acceleration of puberty or, in some instances, precocity in young girls. This being the case, another factor or mechanism associated with and separate from obesity may be involved with regulation of gonadotropin secretion in early puberty.

Oligomenorrhea and PCOS Risk

Among postmenarchal girls with irregular bleeding, it has been estimated that approximately 50% of oligomenorrheic adolescent girls have increased levels of serum LH associated with mild elevations of circulating androgens.[82,83,92] In addition, we and others have shown that these individuals exhibited an increased rate of LH release as determined from frequent sampling studies, which suggested a diagnosis of PCOS.[92,93] Notably, in a long-term follow-up study of oligomenorrheic girls, those with normal serum LH values

eventually developed regular ovulatory function compared to more than half of those with elevated LH levels in which the gonadotropin abnormalities persisted along with hyperandrogenism.[83,94] These intriguing findings suggest that oligomenorrhea in early adolescence may be associated with an endocrinological phenotype of PCOS in the absence of overt signs of hyperandrogenism. Whether the transitory nature of elevated LH and androgens represents an extension of normal hypothalamic-pituitary development remains to be determined.

Abnormal Metabolical Function

Similar to their adult counterparts, hyperandrogenic girls, with likely adolescent PCOS, exhibit abnormal insulin responses to glucose loading.[95] Correspondingly, assessment of 24-hour patterns of insulin revealed greater release in hyperandrogenic girls than that observed for normal girls whereas a reciprocal relationship was found for IGFBP-1 secretion. Pulsatile growth hormone secretion was not altered in adolescent PCOS.[95] The lipid profile in the girls with increased androgen indices demonstrate higher low-density lipoprotein cholesterol (LDLC) to high-density lipoprotein cholesterol (HDLC) ratios in conjunction with lowered SHBG levels.[96] These data suggest that any risk for long-term health consequences may be established early in reproductive life for these girls.

Prepubertal Disposition

It has been reported that girls with premature pubarche are at increased risk for functional ovarian hyperandrogenism and polycystic ovaries following puberty.[96] Moreover, with subsequent development of hyperandrogenism and hyperinsulinemia, there was a corresponding reduction in birth weight of these individuals.[96] The link between low birth weight and insulin resistance in children appears to be persistent throughout life as indicated by studies performed in early and late adulthood.[97,98] This relationship may be of particular relevance to a possible mechanism for PCOS in this population. Low birth weight is commonly associated with hypoplasia of the fetal adrenal and correspondingly low serum DHEA-S levels.[99] DHEA-S secretion also serves as a marker for adrenarche, which is independent of and precedes gonadarche by several years. It has been demonstrated in pairs of discordant siblings who achieved similar weight in childhood that DHEA-S levels were higher in those of low birth weight compared to those born with normal weight.[100] Thus, if as proposed, fetal growth modulates adrenarche, then increased DHEA-S may have reflected an exaggerated adrenarche in these children. The resultant increased androgen pool may set in motion a cycle of altered physiology characteristic of PCOS. This notion is further reinforced by the presence of hyperinsulinemia and insulin resistance, which may enhance androgen production in adolescent girls at risk for PCOS.[101] In addition, elevated levels of circulating insulin coincide with a reciprocal decline of SHBG, thereby allowing for increased availability of free testosterone. Thus, the detection of hyperinsulinemia in postmenarchal girls with hyperandrogenism relates temporally to physiological insulin resistance during puberty and may be of critical importance in the genesis of PCOS.

While premature pubarche may increase the risk for PCOS after puberty, exposure to androgen prepubertally may also provoke features of the disorder. In a study conducted in nonhuman primates, prepubertal female rhesus macaques at 1 year of age were administered silastic testosterone implants resulting in fourfold rise of circulating levels.[102] This level of induced hyperandrogenemia was maintained long-term throughout the course of study. At age 5 when the monkeys were in early adulthood, testosterone-treated animals had a significantly greater LH pulse frequency during the early follicular phase compared with controls given cholesterol filled implants. No differences were seen in insulin sensitivity, although there was suggestion of multiple follicle formation in the ovaries as determined by magnetic resonance. Ovulation rates were similar in both groups. These results show that increased androgen exposure over the course of pubertal development may enhance the neural drive to the reproductive axis that resemble those of obese hyperandrogenemic girls in early adulthood and are characteristic of PCOS. Moreover, the findings suggest that the prepubertal hypothalamic-pituitary-ovarian axis may be vulnerable to endocrinological insult such as excess androgen production.

Fetal Predisposition

It has been shown that adult, female rhesus monkeys exposed to testosterone *in utero*, at concentrations equivalent to those found in males, may exhibit increased LH secretion, impaired insulin secretion, hyperandrogenic anovulation, and enlarged ovaries with multiple cystic follicles.[103] Similar outcomes have been observed in female adult sheep following *in utero* exposure of high doses of testosterone at various phases of pregnancy.[104,105] These observations have led to the hypothesis that the clinical phenotype of PCOS may be the result of intrauterine androgen exposure during pregnancy. In human pregnancy, it has been reported that high maternal serum testosterone levels do not confer this clinical consequence in female offspring.[106] This is likely due to increased circulating levels of SHBG as well as the metabolical capacity of placenta aromatase to neutralize maternal androgen production. Rather, an *in utero* effect of hyperandrogenism may occur in the presence of abnormal steroidogenesis by the fetal ovary or adrenal gland. In support of this concept, it has been reported that women with 21-hydoxylase deficiency also display excessive ovarian androgen production and polycystic ovaries.[35] The possibility exists that androgenic programming in fetal life may predispose select individuals to the clinical features of PCOS.

Altered Physiology

Hypothalamic-Pituitary Interaction

In adult women with PCOS, LH secretion is characterized by increased pulse frequency and amplitude, elevated 24-hour mean serum concentrations, and greater responses to gonadotropin releasing hormone (GnRH) compared to those found in normal women (Fig. 22.9).[107] The mechanism(s) responsible for this increased release of LH are not well understood. A particular characteristic of LH secretion in PCOS is increased pulse frequency, the periodicity of which

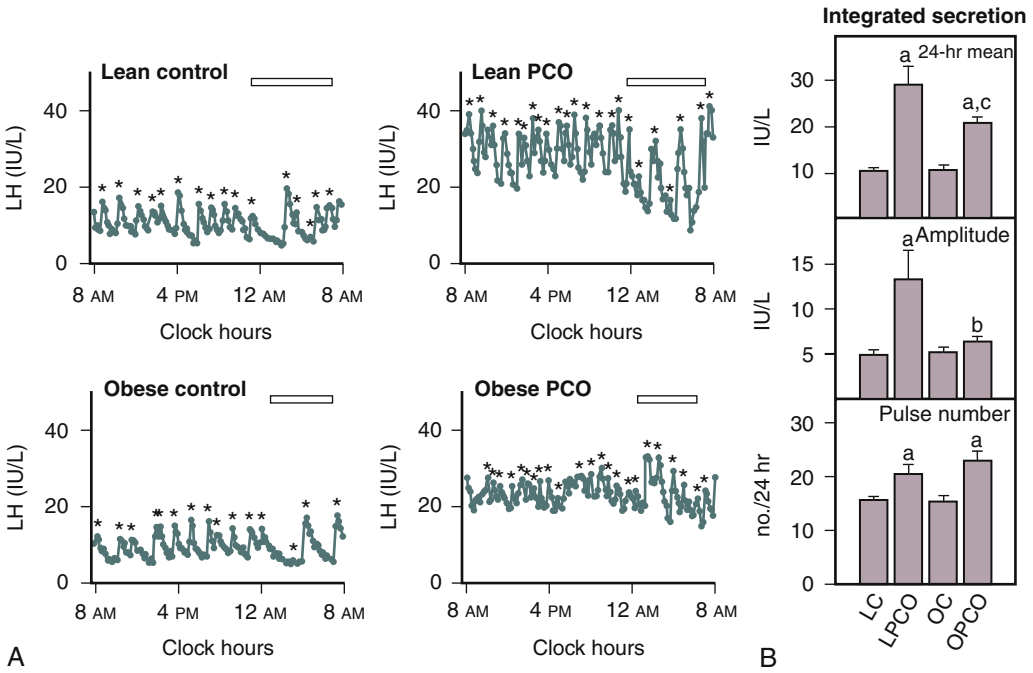


FIGURE 22.9 **A**, Representative 24-hour luteinizing hormone (LH) pulsatile patterns in lean and obese control women during the follicular phase of the menstrual cycle and in lean and obese women with polycystic ovary syndrome (PCOS). *Asterisks* indicate LH pulses. *Open bars* signify sleep times. **B**, Twenty-four-hour mean (±SE) LH levels, pulse amplitude, and pulse frequency in lean and obese women with PCOS (LPCO, OPCO) and their respective controls (LC, OC: n = 8 for each group. a, $P < 0.001$ versus corresponding control group; b, $P < 0.01$; c, $P < 0.001$ versus corresponding lean group. *(Adapted from Morales AJ Laughlin CA, Butzow T, et al. Insulin, somatotropic, and LH axes in lean and obese women with polycystic ovary syndrome: common and distinct features.* J Clin Endocrinol Metab *81:2854–2864, 1996.)*

is approximately 1 hour. This rapid rate of LH release does not appear to be altered by experimental manipulation or physiological change.[108,109] These observations imply that corresponding pulsatile release of hypothalamic GnRH is increased. In PCOS, the relationship between GnRH pulse frequency and gonadotrope responsiveness may be a key issue relative to inappropriate gonadotropin secretion. Previous studies in rodents have demonstrated preference for LHβ gene expression in response to rapid rates of GnRH delivery.[110] Not only does GnRH drive the release of LH but also it has been shown, in normal women, to self-prime the pituitary and thereby contributes to increased LH sensitivity to subsequent GnRH stimulation.[111] Collectively, these findings have led to the suggestion that the profound abnormality of gonadotropin secretion in PCOS may be a primary consequence of increased hypothalamic GnRH activity.[112]

In humans, progressive increases in the frequency of GnRH stimulation have resulted in corresponding increases in the rate of LH release as well as elevated basal concentrations.[113] In GnRH-deficient women, an increase in the rate of GnRH administration from every 90 minutes to every 60 minutes was not associated with corresponding increases in serum LH or changes in pulse amplitude, whereas GnRH given at 30-minute intervals resulted in elevated LH levels and reduction of pulse amplitude.[112] While consistent with the primacy of increased hypothalamic GnRH, an LH pulse frequency of 1 hour may represent a physiological limit beyond which more frequent pulses in women do not occur. In normal ovulatory women during the late follicular phase and at midcycle, as well as in postmenopausal women, it has been documented that LH pulse frequency approximates 60 minutes.[114,115] In addition, previous studies have shown that GnRH release from the medial basal hypothalamus of the fetus and adult has a periodicity of about 1 hour.[116] Thus, in PCOS women, the tempo and to some degree magnitude of pulsatile gonadotropin secretion, are probably established by hypothalamic GnRH activity. However, beyond the requisite need for GnRH, LH responsiveness to GnRH and, accordingly, maximal increases in LH pulse amplitude, are probably reliant upon other factors.

It has been suggested that the positive feedback effects of chronic estrogen secretion associated with this disorder may bring about an increase of LH either by a direct effect on gonadotrope sensitivity to GnRH or indirectly by facilitating GnRH pulse frequency.[107,117] *In vitro*, estrogen has been shown to increase the fraction of individual gonadotropes responding to GnRH which is consistent with amplification of LH responses to GnRH in normal women receiving estradiol benzoate.[118] However, prolonged administration of estrone to PCOS patients failed to raise circulating levels of LH beyond baseline values or increase GnRH stimulated LH responses.[117] Alternatively, in animals it has been demonstrated that estrogen enhances GnRH pulse frequency and in women with PCOS serum GnRH levels are increased.[119,120] That estrogen may exert an effect at the hypothalamus in PCOS is supported by the strong positive correlation of mean serum estradiol levels to GnRH pulse frequency.

Hyperandrogenemia has also been implicated as a potential cause of increased LH secretion in PCOS. *In vitro*, it has been shown that androgen administration resulted in

increased GnRH pulse generator activity.[121] Examination of LH secretion in hyperandrogenic patients with congenital adrenal hyperplasia revealed that mean LH levels and LH responses to GnRH were increased, which tended to normalize with the onset of treatment and corresponding lowering of androgen levels.[35] By comparison, other studies have not been able to detect an increase in LH following the administration of androgen. Short-term infusion of androgen to both normal women and women with PCOS failed to alter basal LH secretion.[122] Moreover, high dose androgen infusion in normal women appeared to result in an acute reduction of serum LH levels.[36,123]

Notwithstanding these past findings, other studies have indicated that excess androgen production may have a profound influence on LH pulse frequency in women with PCOS. Progesterone administration, either alone or in combination with estrogen (oral contraceptive) suppressed mean LH and LH pulse frequency in both PCOS and normal women.[87] The lowering of LH release was more pronounced in normal women compared to those with PCOS and suggested an effect of increased androgen production.[87] In subsequent studies, pretreatment with an androgen blocking agent prior to physiological estrogen and progesterone administration resulted in restoration of LH pulse frequency to that observed in normal women (Fig. 22.10).[88] These findings suggested that in PCOS, high circulating levels of androgen prevent the negative feedback effects of estrogen and progesterone on LH pulse release as noted in earlier studies. Moreover, the physiological relevance in women regards a rapid rate of spontaneous hypothalamic GnRH activity, which is regulated by the feedback effects of ovarian steroids.

Efforts to determine an effect of insulin in PCOS women have not documented consistent alterations in LH secretion or LH release following GnRH stimulation.[50,124] Reduction of hyperinsulinemia by administration of insulin-lowering drugs to PCOS patients resulted in decreased mean serum levels of androgens and LH in some cases, whereas in others an accompanying fall of LH was not found.[50,65-67,125-127] We have explored the role of insulin on gonadotropin secretion in PCOS and normal women.[128] Our results demonstrated that episodic gonadotropin secretion and LH responses to multi-dose GnRH stimulation were not altered by insulin infusion over an interval of 12 hours in both groups. In particular, endogenous serum LH levels were unchanged prior to and immediately following initiation of the insulin clamp (Fig. 22.9). These findings confirm and clarify previous studies, which have been unable to document consistent alterations in LH secretion or LH release following GnRH stimulation following insulin administration in women with PCOS.[50] In addition, the results may explain why changes in serum LH were not observed in PCOS women treated with insulin-lowering drugs despite significant reductions in circulating androgen levels. Alternatively, interpretation of these clinical trials is potentially confounded by several factors. First, most of the patients studied were obese and it has been shown recently that obesity is inversely correlated to LH secretion in PCOS (Fig. 22.11).[109] Second, hyperinsulinemia is positively correlated with the BMI in women with this syndrome. Third, the occurrence of ovulation in PCOS is associated with a lowering of LH levels into the normal range.

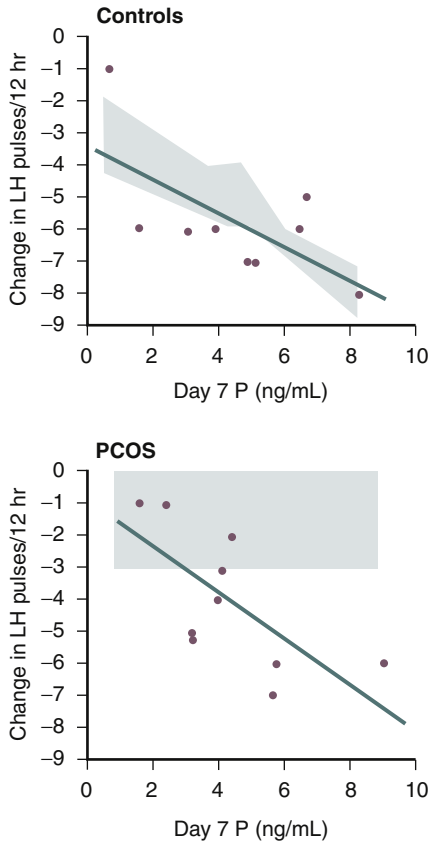


FIGURE 22.10 The change in luteinizing hormone pulse frequency between flutamide treatment alone and after estradiol (E_2) and progesterone (P) administration for 7 days during continued flutamide treatment. Data are shown as a function of the mean plasma P level during E_2 and P administration with flutamide for controls and polycystic ovary syndrome (PCOS) patients. Shaded area shows the range of responses in an identical protocol performed in the absence of flutamide. The slopes for the linear regression analysis are as follows: controls (with flutamide), –0.53; controls (without flutamide), –0.76; PCOS (with flutamide), –0.72; and PCOS (without flutamide), 0.07. *(Adapted from Eagleson CA, Gingrich MB, Pastor CL, et al. Polycystic ovarian syndrome: evidence that flutamide restores sensitivity of the gonadotropin-releasing hormone pulse generator to inhibition by estradiol and progesterone.* J Clin Endocrinol Metab *85:4047–4052, 2000.)*

Recently, we re-examined the relationship of insulin to composite basal LH secretion and LH responses to GnRH in normal and PCOS women using rigorous analytical methods.[129] In normal women, insulin was negatively correlated to mean LH levels. In contrast, in women with PCOS multivariable modeling revealed that insulin had a suppressive effect on baseline LH and LH responses to GnRH dosing. These observations provided evidence for a role for insulin in modulating LH levels.

Increased LH pulse frequency and other features of polycystic ovary syndrome have been described in women with epileptic disorders or women treated with antiepileptic drugs.[130,131] The link between epileptic and postseizure ictal states may involve stimulation of excitatory neurotransmitters, the receptors of which exist in hypothalamic nuclei that influence GnRH release. Thus, the consequences of epileptic activity may result in increased GnRH activity and simulate the pattern of increased LH secretion in PCOS.[132] In addition to altered LH secretion, polycystic ovaries and

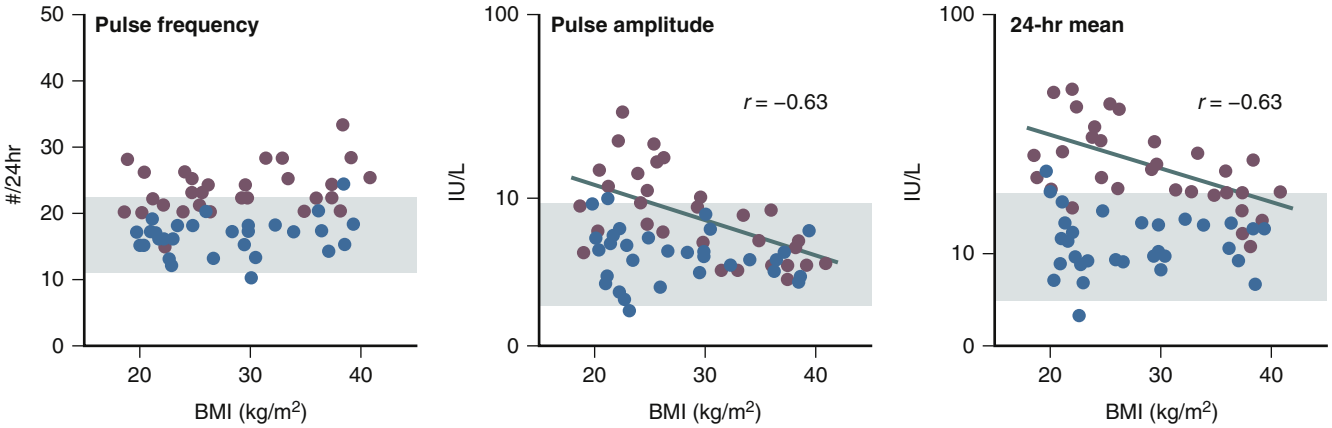


FIGURE 22.11 Regression of body mass index (BMI) for polycystic ovary syndrome (PCOS, purple dots) and normal control (NC, blue dots) women against luteinizing hormone (LH) pulse frequency (PCOS and NC, P = ns), LH pulse amplitude (PCOS: r = –0.63, P < .001; NC: P = ns), and 24-hour mean LH levels (PCOS: r = –0.63, P < .001; NC: P = ns). Values for LH pulse amplitude and 24-hour mean are log-transformed. Shaded area represents 95% confidence interval for NC (frequency, 11-22 pulses/24 hours; amplitude, 2.6-9.2 IU/L; 24-hour mean, 6.1-18.2 JU/L). *(Adapted from Arroyo A, Laughlin CA, Morales AJ, Yen SSC. Inappropriate gonadotropin secretion in polycystic ovary syndrome: influence of adiposity.* J Clin Endocrinol Metab *82:3728–3733, 1997.)*

hyperandrogenism have been reported in untreated and treated women with epileptic seizures, which strengthen the possible association between PCOS and epilepsy.[133] A mechanistic role for antiepileptic medication, including sodium valproate, in the development of excess androgen production or follicle cyst formation in treated subjects has not been elucidated.

In contrast to pituitary LH, FSH secretion in PCOS is decreased as indicated by significantly lower serum concentrations compared those found in normal women during the early follicular phase of the menstrual cycle.[107] In addition, FSH responses to GnRH stimulation are reduced as shown in some, but not all studies.[134,135] The precise underlying basis for decreased FSH secretion in PCOS has not been determined, although the negative feedback effect of chronic unopposed estrogen secretion in these women has been implicated as a mechanism.[117,136] The reduction in serum FSH may also reflect the activity of hypothalamic GnRH. As mentioned earlier, increased frequency of pulsatile GnRH predisposes to a preference for LHβ gene expression at the expense of the FSHβ gene.[110]

Theca Cell Function

The most notable clinical feature of PCOS is hirsutism, which is the result of excessive androgen production. Both the ovary and adrenal glands contribute to the pool of increased circulating androgens. However, in PCOS the major bioactive serum androgens, androstenedione and testosterone are produced by the ovary, whereas elevated concentrations of DHEAS are derived from the adrenal glands.[55] Within the polycystic ovary, numerous antral follicles are surrounded by hyperplastic theca cells which are the predominant site of androgen overproduction. There is firm evidence to indicate that excess ovarian androgen is driven by the abnormally increased secretion of pituitary LH acting on theca cells.[137] Nonetheless, the rather broad range of LH secretion in PCOS suggests that other mechanisms may be instrumental in the excessive production of androgens, including increased theca cell sensitivity to LH and the presence of cogonadotropic growth factors.

In vitro studies have established that theca cells from PCOS ovaries in culture produce significantly more androgen following LH stimulation than that generated by theca cells from normal ovaries.[138,139] In these studies the rate of androgen production in PCOS theca cells was greater than of normal cells but the magnitude of response was similar in both groups, reflecting higher basal production of androgen in PCOS cells. Clinically, stimulation of ovarian androgen production by a GnRH agonist revealed significant increases in serum 17-hydroxyprogesterone and androstenedione concentrations in PCOS compared to those of normal women.[140] Differences in testosterone responses between the two groups were not found. These results suggested an overexpression of *CYP17A1*, which encodes both 17-hydroxylase and C-17-20-lyase activities. Moreover, these findings suggested that differential androgen production in PCOS may arise *de novo* within the theca cell. To further examine this issue, steroid responses to hCG were examined in PCOS subjects and normal subjects prior to and following administration of a GnRH agonist, which effectively suppressed and eliminated any disparity in basal LH secretion and ovarian androgen production.[141] Serum 17-hydroxyprogesterone responses to hCG were not abrogated by agonist treatment which provided further evidence that in PCOS the magnitude of androgen responsiveness to LH may have been due to a primary abnormality of theca cell steroidogenesis.

Rosencrantz and colleagues performed a dose-response study in PCOS women and normal women to determine theca cell androgen production following intravenous administration of multiple doses of human chorionic gonadotropin (hCG).[142] In PCOS women, 17-hydroxyprogesterone responses to hCG increased in a dose dependent manner and were greater than those observed in normal women (Fig. 22.12). A shift in the response curve was not observed, which indicated greater theca cell responsiveness and not increased sensitivity to hCG. Notably, peak androstenedione and testosterone responses in PCOS women occurred

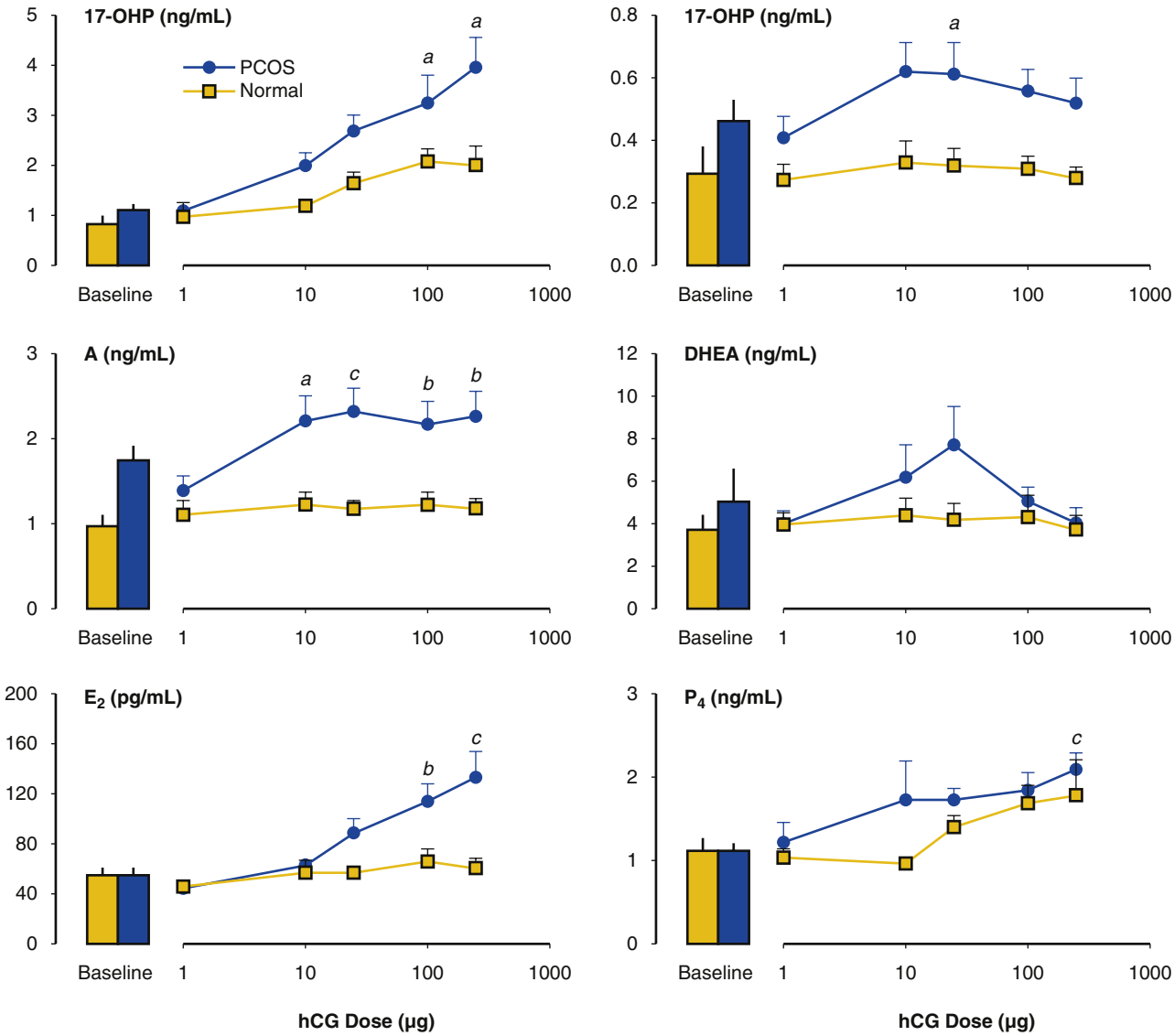


FIGURE 22.12 Mean (±standard error SE) baseline and 24-hour serum 17-OHP, A, T, DHEA, E_2, and P_4 responses to IV administration of 1, 10, 25, 100, and 250 μg of r-hCG in polycystic ovary syndrome (PCOS) and normal women. Using linear mixed-effect repeated measure model (MMRM) analyses, significant increases of 17-OHP, E_2, and P_4 were observed in both groups. By comparison, incremental A responses were significant only in PCOS women. Between groups, 17-OHP and A responses were significantly greater in PCOS women. Serum T responses were also higher compared to normal women, but the difference did not achieve statistical significance. As determined by MMRM, significant differences between groups in response to a fixed dose of human chorionic gonadotropin (hCG) are indicated by a ($P < 0.05$), b ($P < 0.01$), and c ($P < 0.001$). *(Adapted from Rosencrantz MA, Coffler MS, Haggan A, et al. Clinical evidence for predominance of delta-5 steroid production in women with polycystic ovary syndrome,* J Clin Endocrinol Metab *96:1106–1113, 2011.)*

at a low dose of hCG whereas maximal 17-hydroxyprogesterone responses were observed at higher doses of hCG. In contrast, hCG-stimulated responses of androstenedione and testosterone in normal women were not observed. These results suggested exquisite theca cell responsiveness to hCG in PCOS women compared to minimally stimulated androgen production in normal women. Previous clinical studies had suggested a lack of 17, 20-lyase activity based on GnRH-stimulated rises of androstenedione that were relatively less than that of 17-hydroxyprogesterone.[143] However, it was later shown in PCOS theca cells that radiolabeled pregnenolone was more rapidly metabolized to 17-hydroxyprogesterone, pregnenolone and DHEA and, eventually, to androstenedione compared to normal cells.[144] This increased steroid output was accompanied by greater enzymatic activities of cholesterol side-chain cleavage enzyme (CYP11A1), 3-beta, hydroxysteroid dehydrogenase (3β-HSD), and 17β-HSD, reflecting an overall upregulation of ovarian steroid production. Inefficiency of delta-4 17, 20-lyase activity may explain, at least in part, the discordant dose-responses of 17-hydroxyprogesterone compared to those exhibited by androstenedione and testosterone in our PCOS women. The accumulation of androstenedione and testosterone with minimal 17-hydroxyprogesterone production at a low dose of hCG is compatible with hyperactivity of the delta-5 steroid pathway combined with increased 3β-HSD enzyme activity, a stable property of the PCOS theca cell.[144] At higher doses of hCG, incremental changes

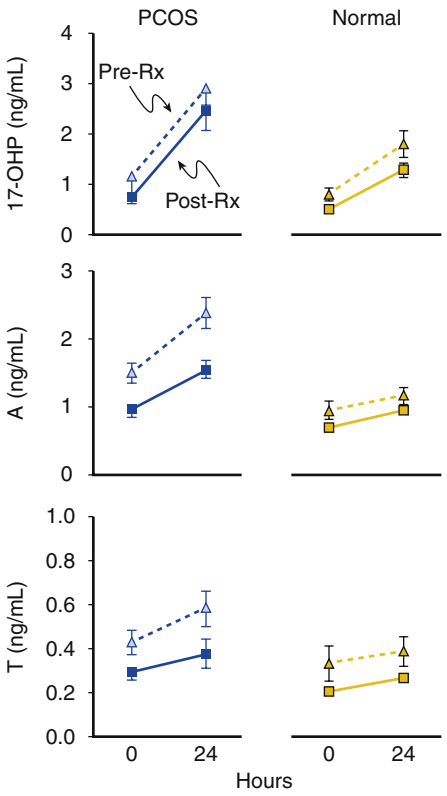


FIGURE 22.13 Mean (±SE) serum androgen levels following administration of hCG, 25 μg IV, prior to and after treatment with gonadotropin releasing hormone (GnRH) antagonist in women with polycystic ovary syndrome (PCOS) and normal controls. The rise from baseline values is significant ($p < 0.05$) for all pretreatment and posttreatment responses in both groups except for the T response in normal women before GnRH antagonist *(Rosencrantz MA, unpublished observation).*

in 17-hydroxyprogesterone responsiveness likely arose from combined over-expression of both 17-hydroxylase and 3β-HSD enzymes.

It is clear that LH is necessary to sustain circulating androgen levels. However, the extent to which endogenous LH contributes to theca cell androgen production is not completely obvious. To address this issue, we examined theca cell response to intravenous hCG stimulation in PCOS and normal women prior to and 2 days following administration of a GnRH antagonist. In both groups, antagonist treatment significantly lowered LH and moderately reduced FSH which was accompanied by decreased basal androgen levels (Fig. 22.13). Corresponding androgen responses to hCG were also lower in each group. However, the incremental fold-change for both PCOS women and normal women was not different from responses observed before agonist treatment. Our results extend the findings of other previously reported studies.[141,145] Collectively, these results suggest that endogenous gonadotropin secretion contributes to basal androgen secretion and, even in the presence of lowered gonadotropin levels, maintains ovarian androgen synthesis and responsiveness to gonadotropin stimulation. This is consistent with primary theca cell dysfunction leading to excessive ovarian androgen production in women with PCOS. Collectively, these results suggest that endogenous gonadotropin secretion contributes to basal androgen secretion and, even in the presence of lowered gonadotropin levels, maintains ovarian androgen synthesis and responsiveness to gonadotropin stimulation. This is consistent with primary theca cell dysfunction leading to excessive ovarian androgen production in women with PCOS.

Exclusive of the direct stimulus-response dynamic of ovarian androgen production, the human theca cell is also subject to the effects of co-gonadotropic growth factors, the most notable of which are insulin and insulin-like growth factors (IGF). Receptors for insulin, IGF-I and IGF-II have been localized to the theca compartment of ovaries from both normal women and PCOS patients.[146] Accordingly, *in vitro* studies of normal human theca tissue have demonstrated that these growth factors are capable of enhancing androgen responses to LH as well as independently stimulating androgen production themselves.[138,147-149] By comparison, studies performed on theca cells from hyperandrogenic women including PCOS have not demonstrated a synergistic effect between LH and insulin or IGF-I.[149] Nevertheless, the observation that reduction of hyperinsulinemia has been associated with significant decreases of serum androgens without corresponding changes in LH in women with PCOS treated with insulin-lowering drugs indirectly suggests a role for insulin in LH stimulated androgen synthesis.

Granulosa Cell Function

In PCOS, the mechanism(s) responsible for the persistence of ovulatory failure are not well understood. Earlier studies had implicated a deficiency of aromatase activity to explain the lack of follicle development as estradiol concentrations were low in follicular fluid and granulosa cells contained little measurable aromatase enzyme.[150] However, subsequent *in vitro* studies have demonstrated that these cells are, indeed, vital and exhibit substantial capacity for steroidogenesis.[13,30,151] We have previously shown that cultured granulosa cells obtained from women with PCOS exhibit a significantly greater rise of estradiol following FSH stimulation compared to that observed in normal granulosa cells, which indicated that the inherent capacity of these cells to respond to FSH was ample.[151] Clinical studies have corroborated these *in vitro* findings. In women with PCOS the capacity for estradiol production in response to the stimulatory effects of FSH was clearly enhanced compared to that of normal women.[152] Notably, this difference was dose-dependent and only manifested at 150 IU, as increases in circulating estradiol after 37.5 and 75 IU of FSH were similar in both PCOS and normal women (Fig. 22.14). The amplification of estradiol responses beyond an apparent FSH threshold dose range indicated greater *in vivo* granulosa cell responsiveness in PCOS in the presence of an abundance of aromatase substrate as profiles of circulating FSH levels at each dose were essentially identical in both groups. This heightened estradiol response may have reflected greater follicle number in the PCOS ovary compared to that of the normal ovary.[32] This explanation is consistent with the report in which comparative estradiol responses to FSH in PCOS and normal women during ovulation induction were more likely related to the number of stimulated ovarian follicles rather than differences in the FSH threshold.[74]

While these results may reflect increased numbers of stimulated follicles or increased granulosa cell sensitivity

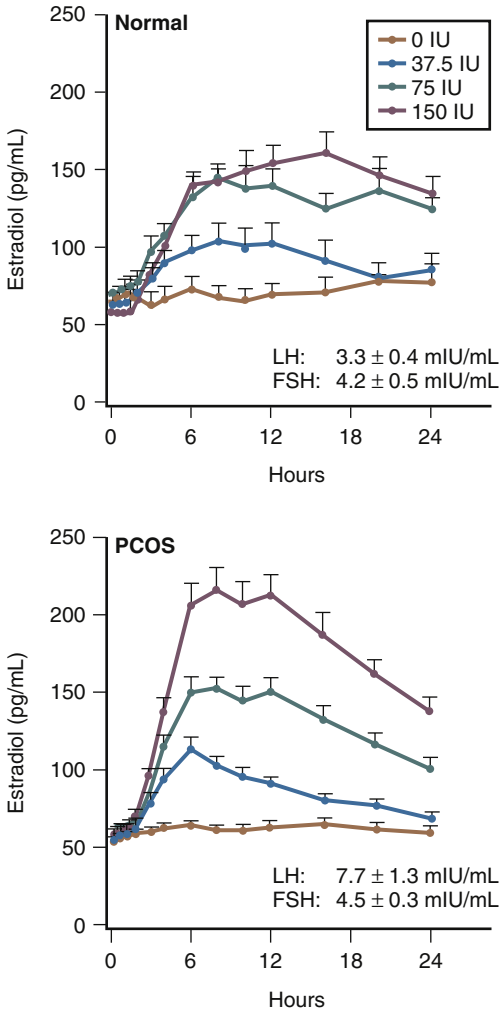


FIGURE 22.14 Mean (±SE) serum estradiol levels following intravenous administration of the indicated doses of recombinant follicle-stimulating hormone (rFSH) to subjects with polycystic ovary syndrome (n = 16) and normal controls (n = 7) at t = 0 hours. The 0-IU dose of rFSH is saline control. Mean (±SE) baseline levels of serum LH and FSH are also shown. *(From Coffler MS, Patel KS, Dahan MH, et al. Evidence of abnormal granulosa cell responsiveness to follicle-stimulating hormone in women with polycystic ovary syndrome,* J Clin Endocrinol Metab *88:1742–1747, 2003.)*

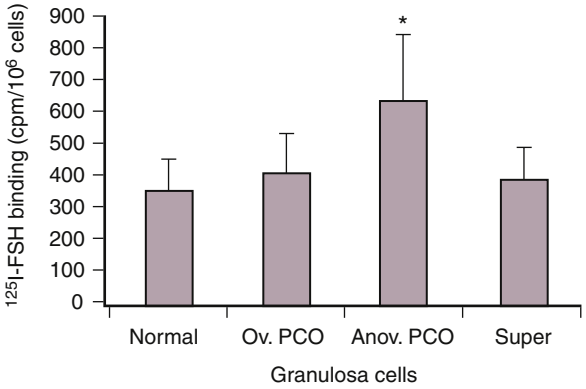


FIGURE 22.15 Binding of human recombinant ^{125}I-follide stimulating hormone to granulosa cells from different groups of patients. Follicle stimulating hormone binding per cell number was significantly (*P < .05) increased in anovulatory polycystic ovary syndrome. The values represent the mean ± standard deviation SD obtained from different cellular preparations, each assayed at least twice in triplicate. *(From Almahbobi C, Anderiesz C, Hutchinson P, et al. Functional integrity of granulosa cells from poly-cystic ovaries,* Clin Endocrinol *44:571–580, 1996.)*

to FSH or both, it is evident that women with PCOS are susceptible to ovarian hyperstimulation in response to gonadotropin stimulation. Whether increased granulosa cell sensitivity may arise primarily or secondarily as a result of intraovarian or extra ovarian factors is unknown. Studies have shown that in granulosa cells obtained by aspiration of follicles from unstimulated anovulatory PCOS ovaries, binding of radio-labeled FSH was significantly higher compared to that detected in cells from ovaries of ovulatory PCOS or normal women (Fig. 22.15).[30] These findings link granulosa cell sensitivity to increased FSH receptor binding in granulosa cells from PCOS ovaries and provide insight into a possible mechanism for increased estradiol responsiveness to FSH stimulation.

Insulin and insulin-like growth factors appear to act as co-gonadotropins within the ovary. Previous studies have shown that these peptides significantly amplify steroid production in PCOS granulosa cells.[153-155] In addition, this facilitory action of insulin was likely mediated through its own receptor.[156] Efforts to clinically demonstrate a role for insulin on granulosa cell function in women with PCOS have been limited. Following treatment with insulin lowering drugs, PCOS women have been shown to improve insulin sensitivity and resume ovulation, which indirectly links insulin resistance to chronic anovulation in this disorder.[64-67] In studies that evaluated granulosa cell function, measurement of serum estradiol levels did not reveal any particular pattern of response in women who became ovulatory or remained anovulatory. In women undergoing weight reduction, initiation of regular menses was associated with improved insulin sensitivity as reflected by increased SHBG.[126] In these studies, because the rate of ovulation improved during treatment, it was unclear whether an alteration of estradiol levels was a direct result of lowered circulating insulin levels or secondary to restoration of ovarian steroidogenesis. Insulin-lowering drugs, such as thiazolidinediones and metformin, have been shown to exert direct effect on ovarian steroid enzymes.[157-159] The potential effect of hyperinsulinemia on granulosa cell function has been examined by assessing estradiol responses to FSH stimulation.[160] In women with PCOS treated with a thiazolidinedione, pioglitazone, for 5 months, estradiol production following FSH during insulin infusion was significantly increased compared to responses observed prior to treatment (Fig. 22.16). Amplification of the estradiol response was associated with an improvement in insulin sensitivity that suggested in PCOS women granulosa cells may be insulin resistant. Moreover, these findings may account for the apparent paradoxical effect of insulin on granulosa cell function in that, *in vitro*, insulin enhances estradiol production whereas, *in vivo*, treatment with insulin-lowering drugs induces ovulation. Whether granulosa cells in PCOS women may be insulin resistant on a primary basis or secondarily has not been addressed.

That PCOS granulosa cells may be insulin resistant, could, at least in part, account for decreased estradiol responsiveness to FSH observed in PCOS women undergoing

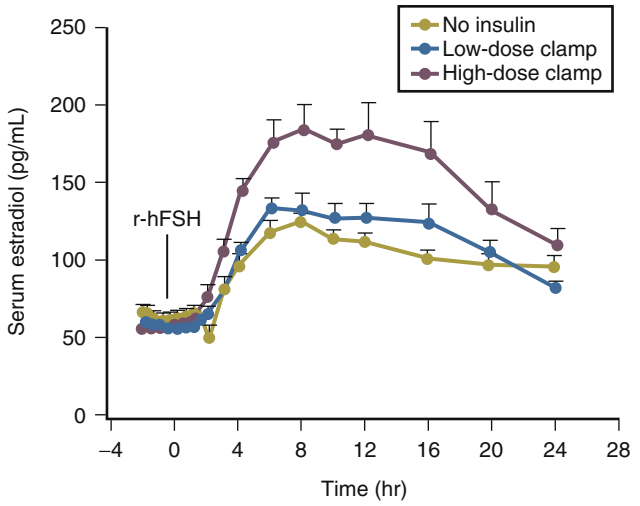


FIGURE 22.16 Time-course of mean (± standard error SE) 24-hour serum estradiol responses following injection of intravenous recombinant follicle stimulating hormone, 75 IU, to women with polycystic ovary syndrome treated with pioglitazone without insulin infusion and 2 hours following initiation of low-dose (30 μ/min/m^2) and high-dose (200 mU/min/m^2) hyperinsulinemic-euglycemic clamps administered for 10 hours. The integrated estradiol response as determined by area under the curve was significantly greater ($P < .02$) in subjects receiving high-dose insulin infusion compared with those observed for women without insulin or with low-dose insulin infusion. *(Adapted from Coffler MS, Patel KS, Dahan MH, et al. Enhanced granulosa cell responsiveness to follicle stimulating hormone during insulin infusion in women with polycystic ovary syndrome treated with pioglitazone,* J Clin Endocrinol Metab *88:5624–5631, 2003.)*

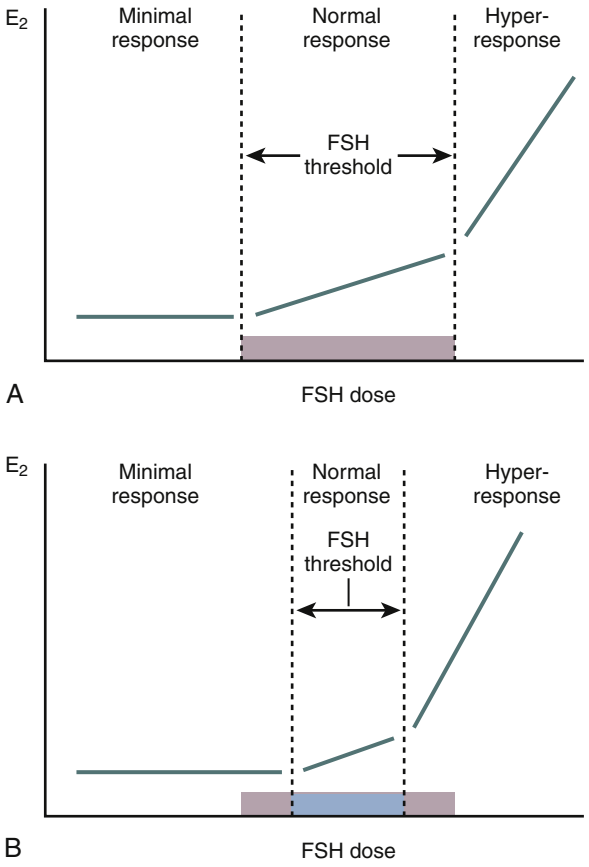


FIGURE 22.17 Conceptualized model of granulosa cell responses to a range of follicle stimulating hormone (FSH) doses administered during ovulation induction in normal women (**A**) and women with polycystic ovary syndrome (PCOS, **B**). Note the narrowed FSH threshold in women with PCOS, which may explain the minimal response to initial gonadotropin stimulation as well as the comparative hyperresponsiveness at higher threshold doses.

controlled ovarian hyperstimulation.[107,161] Clinical experience has indicated that this reduced follicle responsiveness may be overcome with progressive increases in the dose or duration of FSH. Protracted administration of low-dose FSH has been particularly useful as the risk of ovarian hyperstimulation syndrome is considerably diminished with this method of ovulation induction.[162] The gradual accumulation of constant low-dose FSH over time is preferred to an escalation of the daily dose because of follicular hyperresponsiveness to FSH in PCOS women and an increased risk of ovarian hyperstimulation syndrome.[163] These observations combined with our previous report describing greater estradiol responsiveness in PCOS women compared to normal women at a 150 IU dose of FSH suggests a bidirectional narrowing of the FSH threshold range in this disorder (Fig. 22.17).

In the past it appeared that the effect of insulin on FSH stimulated follicle development may have involved the IGF system as both IGF-I and IGF-II were shown to enhance granulosa cell responses to FSH.[151,164] In PCOS granulosa cells, exposure to both IGF-I and FSH resulted in significantly greater estradiol production compared to incubation with each hormone separately. The actions of IGFs are likely mediated by their receptors which have been identified on granulosa cells of both PCOS and normal follicles.[146] However, despite *in vitro* studies in which IGF-I clearly amplified FSH induced aromatase activity, most studies have failed to detect or shown very little of the protein in human granulosa cells.[146,165,166] By comparison, IGF-II mRNA message has been located in all compartments of the human ovary and is strongly expressed in granulosa cells.[146,165] Consistent with this observation are recent studies of human PCOS ovarian tissues which demonstrated that estrogen responses to IGF-II were significantly enhanced in granulosa cells preincubated with insulin than without insulin.[13] These findings point out the potentially critical relationship between insulin and IGF-II in the regulation of granulosa cells function. In particular, in PCOS excess insulin secretion may down regulate insulin receptors, which would prevent translocation of subcellular IGF-II receptors to the plasma membrane and deprive granulosa cells of the stimulatory effect of a potent cogonadotropin.[167,168]

IGFs are complexed with IGF binding proteins (IGFBPs) that regulate their bioactivity. In PCOS serum, IGFBP-1 has been shown to be decreased as a result of hyperinsulinemia which implies an increase in the levels of free IGF-I. This notion is supported by the demonstration of increased circulating levels of free IGF-I in PCOS women.[169] Additionally, IGFBPs may be critical in regulating IGF bioactivity at the level of the ovary. IGFBP-2 and -4 have been shown to be increased in follicle fluid derived from androgenic follicles including those of PCOS patients.[170,171] IGFBP-4 protease was not detectable in these follicles.[172] These results are in stark distinction to those found in healthy antral follicles of

normal women in which IGFBP-2 and -4 were decreased and IGF-II concentrations were increased in the follicular fluid.[173] The impact of the androgenic environment on follicular fluid IGF bioavailability has been documented in transsexuals treated with high-dose androgens. In the follicular fluid of these individuals, IGF-II levels were decreased, IGFBPs increased, and IGFBP-4 protease was non-detectable, thus accounting for decreased bioavilable IGF-II.[174] Whether this IGF-IGFBP profile is a consequence of follicular fluid androgen levels is not clear although the association is compelling and suggests that androgen excess in PCOS, driven in part by insulin mediated theca cell androgen production, may be instrumental.

The potential role of the oocyte in abnormal follicle growth in women with PCOS has not been investigated. Studies have demonstrated that oocyte-derived growth factors may be important to follicle development and function. In particular, growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15) appear to have major roles on folliculogenesis and female fertility.[175] *In vitro* studies have shown that these genes are selectively expressed in developing oocytes during folliculogenesis.[176,177] In addition, in GDF-9 deficient female mice, disruption of reproductive function has been associated with arrested follicle growth at the primary stage, decreased granulosa cell proliferation, inappropriate theca cell development, ovarian cyst formation, and infertility.[178,179] We have examined mRNA expression of GDF-9 and BMP-15 in ovaries obtained from women with PCOS and found that the GDF-9 signal was decreased in oocytes throughout folliculogenesis compared to GDF-9 message in oocytes from normal ovaries.[180] By comparison there was no difference in BMP-15 mRNA expression between groups. These results suggest that the expression of GDF-9 is delayed in oocytes of developing PCOS follicles. Because of the apparent growing importance of GDF-9 in folliculogenesis and fertility, it is suggested that dysregulation of GDF-9 expression may contribute to aberrant folliculogenesis in PCOS women.

Intrafollicular Paracrine Interaction

In 2008, Wachs and coworkers reported that in PCOS women, intravenous administration of FSH resulted in significant increases in serum 17-hydroxyprogesterone, androstenedione, and DHEA levels (Fig. 22.18).[181] By comparison, androgen levels in normal women remained unchanged after FSH stimulation. In PCOS women, serum estradiol responses were also greater than those of the normal group. These findings suggested that theca cell androgen production in women with PCOS may be, in part, regulated by granulosa cells. Both *in vitro* and *in vivo* studies conducted in animals have indicated that FSH may amplify LH-induced ovarian androgen production.[182,183] In cultured human theca cells, androstenedione responses to LH in the presence of inhibin were clearly increased compared to those without inhibin.[184,185] In addition, inhibin was able to negate the inhibitory effect of activin on human theca cell androgen production. Accordingly, in our study the significant increases in ovarian androgens exhibited by PCOS women were accompanied by similar significant increments in FSH-stimulated inhibin B levels compared to those of normal women (Fig. 22.19). A direct effect of inhibin B on theca cell androgen production is unlikely as a receptor for this protein has not been identified. Rather, it has been shown that inhibin B binds to membrane bound binding protein, β-glycan, to form a complex that has high affinity for activin type II receptors.[186] The sequestration of activin type II receptors prevents formation of the activin type II

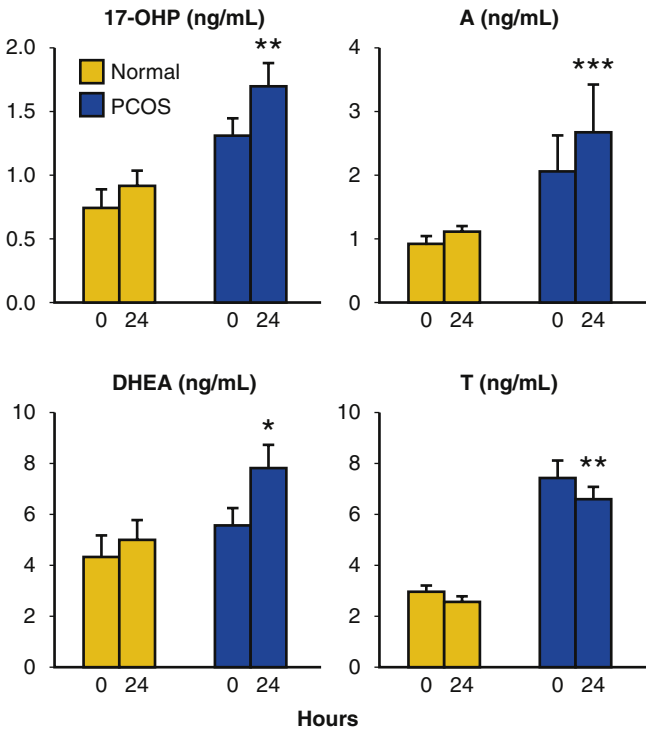


FIGURE 22.18 Mean (± SE) serum androgen levels before and 24 hours after administration of r-hFSH (150 IU) in PCOS and normal women. A significant change from baseline is denoted by an asterisk. *, P < 0.05. **, P < 0.02. ***, P < 0.001. *(Adapted from Wachs DS, Coffler MS, Malcom PJ, et al. Increased androgen response to follicle-stimulating hormone administration in women with polycystic ovary syndrome,* J Clin Endocrinol Metab *93:1827–1833, 2008.)*

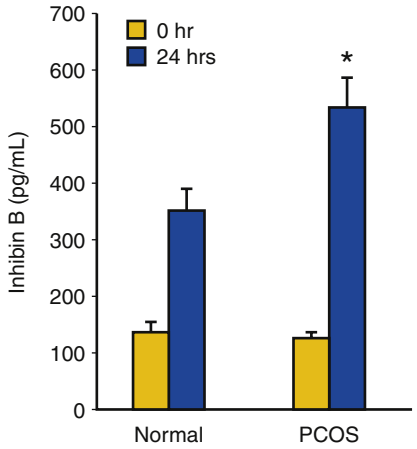


FIGURE 22.19 Mean (± SE) serum Inh B levels before and 24 h after administration of r-hFSH (150 IU) in PCOS and normal women. *, P value for between-group comparison less than 0.001. *(Adapted from Wachs DS, Coffler MS, Malcom PJ, et al. Comparison of follicle-stimulating-hormone-stimulated dimeric inhibin and estradiol responses as indicators of granulosa cell function in PCOS and normal women,* J Clin Endocrinol Metab *91:2920–2925, 2006.)*

or type I receptor complex required for activin signaling and eventual inhibition of CYP17A1 within the theca cell.

Another granulosa cell-derived growth factor, kit ligand, has also been shown to stimulate theca cell androgen production. In cultured bovine theca cells, kit ligand significantly increased androstenedione synthesis in the absence of gonadotropins.[187] Moreover, kit ligand is stimulated by FSH and its receptor, c-kit, is increased by LH which potentially creates a vigorous forward feedback loop for androgen production.[188,189] Together, these findings provide a possible mechanism for FSH-stimulated androgen production in PCOS women. Consideration of an intrafollicular paracrine model of androgen production challenges the long-held two-cell, two-gonadotropin concept of ovarian steroidogenesis. However, documentation of this interaction in human ovarian tissue and whether inhibin or activin signaling and kit ligand or c-kit interaction is altered in theca cells of PCOS women has not been studied.

Adrenal Function

The role and significance of increased adrenal androgen production in women with PCOS continues to be vague. Previous studies have not consistently shown that excess adrenal androgen contributes primarily to increased T and A4 production in PCOS women despite increased serum DHEAS in 20% to 30% of cases.[55,190,191] This suggests that the mechanism for adrenal hyperandrogenemia may arise from either altered adrenal responsiveness to ACTH or abnormal adrenal stimulation by factor(s) other than ACTH. Studies to address this issue have produced varied results. Increased 17-hydroxyprogesterone responses to ACTH, following dexamethasone, have been observed in women with PCOS and functional ovarian hyperandrogenism, which suggested dysregulation of P450c17.[192,193] However, other studies have not been able to confirm these results.[194,195] These latter studies did not employ dexamethasone suppression prior to ACTH stimulation, which may have accounted for the disparate results. Alternatively, separation of PCOS women on the basis of 17-OHP responses to GnRH agonist revealed that basal androgen levels of high responders were minimally affected by dexamethasone while the percent inhibition in normal responders was equivalent to that of normal women.[196] This suggested that adrenal androgen production may contribute significantly to hyperandrogenemia in the normal responder group although studies that address this notion have not been performed. In PCOS women, *in vitro* and *in vivo* studies have shown that serum 17-hydroxyprogesterone and androstenedione responses to ACTH were significantly greater in the presence of hyperinsulinemia compared to those in the absence of elevated insulin levels.[197,198] In addition, it has been demonstrated that in individuals with PCOS, ACTH administration during insulin infusion was associated with a significantly higher 17-hydroxypregnenolone and 17-hydroxyprogesterone responses than those measured during saline infusion.[199] This facilitory effect of insulin appeared to result in a relative lowering of 17-20 lyase activity as indicated by higher 17-hydroxypregnenolone to DHEA and 17-hydroxyprogesterone to androstenedione ratios. These findings are consistent with reports that demonstrate a reduction in serum DHEA-S in women during administration of insulin infusion or a glucose tolerance test.[124,200] Whether adrenal hyperandrogenemia is contributory to the perpetuation of PCOS is not clear. However, that an exaggerated adrenarche may be an inciting event in the pathogenesis of PCOS warrants further investigation of adrenal gland function in adolescent girls with hyperandrogenism.

Pancreatic β-Cell Function and Insulin Receptor Binding

In women with PCOS, severe peripheral insulin resistance is accompanied by compensatory hyperinsulinemia. That insulin responses are exaggerated in women with PCOS following a glucose load belies a distinct abnormality of beta cell function in this disorder. Early studies had shown that in PCOS first-phase insulin secretion was equivalent to that of normal women.[201] However, first-phase insulin release is relevant to the magnitude of ambient insulin resistance in each individual. Consequently, the product of these measures can be calculated (disposition index) and related to the hyperbolic relationship of these measures established in normal women.[202] Employing this technique, it has been reported that the disposition index was significantly decreased in both obese and nonobese PCOS women compared to BMI matched normal controls (Fig. 22.20).[203] In addition, in a subset of PCOS women with a family history of diabetes the disposition index as quantitated by the

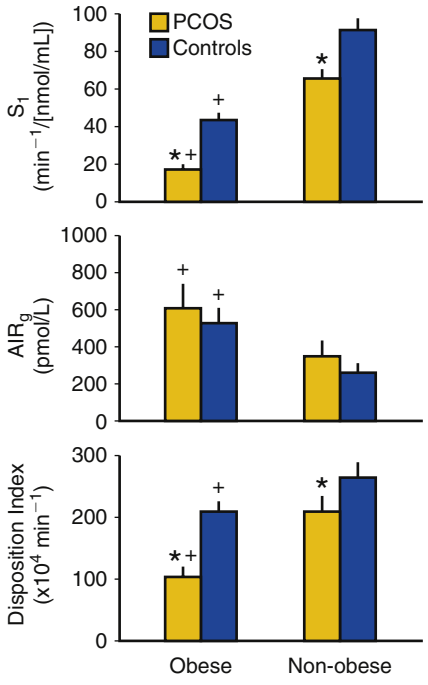


FIGURE 22.20 Insulin sensitivity index (S1) acute insulin response (AIRg), and disposition index in obese and nonobese PCOS and control women. *, Significantly different in PCOS compared to normal women; +, significantly different in obese compared to nonobese women. The S_1 is decreased in PCOS and obese women (both $P \leq 0.001$). The AIR_g is significantly increased in obese women ($P \leq 0.005$), and the disposition index (S_1 X AIR_g) is significantly decreased in PCOS and obese women (both $P \leq 0.005$). *(Modified from Dunaif A, Finegood DT. Beta-cell dysfunction independent of obesity and glucose intolerance in the polycystic ovary syndrome,* J Clin Endocrinol Metab *81:942–947, 1996.)*

insulin secretory response to graded doses of insulin infusion was in the eighth percentile whereas in those without a similar family history, the index was in the 33rd percentile.[204] Thus, women with PCOS and a family history of type 2 diabetes exhibit impaired insulin responses compared to those without a family history, particularly when expressed in relation to the degree of insulin resistance.

In PCOS, insulin receptor binding and affinity have been shown to be normal. However, studies of adipocytes from PCOS women have revealed decreases in glucose transport and lipolysis indicating an impairment of insulin signaling (Fig. 22.18).[205,206] Evidence for decreased tyrosine phosphorylation and increased insulin-independent serine phosphorylation has been demonstrated in cultured skin fibroblasts removed from women with PCOS.[207] Serine phosphorylation inhibits tyrosine kinase activity of the insulin receptor and may be responsible for the observed insulin resistance in PCOS.[208,209] Notably, increased serine phosphorylation was observed in only 50% of PCOS women studied as the remainder exhibited insulin-stimulated insulin receptor autophosphorylation which was similar to that found in normal control women.[207] Thus, these findings may be applicable to a subset of women with PCOS, whereas in others, the mechanism still remains unknown. Importantly, while a defect in insulin receptor phosphorylation may exist for some PCOS women, the potential for abnormalities beyond the receptor is suggested by apparent alterations in other downstream signaling events.[210] Moreover, different pathways may be affected to greater or lesser degrees in various insulin-sensitive tissues which reflect the complexity of the mechanism of insulin resistance in this disorder.

Genetics of PCOS

The familial predisposition of PCOS implies a disorder of inheritance, although the mode of transmission has not been established despite a number of studies. Much of the difficulty resides with methodological limitations due to small numbers of families and the inability to establish phenotypes among family members. The latter reflects the variable criteria used to designate individuals with PCOS. Studies of twin sisters have not demonstrated a substantial genetic component for polycystic ovaries, although concordance was found in affected twins relative to biochemical markers including fasting insulin levels and serum androgen concentrations.[211-213] Similarly, efforts to detect chromosomal abnormalities among PCOS subjects have not revealed alterations in number or structure.[214-216] In pursuit of the genetics of PCOS, most studies to date have focused on identifying candidate genes that are linked to recognized abnormalities of steroid hormone production and action, carbohydrate and fuel metabolism, and gonadotropin secretion. To date, only a few genes have emerged that may be reasonably related to PCOS.

Of the genes involved in steroidogenesis, there is evidence to suggest that allelic variants of *CYP11A1* may have a potential role in excess androgen production and hirsutism in PCOS.[217-220] Gharani and colleagues demonstrated association between an allele of *D15S520* that is located in the promoter region of *CYP11A1* and PCOS.[217] However, these findings were not replicated in other studies.[219,220] The androgen receptor (AR) gene persists as a possible candidate for PCOS in light of the inverse relationship between the number of trinulceotide (CAG) repeats and androgen action.[221] The AR gene is X-linked and in women one copy of the X chromosome is inactivated. Thus, the manner in which X-inactivation occurs may influence AR bioactivity. This was suggested in a study by Hickey and colleagues which showed sister pairs with the same AR CAG repeats genotype were more often associated with similar patterns of X-inactivation and like phenotypes compared to sister pairs with the same genotype, but different X-inactivation and varying clinical phenotypes.[222]

Given the predisposition of PCOS women to insulin resistance, genes that are related to carbohydrate metabolism have been the subject of considerable investigation. Of particular note is the insulin receptor gene, a mutation of which leads to severe insulin resistance, marked hyperandrogenism, and acanthosis nigricans (type A syndrome of insulin resistance). Two studies have identified the region near the insulin receptor gene locus as being associated with PCOS.[219,223] In contrast, abnormalities in the tyrosine kinase domain of the insulin receptor gene in PCOS women were not detected.[224] Additionally, molecular analysis of the coding region of the insulin receptor gene conducted on the deoxyribonucleic acid (DNA) of women with PCOS failed to detect any missense or nonsense mutations suggesting that mutational defects on the insulin receptor gene are highly unlikely.[225] As for the insulin gene or *VNTR*, there are conflicting reports linking this marker to PCOS.[219,226,227] As a regulator of transcription, *VNTR* is related to hyperinsulinemia, risk of type 2 diabetes, and development of childhood obesity.[228] Several studies have shown a modest association between *VNTR* and women with PCOS.[226,229] However, other investigators have been unable to demonstrate evidence of a link between PCOS and the insulin gene.[219,230,231] Collectively, these findings suggest that involvement of this gene locus with PCOS is uncertain.

It has been shown that variation in the gene encoding the cysteine protease, calpain-10, influences susceptibility to type 2 diabetes.[232,233] However, two subsequent studies in women with PCOS of African-American and European descent were unable to establish associations with multiple DNA polymorphisms in the calpain-10 gene.[234,235] Nevertheless, the 112/121 haploid combination in African-Americans with PCOS was found to be significantly associated with increased circulating insulin levels in response to a glucose challenge. Another potential susceptibility locus was identified from a screen of 37 PCOS candidate genes that found evidence for a strong association of an allele of *D19S884* on chromosome 19p13.2.[219] This association was strengthened by a similar outcome in a subsequent study by the same investigators involving a larger number of families.[236] While the *D19S884* allele may be a distal enhancer of the insulin receptor gene, its functional role has not yet been determined. Recently, other susceptibility variants in the gene for transcription factor-7 like-2, which is a significant risk factor for type 2 diabetes, were associated a PCOS cohort. In addition, these alleles were linked with β-cell dysfunction in PCOS women with impaired glucose tolerance.[237,238]

All these studies reflect efforts to assign a single gene marker to PCOS. However, the phenotype of this syndrome

has multiple clinical alterations that imply a highly complex genetic disorder. Thus, it was anticipated that genome wide association studies (GWAS) would provide a more comprehensive approach with fruitful outcomes. Indeed, in a large GWAS conducted in Han Chinese involving 744 PCOS cases and 895 controls in the discovery set and 3338 PCOS and 5792 controls in the replication set, there was evidence of strong associations between three PCOS susceptible loci on chromosomes 2p16.3 (luteinizing hormone-chorionic gonadotropin receptor *[LHCGR]*), 2p21 (thyroid adenoma associated protein *[THADA]*), and 9q33.3 (DENN-MADD domain containing 1A *[DENND1A]*).[239] In a subsequent large study of women with PCOS of European descent, similar findings emerged for *DENND1A* and *THADA*, which gave credence to the previous GWAS study.[240] Almost simultaneously, a third study largely confirmed the association of *DENND1A* susceptibility loci in a cohort of PCOS women from Iceland and the United States.[241] These findings warrant further investigation, although currently it appears that *DENND1A* and, possibly, *THADA* confer increased risk for PCOS.

Pathophysiological Concept

A clear explanation of the pathogenesis for PCOS has not been established. Several concepts have emerged that await confirmation. A growing body of evidence suggests that excessive androgen production may be essential in the evolution of this disorder (Fig. 22.21). Notably, it has been demonstrated that administration of androgen to nonhuman primates alters ovarian morphology by increasing the size of the ovary, thickness of the capsule, and number of preantral and antral follicles.[37] These data are consistent with the polycystic ovaries of hyperandrogenic women with 21-hydroxylase deficiency and female to male transsexuals administered long-term androgen therapy.[154,165-168,170,171] The mechanism that dictates these morphological changes has not been elucidated, although in rhesus monkeys, androgen receptor mRNA is colocalized with that of the FSH receptor in granulosa cells and androgen treatment increases the expression of FSH message.[39] The presence of increased FSH binding in granulosa cells of antral follicles from women with PCOS compared to binding in normal follicles suggests that local exposure of excess androgen may drive this process and account for increased follicle number and size in this disorder.[30]

An androgen-induced increase of FSH receptors on granulosa cells of PCOS women may also explain the robust follicular response to FSH observed *in vitro* and relate to heightened granulosa cell responsiveness to gonadotropin stimulation.[29,151,152] Similar to studies in nonhuman primates, women with PCOS treated with androgen exhibited intense nuclear staining for androgen receptor in granulosa cells.[172] In rat granulosa cell culture, studies have shown that testosterone acts at a site upstream from cyclic adenosine monophosphate c-AMP, which suggests involvement of the FSH receptor, whereas other studies have been unable to determine whether changes in c-AMP are induced by androgen treatment.[141,147,148] Despite these inconsistent findings, it is likely that the effect of androgen on the FSH receptor and subsequent granulosa cell function is mediated through its receptor. However, the precise mechanism for this interaction remains to be defined. An

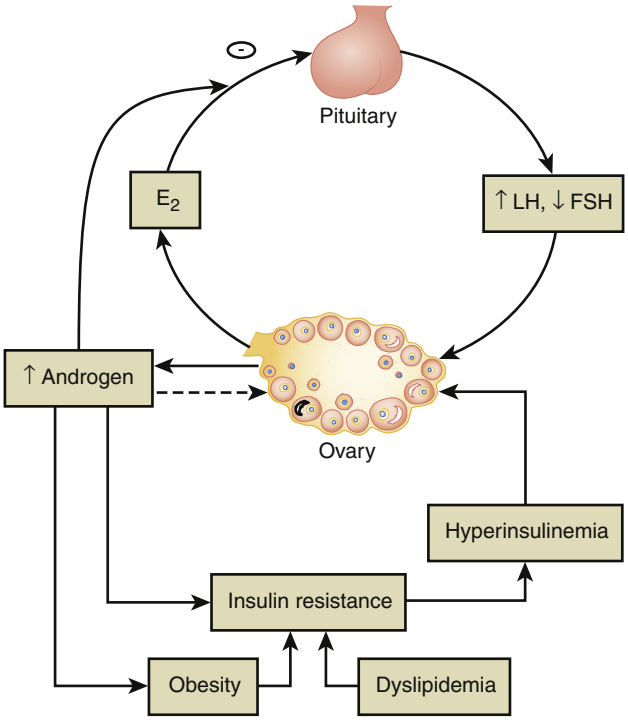


FIGURE 22.21 Pathophysiological concept of polycystic ovary syndrome (PCOS): Increased luteinizing hormone (LH) secretion together with enhanced theca cell responsiveness drives the production of excess ovarian androgen. Increased androgen production may inhibit steroid negative feedback effects on hypothalamic gonadotropin-releasing hormone pulse generation to account for the rapid LH pulse frequency observed in PCOS women. In addition, increased androgens are associated with android obesity, visceral fat deposition, and dyslipidemia, all of which may contribute to insulin resistance. Independently, hyperandrogenemia, obesity, and hyperinsulinemia may decrease sex hormone-binding globulin, thereby increasing bioactive testosterone. Finally, increased androgen may have direct effects on the ovary to increase follicle number, follicle size, and, possibly, enhance granulosa cell responsiveness to follicle stimulating hormone.

amplification of granulosa cell responsiveness to FSH is not unique to androgens as estrogens have long been known to enhance FSH-stimulated estradiol production.[242] In rodent granulosa cells, synergy between estradiol (E_2) and FSH has been demonstrated with regards to increased FSH receptor binding, induction of LH receptors, increased aromatase activity, and progestin synthesis.[243-245] The mechanism for this synergy has not been completely elucidated, although estrogen-induced granulosa cell proliferation or increased FSH-binding capacity per granulosa cell have been suggested.[244,246,247] Alternatively, both processes may serve to amplify FSH receptor number in granulosa cells of developing follicles. Thus, these findings strongly suggest that in normal and PCOS granulosa cells, estrogen as well as androgen may enhance FSH-stimulated estradiol release through a mechanism that involves the FSH receptor.

Increased androgen production may have a significant impact on inappropriate gonadotropin secretion in PCOS. The critical feature of LH secretion in PCOS is increased LH pulse frequency, which remains intact in the face of physiological and most pharmacological manipulations. Until recently, it was difficult to exclude altered GnRH

release as an instigating factor in PCOS. However, that the rate of rapid LH release in PCOS women may be normalized with administration of physiological doses of estrogen and progesterone following treatment with an anti-androgen suggests an inhibitory effect of increased androgen bioactivity on steroid negative feedback.[88] This notion is underscored by the appearance of increased LH pulse release in young adult female monkeys that had received chronic androgen administration prior to and through puberty.[102] These results suggest that hyperandrogenism may be responsible for or, at least, contribute to increased LH pulse frequency in this disorder. The prevention of steroid negative feedback may be a unique effect of androgen as estradiol and insulin, the secretion of which are both abnormal, have not been shown to alter LH pulse frequency. Further studies are necessary to elucidate primary and secondary mechanisms of increased GnRH pulse generator activity in PCOS.

The apparent central role of androgen excess in PCOS underscores the importance of the process by which hyperandrogenism is achieved. That the theca cell is the primary source of hyperandrogenism in PCOS is undeniable. Furthermore, there is evidence to demonstrate increased theca cell responsiveness to LH stimulation compared to normal theca tissue, which suggests a primary defect in this cell.[138,139] As previously mentioned, in PCOS women, increased 17-hydroxyprogesterone dose-responses to hCG were accompanied by significantly greater increases of both androstenedione and testosterone.[142] The androgen responses were achieved at a much lower stimulatory dose of hCG than that required to induce significant increments of 17-hydroxyprogesterone. These results were consistent with *in vitro* studies using propagated PCOS theca cells in which stimulated progesterone, 17-hydroxyprogesterone, and testosterone production were greater than that observed for normal cells.[144,248] The pronounced increase of *CYP11A1* expression and 3β-HSD and 17β-HSD enzyme activities subserve an overall upregulation of steroid formation in the PCOS ovary. In addition, the production of androgen is driven by pituitary LH, which has a bimodal dose effect on the enzyme activities encoded by *CYP17A1*.[249,250] At low doses, LH primarily stimulates 17-hydroxylase activity, whereas at high doses LH down-regulates the 17-20 lyase activity of *CYP17A1*, which would account for the greater incremental change in 17-hydroxyprogesterone.

Notwithstanding the physiological impact of increased theca cell production of androgens, the bioavailability of testosterone is largely influenced by the production of sex hormone binding globulin, the reduction of which may lead to significant hyperandrogenism. Decreases in circulating SHBG concentrations have been observed in women with hyperinsulinemia, excessive weight gain, or hyperandrogenism with the clinical outcome resulting in various degrees of hirsutism. In PCOS, all of these conditions may coexist simultaneously, thereby amplifying the clinical manifestations of excess androgen production. To illustrate the impact of SHBG, marked elevation of serum free testosterone levels and severe hirsutism have been recently reported during pregnancy in a woman who essentially lacked the capacity to produce bioactive SHBG. The deficiency in SHBG production was caused by a single nucleotide polymorphism within an allele that encoded a missense mutation.[26]

As previously stated the role of the adrenal in the genesis of PCOS is unclear. Nevertheless, elevated DHEAS in approximately 20% to 30% of cases indicates an adrenal contribution to excess androgen production that may impact pathophysiological alterations. Moreover, it remains to be determined whether the emergence of adrenal hyperactivity at the time of or just prior to puberty provokes the onset of this syndrome. In girls that experience pubic hair growth prior to age 8, premature pubarche, the risk of PCOS is increased.[251] Similarly, it has been hypothesized that an exaggerated adrenarche at puberty, marked by increased production of DHEA and its sulfate, leads to abnormal androgen exposure and eventual PCOS.[252] These considerations raise the possibility that the genesis of PCOS may be critically linked to the onset of adrenal androgen production at or shortly before puberty.

The role of insulin resistance and hyperinsulinemia in PCOS as a primary cause has not been established. However, there is clear evidence to suggest that hyperinsulinemia may perpetuate the altered reproductive and metabolical physiology in this syndrome. *In vitro* studies have demonstrated that insulin enhances LH-induced androgen production from normal theca cells.[138,147,148,253] Moreover, improvement in insulin sensitivity through the administration of insulin lowering drugs has been accompanied by reduction of serum androgen levels without an effect on circulating LH concentrations. In the granulosa cell, insulin has been shown to enhance estradiol responses to FSH *in vitro* and there is indirect *in vivo* evidence to suggest that insulin may facilitate FSH stimulated estradiol release.[64-67,125,155,254] At the level of gonadotropin secretion, evidence for a role of insulin is inconsistent and remains to be determined. Insulin may have a suppressive or minimal effect on pituitary LH and FSH release whereas results obtained *in vitro* in the rat model would suggest otherwise.[128] However, overall these data suggest that the role of insulin resistance in PCOS is directed primarily, but not exclusively, at the level of the ovary. Concomitantly, hyperinsulinemia may also effect a reduction of serum SHBG as well as reflect a consequence of obesity in these individuals. Whether insulin resistance is etiological is unclear as not all women with this disorder have been found to exhibit abnormal insulin secretion. In addition, defining cellular defects in insulin signaling and insulin related cell function have not been consistent within similar or different tissues of women with PCOS.[255]

Aside from the effects of increased LH secretion on theca cell androgen production, it has been suggested in PCOS that ovarian follicles undergo premature luteinization as a result of the atretogenic action of LH on the granulosa cell. This concept has been supported by evidence that demonstrates increased LH-induced progesterone production from medium-sized follicles (<9 mm diam.) of granulosa cells from PCOS ovaries compared to those observed with normal granulosa cells.[256] In addition, PCOS granulosa cells have been documented to express significantly greater LH receptor mRNA message than cells from normal follicles.[257] These findings together with the increase in LH secretion provide a compelling argument for such a process. Whether this mechanism can explain the arrest of follicle development at the midantral stage of growth in women with PCOS remains to be shown. Moreover, it is not clear how pervasive is the effect of increased LH secretion on granulosa

cell viability. Indeed, it has been demonstrated that most granulosa cells derived from unstimulated follicles of PCOS ovaries are extremely vital and exhibit great capacity for steroidogenesis.[30] Moreover, in 2008 it was reported that in PCOS granulosa cells that markers for apoptosis were decreased and anti-apoptotic gene expression increased compared to those of normal granulosa cells.[31] It may be the case that only the very terminal granulosa cells become quickly atretic and suffer demise.

PCOS continues to remain a pathophysiological enigma with several components of altered physiology unexplained. While a unifying concept seems currently elusive, there is reason to invoke a crucial role for excess androgen exposure in the perpetuation and, perhaps, pathogenesis of this disorder. This approach would account for the diversity of clinical situations which may give rise to the PCOS phenotype including primary and secondary causes of increased androgen production as well as conditions in which androgen bioactivity is increased.

Long-Term Consequences

In PCOS the reproductive-metabolical alterations that are responsible for the immediate concerns of hirsutism, acne, and anovulatory infertility may also pose significant long-term risks to a woman's general health and well-being. These concerns have principally been attributed to chronic anovulation, insulin resistance, and obesity, all of which are salient features of the disorder.

Cancer

Beyond the immediate problem of anovulatory infertility in PCOS, persistent stimulation of the endometrium by chronic unopposed estrogen may lead to endometrial hyperplasia or adenocarcinoma in some women, particularly those with obesity. In a long-term follow-up study of women with PCOS based on ovarian wedge resection, the odds ratio for developing endometrial carcinoma was 5.3 (95% CI: 1.55-18.6) compared to controls.[258] The relationship of endometrial cancer with PCOS was evident in a case-control study, which showed that elevated androstenedione levels were associated with 3.6-fold and 2.8-fold increased risks of carcinoma in premenopausal and postmenopausal women, respectively.[259] Conversely, it has been documented that in young women with endometrial cancer a history of menstrual irregularities consistent with anovulation and the diagnosis of PCOS is common.[260] This problem is compounded by the presence of obesity and the associated decrease of sex-hormone binding globulin resulting in an increase of circulating free estradiol. In cases of endometrial cancer found in PCOS women, the lesion is usually well-differentiated as compared to poorly-differentiated or undifferentiated forms noted for older postmenopausal women. It is not clear whether the histological pattern of endometrial cancer in young PCOS patients might alter consideration of definitive therapy. However, expression of estrogen and progesterone receptors was more commonly encountered in the endometrial tissues of premenopausal versus postmenopausal women.[260]

Studies of the association between PCOS and breast cancer have generally not revealed increased risk. However, many of these studies were limited by conditions of design or subject selection. For instance, increased risk was not found among a cohort of chronic anovulatory women from the Mayo Clinic.[261] It was presumed that these women had PCOS and the study was retrospective. Subgroup analysis of postmenopausal breast cancer showed a significantly increased risk, although the affected cohort was comprised of only five subjects. The Cancer and Steroid Hormone Study, involving 4730 women with breast cancer and 4688 controls failed to identify an increased risk for breast cancer.[262] Moreover, there was actually a 50% reduced likelihood of developing breast cancer. Unfortunately, the diagnosis of PCOS was self-reported and no laboratory documentation was performed. Similarly, the Iowa Women's Health Study, which did not demonstrate increased risk for breast cancer also relied on self-reporting of PCOS.[263] In a study in which the diagnosis of PCOS was based on histological appearance and clinical features, no association could be determined in a cohort of 786 women.[264] In 2003 it was reported that PCOS women were found to have a statistically significant family history of breast cancer compared to that of controls, suggesting a familial association.[265] This study suffered from a lack of enrolled participants, as there were 41 women with PCOS and 66 controls. Thus, of the studies conducted to determine the relationship of PCOS to breast cancer, the vast majority have not been able to define a positive association.

A link between PCOS and ovarian cancer has been suggested from the findings of the Cancer and Steroid Hormone Study, in which women with epithelial ovarian cancer had a significantly higher likelihood of reporting a diagnosis of PCOS compared to that of controls.[266] Moreover, after adjusting for age, parity, oral contraceptive use, infertility and education, the odds ratio, 2.4 (95% CL, 1.0-5.9), for a history of PCOS remained statistically significant. In contrast, longitudinal follow-up studies have not been able to corroborate an increased risk of ovarian cancer in PCOS women. The Mayo Clinic cohort of 1270 subjects with presumed PCOS revealed no concurrent or subsequent risk of ovarian cancer.[261] Similarly, the mortality from ovarian cancer in 768 women with histologically identified PCOS ovaries followed for an average of 30 years was comparable to an expected rate of 2.6 deaths.[264] Recently, in a large population-based case-control study from Australia, women with a history of PCOS or excess androgen exposure were not associated with an increased rate of ovarian cancer.[267]

Diabetes Mellitus

The discovery that women with PCOS have insulin resistance and are at risk for diabetes mellitus has had an enormous impact on the long-term health implications of this disorder. While insulin resistance has been described as being relatively mild, it has been estimated that 20% to 40% of these patients will develop glucose intolerance or type 2 diabetes mellitus by the fourth decade of life.[268,269] Conversely, premenopausal women with type 2 diabetes appear to be at increased risk for PCOS. It is apparent from several studies that PCOS is a leading factor for type 2 diabetes in women.[57,255,270] Not only are women with PCOS at increased risk for impaired glucose tolerance and diabetes, but increased circulating concentrations of insulin have been

reported to be common in first degree relatives.[271] Recently, it has been reported that the combined prevalence rates for impaired glucose tolerance and diabetes in mothers and fathers of women with PCOS were 46% and 58%, respectively, which are considerably greater than rates encountered in families without a history of PCOS.[21] In addition, affected sisters of PCOS women were shown to have higher insulin to glucose ratios than their unaffected female siblings.[272] Collectively, these findings illuminate the trend towards abnormalities of glucose metabolism and diabetes in PCOS women and their immediate family members.

Dyslipidemia

Lipid abnormalities in PCOS are characterized by significant increases of circulating total cholesterol, LDL-cholesterol and triglycerides compared to normal matched controls.[273] Conversely, serum levels of total HDL cholesterol and HDL_2 are significantly lower in PCOS than that of normal women. While it has been shown that in PCOS this lipid profile may exist independently of several risk factors, the impact of these factors on lipid metabolism may be considerable. Previous studies have clearly demonstrated that obesity and impaired glucose tolerance are associated with adverse lipid profiles. The effect of androgen excess on lipid metabolism is less well understood in PCOS.[274] Compared to age-matched normal women, the dyslipidemia of PCOS appeared to be less distinctive when the data were adjusted for body weight. Moreover, serum concentrations of lipoprotein-a and possibly PAI-1 were reduced by testosterone whereas DHEA increased insulin sensitivity.

Regardless of the mechanisms which predispose to the lipid abnormalities in PCOS, these patients should be at risk for plaque generation in coronary vessels.[275] Through the action of hepatic triglyceride lipase, very low density lipoprotein (VLDL) and intermediate density lipoprotein (IDL) are converted to LDL cholesterol. LDL cholesterol appears to be the most atherogenic lipoprotein. Another lipoprotein, lipoprotein-a, is also atherogenic and has the most pronounced effect when LDL cholesterol is elevated. Hepatic triglyceride lipase is also responsible for converting HDL_2, which is rich in cholesterol, to cholesterol poor HDL_3. While the atherogenic properties of LDL cholesterol are well established, there is evidence to suggest that low HDL cholesterol and high triglycerides may be more predictive of coronary artery disease in women than men.

Cardiovascular Disease

It is generally believed that women with PCOS are at increased risk for the development of cardiovascular disease. This is based on the presence of several risk factors which predispose to heart disease. These include impaired glucose tolerance, android obesity, hyperandrogenism, dyslipidemia, and hypertension. Whether PCOS itself constitutes a risk factor independent of known risk factors is not absolutely clear. Evidence to support this association was provided by a case-control study in which 206 women with PCOS and corresponding controls matched for age and race were compared.[276] The PCOS group was found to have significantly higher levels of total cholesterol, LDL-cholesterol, and triglycerides than normal controls. Adjusting for confounding variables such as BMI, fasting insulin, exogenous hormones, oral contraceptives, and age by multiple regression analysis, the lipid abnormalities in PCOS women remained highly significant. In addition, the dose-response relationship between insulin and PCOS was examined within individual cases and only about 20% of the variance could be attributed to insulin. Of note, androgens also did not contribute to the variance in lipids. These findings suggest that dyslipidemia in PCOS involves pathways separate from those of insulin and androgens. To determine whether the risk of cardiovascular disease may be demonstrated clinically in PCOS, a comparison of carotid ultrasonography was performed in PCOS patients and their matched controls.[277] The intima-media thickness, which has been directly correlated with cardiovascular disease, was significantly greater in PCOS than in controls. In addition, atherosclerotic plaque formation was found to be twofold greater in the PCOS group.

Retrospective studies exist which have suggested that women with PCOS or at least the stigmata of PCOS have increased arterial lesions in their coronary vessels.[275,278] In an analysis that employed a statistical risk factor model, it was determined that PCOS women had a significant increased risk of myocardial infarction compared to controls.[279] Unfortunately these studies were limited by their retrospective design, lack of controls for obesity, and insufficient categorization of patient groups. Nevertheless, the current literature clearly indicates that women with PCOS cluster risk factors for premature morbidity and mortality regarding heart disease.[280] Despite the suggestive evidence of greater cardiovascular risk, measurement of actual death from myocardial injury has yet to be established. In 2 studies which examined the long-term health consequences in 786 women with PCOS, increased risk for coronary heart disease was identified, but mortality and morbidity did not differ from the age-matched control group.[258,264]

Hypertension

It has been reported in a retrospective study that postmenopausal women with a history of PCOS, corroborated with pathological conformation, have an approximate fourfold increase in rate of hypertension compared to control subjects.[281] In addition, PCOS women tended to exhibit elevated blood pressure when compared to age-matched controls. However, increases in blood pressure in both obese and nonobese PCOS do not appear to be greater than that of control subjects after adjusting for weight and body composition.[282,283] Thus, despite the presence of insulin resistance in PCOS, particularly in obese women, hypertension may not be a common accompaniment of the disorder. Currently, the relationship of hypertension to PCOS is under investigation.

Differential Diagnosis

The lack of a specific diagnostic test for PCOS combined with the broad clinical spectrum resulting from anovulation and hyperandrogenism warrant consideration of related conditions with similar presentations. These include both functional and neoplastic processes. Among the functional disorders are ovarian hyperthecosis, congenital adrenal hyperplasia, and Cushing disease. Included in the neoplastic

group are androgen producing tumors of the ovary and adrenal gland.

Ovarian Hyperthecosis

Hyperthecosis refers to an unusual proliferative condition in which the ovary contains nests of luteinized theca cells scattered throughout the stroma.[284] The extent of theca cell involvement may vary from minimal to extensive. Severe hyperthecosis may be accompanied by extensive and dense fibroblast growth resulting in an enlarged ovary of extremely firm texture, findings which are clearly distinct from those found in PCOS. Interestingly the degree of hyperthecotic transformation in the ovary is not correlated to the severity of disease.[285] This observation would suggest that the hyperthecotic tissue may be hypersensitive to gonadotropin stimulation since serum LH levels are commonly in the normal range of values. Due to markedly high serum androgen concentrations, these individuals suffer from severe hirsutism and a significant percentage of patients exhibit virilizing signs such as clitoromegaly, temporal balding, a male body habitus and a deepening of the voice. The androgen production may be resistant to conventional forms of long-term ovarian suppression such as oral contraceptive therapy, although administration of gonadotropin releasing hormone agonists have been shown to dramatically decrease androgen production. There usually is marked insulin resistance with substantial elevations of circulating insulin levels. In addition, these patients are often obese and exhibit acanthosis nigricans.

Congenital Adrenal Hyperplasia

Among the several enzymatic defects that comprise congenital adrenal hyperplasia (CAH), the incomplete form of 21-hydroylase deficiency best simulates PCOS (see Chapter 25). This deficiency is manifested by an accumulation of 17-hyroxyprogesterone which leads to abnormal elevations of the hormone compared to circulating values found in the follicular phase of the menstrual cycle. As 17-OHP is an androgen precursor, expression of this defect is associated with increased production of androstenedione and testosterone with resultant hyperandrogenism. Notably, the clinical presentation may be indistinguishable from that of PCOS. However, there are several aspects of CAH-21-hyroxylase deficiency which may suggest the diagnosis. These include severe hirsutism, clitoromegaly, familial tendency and short stature. The condition is transmitted by an autosomal recessive inheritance pattern whereas an explanation for short stature is unknown. Morphologically, the ovaries have been reported to appear similar to those of PCOS. The capsule generally is dense and thickened although the presence of peripheral cystic follicles has been an inconsistent finding. The second most common enzyme deficiency is 11-β-hydroxylase which gives rise to a mild hirsutism due to increases in 17-hydroxyprogesterone as well as 11-deoxycortisol, the immediate precursor for this enzyme. The accompanying hypertension often distinguishes this disorder from the 21-hyroxylase form of CAH.

Cushing Syndrome

The clinical features of Cushing syndrome primarily result from excessive cortisol production by an adrenal neoplasm or from excessive adrenocorticotropin (ACTH) production. In most cases, ACTH overproduction is due to a pituitary tumor, although, rarely, ectopic sources of ACTH may be encountered such as in adenocarcinoma of the lung. The preponderant findings are obesity, hirsutism, acne, and menstrual irregularity. These suggest the diagnosis of PCOS. However, additional evidence of moonlike facies, buffalo hump, hypertension, muscle wasting, abdominal striae, and osteoporosis indicate a primary problem of cortisol excess. While circulating androgen levels are elevated, there is also abnormal cortisol secretion characterized by increased basal levels, loss of circadian rhythmicity and failure of suppression in response to dexamethasone. In contrast to CAH, careful examination of the ovaries does not reveal changes typical of PCOS in the vast majority of cases.

Androgen Producing Neoplasms

Androgen producing tumors may arise from the ovary and the adrenal gland. In contrast to the gradually evolving clinical presentation associated with functional hyperandrogenism, the neoplastic process can be quite dramatic. Within a matter of months these lesions may induce severe hirsutism, a male body habitus and virilization marked by clitoromegaly. In addition, there may be acne and a lowering of the voice. Despite the severity of androgenic manifestations, the early stages of development of these tumors can mimic PCOS or other functional hyperandrogenic syndromes. Occasionally, the hormone production may be mixed to include excess production of cortisol and progesterone.[286] Disruption of menstrual cyclicity varies from irregular bleeding to amenorrhea. The rapid onset of symptoms provides an important clue to the diagnosis. In some instances, a pelvic or abdominal mass can be palpated, which suggests an ovarian tumor.

Evaluation

Laboratory Evaluation

Basically the laboratory diagnosis of PCOS is one of exclusion. Traditionally, it has been recommended that patients suspected of having PCOS on a clinical basis undergo a minimum endocrine evaluation consisting of serum total testosterone or free testosterone and, if indicated, 17-hydroxyprogesterone (see Chapter 25). Measurement of DHEAS is likely to be of low yield unless indicated by clinical findings of severe hirsutism and suspicion of a neoplasm. Individuals with moderate to severe hirsutism may be harboring a hyperplastic or neoplastic lesion. Ethnic considerations are also important as Asians often do not or only minimally manifest androgen excess. Measurements of testosterone and DHEA-S that exceed 200 ng/dL and 7000 ng/dL, respectively, may be associated with an androgen-producing tumor of the ovary or adrenal gland. Should circulating concentrations exceed these threshold levels for tumor, then imaging studies such as ultrasound and MRI are warranted to locate the lesion. Occasionally, high circulating androgen levels may not be associated with a distinct lesion, but rather bilateral non-cystic ovarian enlargement. If accompanied with a gradual onset of symptoms, this presentation would suggest the diagnosis of hyperthecosis.

Commonly, these patients will exhibit severe insulin resistance and acanthosis nigricans.

Determination of 17-hydroxyprogesterone is useful as a screening test for the detection of CAH due to 21-hydroxylase deficiency. It has been proposed that a threshold concentration of basal 17-hydroxyprogesterone of 3 ng/mL provides the maximal cost-benefit.[287] A very high level of 17-hydroxyprogesterone is indicative of the disorder. A circulating level of less than 3 ng/mL obtained at random in anovulatory individuals or during the follicular phase in women with regular menstrual cycle excludes the diagnosis. Values in excess of 3 ng/mL warrant further evaluation by an ACTH stimulation test. Following an overnight fast, ACTH (1-24), 250 μg, is injected intravenously. After 1 hour, a serum 17-hydroxyprogesterone is measured and an increase of greater than 20 ng/mL is indicative of the enzyme defect. Genetic testing may also be used, but this is usually for cases in which the diagnosis is uncertain.

Women with Cushing syndrome may also present with a clinical picture consistent with PCOS. The optimal screening test is a 24-hour urinary free cortisol, for which the normal value is less than 100 mcg/24 hour. Abnormal responses require further testing to determine the mechanism and site of excess cortisol production. These include low dose, high dose dexamethasone suppression tests as well as imaging studies to determine the existence of adrenal hyperplasia, Cushing disease, adrenal adenoma or ectopic ACTH production.

As part of the assessment of oligoamenorrhea due to anovulation, measurements of prolactin and TSH may be desirable. In PCOS, serum elevations in prolactin have been reported to range between 20% and 40% and probably relates to lactotrope stimulation by chronic estrogen exposure.[288,289] Coexistence of a prolactinoma and PCOS is uncommon. Disorders of thyroid secretion have been associated with irregular menstrual bleeding, although there usually are other accompanying clinical features that suggest the diagnosis.

Some comment is warranted regarding the measurement of serum gonadotropin levels and free testosterone concentrations. Despite the widespread practice of measuring serum LH and FSH, the circulating levels of these glycoproteins really do not contribute significantly to the diagnosis of PCOS. Increased pituitary LH secretion cannot always be determined by measurement of the serum concentration, as approximately one-third of patients will have circulating levels of LH in the normal range. Circulating endogenous LH levels are positively correlated to body mass index, which suggests that normal LH levels are not uncommon in obese women with the disorder. Similarly, the LH to FSH ratio probably has little additive value in determining the diagnosis. The measurement of free testosterone does not contribute significantly to the identification of women with PCOS. Rather, its principle utility may be applied to determining the efficacy of treatment for hirsutism.

The observation that women with PCOS are insulin resistant and have compensatory hyperinsulinemia raises the question of whether assessment of glucose metabolism and insulin secretion should be evaluated in these patients. Unfortunately, at the present time, the ability to determine insulin resistance is limited by tests, which lack sensitivity or are impractical for implementation. Based on fasting levels of glucose and insulin, a variety of indices have been designed to establish insulin resistance.[60,290,291] While a reasonable correlation exists between each model and provocative glucose tolerance tests, normal values do not preclude the presence of insulin resistance. However, the fasting level of glucose may be used to distinguish glucose intolerance or diabetes and an elevated fasting insulin level will confer insulin resistance. An oral glucose tolerance test also provides valuable information, particularly if glucose measurements are accompanied by circulating insulin values. However, a reliable and reproducible assay for insulin is mandatory. Whether determination of insulin resistance is essential to the diagnosis of PCOS seems unlikely. Nevertheless, with the availability of insulin lowering drugs, ascertainment of insulin resistance is warranted, particularly in high-risk individuals.

Imaging Studies

In women with PCOS, ultrasound imaging of the ovaries usually reveals bilateral or unilateral enlargement and 12 or more antral follicles per ovary.[48] As this appearance is unique to the syndrome, ultrasound evidence of polycystic ovaries virtually confirms the diagnosis in women with anovulation and hyperandrogenism. However, that the vast majority of PCOS may be determined solely from clinical symptomatology and polycystic ovaries may be demonstrated in normal women makes the routine use of pelvic ultrasound for the diagnosis optional. Considerable information regarding the endometrial response to chronic estrogen exposure may be achieved with ultrasound.

Treatment

Oral Contraceptives

Notwithstanding infertility, the most problematic issue for women with PCOS is excessive hair growth. Thus, one of the primary goals of treatment includes ameliorating the clinical effects of hyperandrogenism, which may be achieved by suppression of ovarian steroidogenesis, interruption of androgen action at the target tissue, and reduction of hyperinsulinemia. Administration of an oral contraceptive containing combination estrogen-progestin has proven to be an effective treatment for hirsutism, although the range of response is variable depending on the severity of hair growth at the time of treatment. In addition to suppression of ovarian androgen production, oral contraceptives increase SHBG as well as facilitate the metabolical clearance rate of testosterone. This modality of treatment also has the advantage of instituting regular cyclic withdrawal bleeding and providing sufficient progestin to prevent excessive endometrial proliferation and hyperplasia.

Anti-Androgens

In many instances, anti-androgenic agents have been in conjunction with oral contraceptives to maximize clinical benefit. Spironolactone is an aldosterone antagonist which, along with its major metabolite, canrenone, competes for testosterone binding sites, thereby exerting a direct anti-androgenic effect at the pilosebaceous unit. In addition,

spironolactone appears to interfere with cytochrome P450, thereby inhibiting steroid enzyme action and resultant androgen production.[292] In the past, it was used to treat mild hypertension and may exert a mild diuretic effect. Because this medication opposes the action of aldosterone, serum potassium levels may increase and therefore should be monitored. Other anti-androgens include flutamide and finasteride. Flutamide competes for the androgen receptor whereas finasteride inhibits 5α-reductase. Both agents have been shown to be effective for the treatment of hirsutism.[293] In a few cases, flutamide has been associated with liver toxicity particularly in women that are obese.[294,295] Clinical studies have determined that these compounds exhibit comparable effectiveness in reducing hair growth.

Insulin Lowering Drugs

Insulin lowering drugs have been shown to improve insulin sensitivity in PCOS women with insulin resistance, which warrants their consideration in the management of this disorder. Most studies have detected a significant decrease in serum testosterone levels whereas others, which have included patients with severe obesity, were unable to demonstrate a similar effect.[64-67,125,254] Metformin, a biguanide, increases insulin sensitivity in the liver to reduce gluconeogenesis and hyperinsulinemia. Clinical studies have shown that administration of metformin to PCOS women resulted in decreased androgen levels, increased rates of spontaneous ovulation, and enhanced ovulatory responses to clomiphene.[65,67] Studies have shown that metformin may have direct effects on ovarian steroidogenesis independent of insulin action. Incubation of human ovarian theca-like tumor cells with metformin inhibited the mRNA expression of steroidogenic regulatory protein and 17α-hydroxylase, whereas no effect was detected for 3β-hydroxysteroid dehydrogenase (3βHSD) or cholesterol side chain cleavage.[296] In contrast, metformin was not associated with changes in 17α-hydroxylase or 3βHSD in studies of yeast cells.[158] The disparity between results may reflect differences in the cell systems employed. However, evidence for metformin ameliorating excessive hair growth is lacking. Side effects of metformin include gastrointestinal symptoms which are dose-related and tend to resolve after several weeks. A rare adverse effect of metformin therapy is lactic acidosis which may occur in individuals with systemic and debilitating diseases. Therefore, metformin should not be prescribed to patients with renal, hepatic, or major cardiovascular disease or hypoxia because these patients have a predisposition to elevated lactate levels. Precautionary temporal withdrawal of metformin is advised in patients undergoing radiological procedures involving intravascular iodinated contrast materials and surgery.

Thiazolidinediones comprise another group of insulin lowering drugs that include rosiglitazone and pioglitazone. These drugs act by binding to perioxisome proliferation activator receptor gamma which forms a heterodimer with retinoic acid receptor and binds to a promoter to increase the expression of genes that regulate glucose homeostasis. It has been well-documented that thiazolidinediones decrease androgen levels in women with PCOS.[125,127] In addition, in a large multi-center clinical trial, it was shown that improved insulin sensitivity was associated with resumption of ovulation following long-term treatment with troglitazone.[64] This effect was dose-dependent as determined by the rate of ovulation and the length of time required to achieve ovulation. Similar to metformin, thiazolidinediones have also been shown to have direct effect on steroidogenesis. In studies using yeast, the steroidogenic enzymes, 17α-hydroxylase and 3βHSD, were inhibited by troglitazone and to a lesser extent rosiglitazone and pioglitazone.[158] Similar results have been achieved in human granulosa cells. However, studies to determine whether troglitazone influences aromatase enzyme have not produced consistent results in human granulosa cells.[157,159] Liver toxicity was associated with first generation drugs of this class of compounds. However, both rosiglitazone and pioglitazone have been virtually devoid of liver effects. Nevertheless, thiazolidinediones should not be initiated in patients with evidence of liver disease. There have been some reports of increased cardiovascular risk with rosiglitazone whereas others have been unable to substantiate similar findings. Currently, it remains on the market in the United States and Europe.

Surgical Approach to Anovulation

In the formal description of PCOS in 1935, it was reported that several women undergoing wedge resection experienced a resumption of ovulation.[4] This was attributed to the removal of hyperplastic central stroma. As a result, for many years this technique was employed to treat anovulatory infertility in these women until the discovery and development of medical agents that stimulated the ovary and induced ovulation (clomiphene citrate, gonadotropins). Accordingly, enthusiasm for wedge resection waned and it was used sparingly. In 1984 it was reported that in women with PCOS, electrocautery of the ovarian capsule during laparoscopy resulted in a high rate of ovulation and pregnancy.[297] This led to multiple papers examining the utility of this method that showed ovulation rates of 70% to 90% and pregnancy rates of 50% to 70%.[298] The duration of spontaneous ovulation following LOD is variable, although some reports suggest an ovulatory pattern may persist for several years.[299]

Penetration of the ovarian capsule by diathermy or laparoscopic ovarian drilling (LOD) may be achieved using either cautery or laser, although the former appears to result in fewer post-operative pelvic adhesions. The technique involves puncture of the capsule with a unipolar coagulating current at about three to six different areas to a depth of 4 to 10 mm. The tendency is to perform this technique on both ovaries although one report showed that unilateral and bilateral ovarian drilling exhibited similar rates of ovulation.[300] The mechanism by which LOD induces ovulation is unknown. During the procedure there is a decrease of androgens and steroid hormones that have led to several theories including disruption of estrogen negative feedback on gonadotropin secretion, decrease of intraovarian androgens that inhibit follicle development, or activation of intraovarian growth factors that stimulate follicle growth. However, evidence for these considerations has not been convincing.

The application of LOD appears to be best suited for anovulatory women that are resistant or fail to respond to clomiphene citrate treatment. In contrast, recently it was

shown that clomiphene-resistant women exhibited slightly higher rates of ovulation with letrozole therapy compared to LOD.[301] These findings indicate that initial attempts at ovulation induction in untreated anovulatory PCOS women should employ medical treatment rather than surgery. Not all clomiphene-resistant women will respond to LOD as an estimated one-third will exhibit persistent anovulation. In these patients, past LOD may sensitize the ovary such that responsiveness to subsequent clomiphene citrate CC therapy may restore ovulatory function.[302]

Ovulation Induction

See Chapter 30 for a discussion of ovulation induction.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 23

Female Infertility

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Fertility is defined as the capacity to conceive and produce offspring. *Infertility* is the state of a diminished capacity to conceive and bear offspring. In contrast to sterility, infertility is not an irreversible state. The current clinical definition of infertility is the inability to conceive after 12 months of frequent coitus. Infertility prevalence among women aged 15 to 44 years was 8.5% in 1982 and 7.4% in 2002. More recent population-based studies from 2009 and 2012 reported a higher prevalence of infertility with estimates ranging from 12% to 24%.[1-3] Infertility prevalence is greater in older women, a factor which may contribute to the rising rate of infertility.[4] Since the fertility potential of the female partner decreases after 35 years of age, most authorities recommend initiating an infertility evaluation after 6 months of attempting conception in women 35 to 40 years of age and immediately in women over 40 years of age.[5,6] Women with known causes of infertility, such as amenorrhea, should immediately start an evaluation to assess the cause of the endocrine disorder.

A Statistical Model of Infertility

The clinical definition of infertility is relatively crude because it does not reflect the wide range of fertility potential in couples that have not conceived after 12 months. The clinical definition of infertility implies the existence of a dichotomous state, either a pregnancy is achieved in 12 months (infertility is not present) or a pregnancy is not achieved in 12 months and, by definition, infertility is present. The current clinical definition of infertility is similar to analyzing a continuous variable, such as height, by using a dichotomous variable: "short" and "tall." Height is clearly much better described by a continuous measure, such as centimeters, rather than by using a dichotomous variable like "short" and "tall."

Our clinical approach to fertility and infertility would be best advanced by the use of the statistical concept of fecundability. *Fecundability* is the probability of achieving a pregnancy in one menstrual cycle (approximately 0.25 in healthy young couples). A related concept, *fecundity*, is the ability to achieve a pregnancy that results in a live birth in one menstrual cycle. Fecundability, a population estimate of the probability of achieving pregnancy in one menstrual cycle, is a valuable clinical and scientific concept because it creates a framework for the quantitative analysis of fertility potential. Based on the clinical characteristics of a population of infertile couples, the estimated fecundability may range from 0.00 in couples with an azoospermic male partner to approximately 0.04 in couples where the female partner has early stage endometriosis.

In addition, fecundability provides a convenient quantitative estimate of the efficacy of various fertility treatments. An infertile couple with an estimated fecundability of 0.04 if left untreated, may have the choice of two approaches to the treatment of their fertility: a low-cost treatment (clomiphene plus intrauterine insemination) that will increase fecundability to 0.08, or an expensive treatment (*in vitro* fertilization) that will increase fecundability to 0.25. A clear quantitative presentation of the potential effect of each treatment on fecundability should assist the couple in choosing an optimal treatment plan. A practical problem with using fecundability as a central concept in infertility care is that the prediction models for estimating the fecundability of a couple are not well developed or validated. Factors that are important in estimating fecundability of a couple are age of the female partner, number of motile sperm, duration of subfertility, and presence of primary or secondary infertility.[7] Clinical care for infertile couples would be significantly advanced if discussions of infertility used the concept of fecundability. The "per cycle pregnancy rate" is the fecundability multiplied by 100. In this chapter, the terms "fecundability" and "per cycle pregnancy rate" are both used to mean the proportion of couples achieving pregnancy in one cycle. The concept of fecundability can be used to derive a simple statistical description of the fertility process.

Fecundability (f) is defined as the probability of conceiving during any one cycle. The probability of failing to conceive during any one cycle is 1–f. Over a short period of time, the fecundability of a population is often stable.[8] For a large group of couples, the probability of conception is f for the first month, $f \times (1–f)$ for the second month, $f \times (1–f)^2$ for the third month, and $f \times (1–f)^{N–1}$ for the Nth month. Using this model, the mean number of months required to achieve conception is 1/f. The cumulative probability of conception, F, through month N is calculated as follows: $F = 1–(1–f)^N$. Based on this simple statistical model, assuming a normal menstrual cycle fecundability of 0.25 and starting with 100 couples, approximately 98 couples should conceive within 13 cycles. If each cycle is 28 days, then 98% of couples should conceive within one calendar year (13 cycles × 28 days/cycle = 364 days).

Over short periods of follow-up, a population of couples attempting pregnancy behaves in a statistically stable manner, with a fixed proportion of the cohort becoming pregnant with each additional cycle of follow-up. As the follow-up

Table 23.1 Observational Studies Often Demonstrate That the Fecundability of the Cohort Decreases as Follow-up Progresses

Cycle	Number of Women Available for Study at Start of Cycle	Number of Pregnancies in Cycle	Per-Cycle Pregnancy Rate
1	200	59	0.30
2	137	41	0.30
3	95	16	0.17
4	78	12	0.15
5	66	14	0.21
6	52	4	0.08
7	48	5	0.10
8	43	3	0.07
9	40	2	0.05
10	38	1	0.03
11	37	2	0.05
12	35	1	0.03

From Zinaman MJ, Clegg ED, Brown CC, et al. Estimates of human fertility and pregnancy loss. *Fertil Steril* 65:503–509, 1996.

Table 23.2 Pregnancy Occurrence and Outcome During Three Consecutive Menstrual Cycles in 200 Healthy Couples Desiring Pregnancy

	Cycle Numbers			
Cycle Outcome	1	2	3	Total
Not pregnant at start of cycle	200	137	95	
Pregnant during cycle	59	41	16	116
Chemical pregnancy	7	7	1	15
Spontaneous abortion	12	5	4	21
Live births	40	29	10	79
Dropped out, not pregnant	4	1	1	6
Lost to follow-up			1	

Age of female partner, 30.6 ± 3.3 (mean ± standard deviation [SD]).
From Zinaman MJ, Clegg ED, Brown CC, et al. Estimates of human fertility and pregnancy loss. *Fertil Steril* 65:503–509, 1996.

is extended, however, the fecundability of the nonpregnant couples declines and the cumulative pregnancy rate approaches an asymptote, which is less than 100%. This pattern is likely due to heterogeneity in the fecundability of the initial population. The couples with the highest per cycle pregnancy rate are rapidly culled from the population (they became pregnant), leaving the couples with the more severe infertility problem in the residual pool.

Conceptually, this issue can be managed by assuming that there is an asymptote to the cumulative pregnancy rate of the population, or by using complex mathematical modeling of the population fecundability based on the assumption that couples in the population have a range of per cycle pregnancy rates. This issue is of special importance in the analysis of fertility rates in populations over long periods of time, such as 2 years. This issue is of less practical importance in studies where the time period for analysis is three cycles or less.

Many studies report that the observed fecundability of a population diminishes with long-term follow-up. For example, Guttmacher[9] assessed the number of months to conception in 5574 women who achieved pregnancy between 1946 and 1956. During the first 3 months of observation, the fecundability was 0.25. During the next 9 months of observation, the fecundability was 0.15. Zinaman and colleagues[10] studied 200 healthy couples who desired to conceive. During the first 3 months of observation, the fecundability was 0.25. During the next 9 months of observation, the fecundability was 0.11 (Table 23.1). Other investigators have reported similar results.[11]

The drop in fecundability suggests that each large population consists of a heterogeneous mixture of couples. Some couples have completely normal fertility and achieve pregnancy at a high rate (>0.25 per cycle). The remaining couples have a lower fecundability (ranging from 0.00 to 0.15). Some of the couples in this pool will eventually present to a clinician for the treatment of infertility. At the end of 12 months of attempting conception, the couples that have not achieved conception have a fecundability in the range of 0.00 to 0.04 if left untreated (Table 23.1). Successful interventions to improve fertility must increase the per-cycle pregnancy rate over this background pregnancy rate.

Unfortunately, not all pregnancies produce a live birth. Many pregnancies are lost soon after implantation. The terms occult pregnancy and chemical pregnancy are often used to describe these early pregnancy losses. *Occult pregnancy* was defined by Bloch[12] as a pregnancy that terminates so soon after implantation that there was no clinical suspicion of its existence. In one recent study, approximately 13% of pregnancies were occult. Unlike occult pregnancies, a *chemical pregnancy* typically occurs in the presence of a clinical suspicion that a pregnancy may exist. A blood or urine human chorionic gonadotropin (hCG) assay demonstrates the presence of a pregnancy, but no clinical evidence of the pregnancy is detectable by ultrasound. Of all clinical pregnancies, approximately 20% result in a spontaneous abortion. Of all pregnancies, approximately 30% are lost—either as occult, chemical, or clinical spontaneous abortions (Table 23.2).[10] Women older than 40 years of age who become pregnant have a spontaneous abortion rate more than double that observed in women younger than 30 years of age.[13]

Diseases Associated with Infertility

Pregnancy is the result of the successful completion of a complex series of physiological events occurring in both the male and female that allows for the implantation of an embryo in the endometrium (Fig. 23.1). At a minimum, pregnancy requires ovulation and the production of a competent oocyte, production of competent sperm, proximity of the sperm and oocyte in the reproductive tract, transport of the embryo into the uterine cavity, and implantation of the embryo into the endometrium. Many disease processes can result in subfertility.

Some diseases, such as those that cause azoospermia, clearly have a *cause-effect relationship* with infertility. For other disease processes, such as stage I endometriosis as defined by the revised American Society for Reproductive Medicine criteria, there is no clear cause-effect relationship between the disease and the infertile state. In these

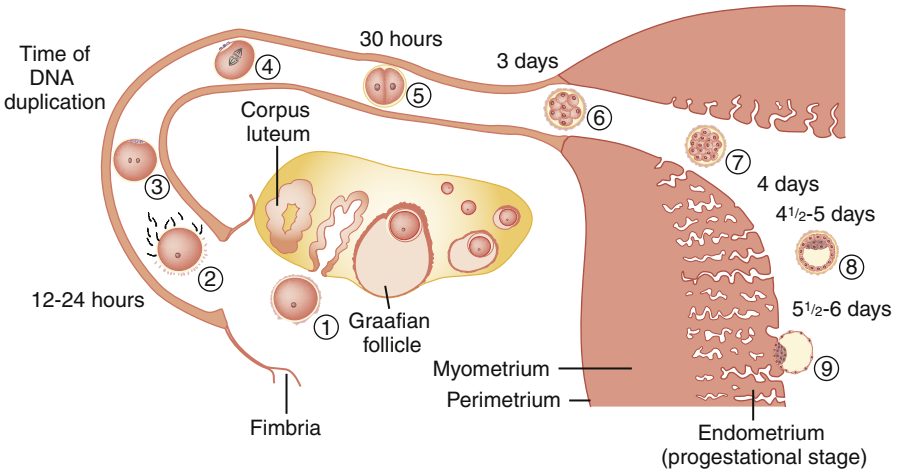


FIGURE 23.1 Schematic representation of the transport of the oocyte, sperm, and embryo in the female reproductive tract.

situations it is preferable to state that there is an *association* between the disease condition and the infertile state, but that causality has not been definitively established. Due to the limits of our current understanding of fertility in humans, it is often difficult to categorize disease conditions as either causal factors (azoospermia) or associated factors (stage I endometriosis) with infertility. Consequently, a discussion of the distribution of reproductive diseases that are diagnosed in infertile couples is not necessarily based on hard scientific data, but rather descriptive observations and assumptions about diseases that might *cause* or might be *associated* with infertility.

Most tabulations of the medical conditions that "cause" infertility divide the problem into male factors and female factors. The World Health Organization (WHO) task force on Diagnosis and Treatment of Infertility conducted a study of 8500 infertile couples using a standardized diagnostic protocol.[14] In developed countries, diseases that were identified as contributing to the infertile state were attributed to the female partner in 37% of couples, to the male partner in 8% of couples, and to both partners in 35% of couples. Five percent of the couples had no identifiable cause of infertility (unexplained infertility) and 15% of the couples became pregnant during the investigation. The diseases in the female most often identified were: ovulatory disorders (25%), pelvic adhesions (12%), tubal occlusion (11%), other tubal abnormalities (11%), hyperprolactinemia (7%), endometriosis (15%), and no identifiable disease in 20%.

In a review of 21 published reports containing 14,141 infertile couples, Collins[15] reported that the primary diagnoses in the couples were: ovulatory disorders (27%), abnormal semen parameter (25%), tubal defect (22%), endometriosis (5%), unexplained (17%), and other (4%), as shown in Figure 23.2. In another data set of 2198 infertile couples the distribution of primary diagnoses was: ovulatory disorders (18%), abnormal semen parameter (24%), tubal disease (23%), endometriosis (6.6%), unexplained (26%), and other (3%).[16] These observations can be broadly grouped into five major conditions that influence fecundability:

1. Abnormalities in the production of a competent oocyte (ovulatory factor, depletion of the oocyte pool, or poor oocyte function/quality)

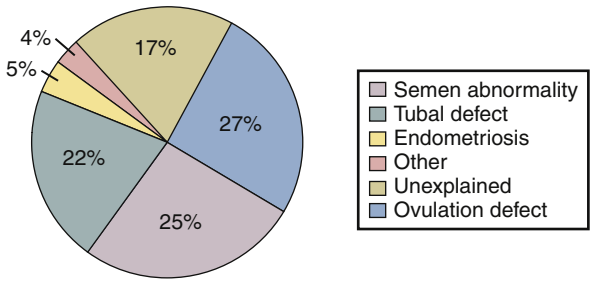


FIGURE 23.2 Primary clinical diagnoses in infertile couples. *(From Collins JA. Unexplained infertility. In Keye WE, Chang RJ, Rebar RW, Soules MR [eds].* Infertility: Evaluation and Treatment. *Philadelphia: WB Saunders, 1995, p 250, with permission.)*

2. Abnormalities in reproductive tract transport of the sperm, oocyte, and embryo (tubal, uterine, cervical, and peritoneal factors)
3. Abnormalities in the implantation process, including early defects in embryo development and embryo–endometrial interaction (embryo–endometrial factors)
4. Abnormalities of sperm production (male factor)
5. Other conditions, including immunological factors that can affect multiple components of the process

The initial infertility evaluation focuses on these five major processes (Box 23.1).

The Initial Infertility Evaluation

The standard components of the infertility evaluation include: a thorough history and physical examination (Table 23.3), a semen analysis (Box 23.1), documentation of competent ovulation, documentation of female reproductive tract and tubal patency, and assessment of the uterine cavity. The evaluation of the semen analysis is discussed in Chapter 24. As discussed in more detail below, ovulation may be presumptively documented based on a history of regular menses every 28 days or observation of a luteinizing hormone (LH) surge in the urine using an immunochemical method, and definitively diagnosed by a serum progesterone greater than 3 ng/mL or the histological demonstration of secretory changes on an endometrial biopsy. For women ≥35 years of age, a test of the size of

the ovarian follicle pool, also known as ovarian reserve, is warranted. Tests to assess the ovarian follicle pool include a menstrual cycle day 3 follicle stimulating hormone (FSH), antral follicle count, or antimüllerian hormone (AMH) measurement.[17]

Documentation of tubal patency should be accomplished early in the infertility evaluation by a hysterosalpingogram (HSG), a hysterosalpingo-contrast sonogram (HyCoSy), or a laparoscopy. Detection of uterine abnormalities can be accomplished by an HSG, HyCoSy, or hysteroscopy. An HSG is an excellent test of distal tubal patency with few false-positives, but it is associated with an approximately 15% false-positive rate for proximal tubal occlusion.[18] This means that if the HSG demonstrates that there is proximal tubal blockage, the finding should be confirmed by a second test (selective catheterization of each tube or laparoscopy). The HSG also provides evidence of the shape of the uterine cavity and will identify large intrauterine defects. As noted below (anatomical factors in the female), the hysterosalpingo-contrast sonogram is gaining in popularity as an initial test in the fertility work-up because it can detect tubal occlusion and is very sensitive for identifying small defects in the uterine cavity.[19] For the detection of uterine abnormalities, hysteroscopy remains the gold standard.[20]

Infertility tests that are probably not needed as part of the initial infertility evaluation include: the postcoital test, endometrial biopsy for luteal phase dysfunction, the hamster egg penetration test, routine *Mycoplasma* culture, and anti–sperm antibody testing. A major problem with the postcoital test is that it has low reproducibility, low interobserver reliability, and has not been reliably shown to help guide treatment recommendations.[21-23] In addition, there is little consensus on what constitutes an abnormal postcoital test. Given these limitations, there is little scientific rationale for performing a postcoital test. However, clinical experience suggests that clomiphene, acting as an anti-estrogen in the cervix, can cause abnormal cervical mucus production, both in quantity and quality. Some clinicians recommend performing a postcoital test to assess the impact of clomiphene on cervical mucus production in women receiving this drug for ovulation induction.

The endometrial biopsy is abnormal in many infertile women and in the past some clinicians believed that it was the gold standard for documenting ovulation and assessing endometrial preparedness for implantation. However, prospective studies demonstrate that the rates of abnormal (out-of-phase) endometrial histology are similar in fertile and infertile women.[24] Given the weak correlation between abnormal (out-of-phase) biopsies and fertility, most clinicians are not performing endometrial biopsy as a first line fertility diagnostic test.

Box 23.1 Initial Laboratory Approach to the Infertile Couple

PRIMARY TESTS FOR INFERTILITY

- Documentation of competent ovulation
  - Midluteal progesterone >3 ng/mL
  - Day 3 FSH or clomiphene challenge test (if female partner >35 years old)
- Semen analysis*
  - Volume ≥1.5 mL
  - Concentration ≥15 million/mL
  - Motility ≥32%
  - Morphology ≥4% (using "strict" criteria)
  - Terminology used to describe abnormal semen analysis: Low sperm concentration, oligospermia; low sperm motility, asthenospermia; sperm morphology abnormal, teratospermia; elevated white blood cells, leukocytospermia
- Documentation of tubal patency
  - Hysterosalpingogram or hysterosalpingo-contrast sonography
  - Assessment of the uterine cavity
  - Hysterosalpingogram, hysterosalpingo-contrast sonography, or hysteroscopy

SECONDARY TESTS FOR INFERTILITY

- Laparoscopy
- Hysteroscopy

*From Cooper TG, Noon E, von Eckardstein S, Auger J, et al. World Health Organization reference values for human semen characteristics. *Hum Reprod Update* 2010; 16: 231–245.

Table 23.3 History and Physical Examination Findings Relevant to the Infertility Evaluation in the Female Partner

History	Physical Exam Findings
Duration of infertility and results of previous tests and treatments.	Weight, height, body mass index.
Prior pregnancies and outcomes.	Evidence for hirsutism, acanthosis nigricans.
Pubertal Milestones: Adrenarche, Thelarche, and Menarche.	Thyroid size. Presence of thyroid nodules.
Menstrual cycle history from Menarche to present. Evidence for cycle irregularity.	Breast exam including palpation for breast masses and expression of nipple secretion.
Previous gynecological and abdominal surgery.	Tanner stage of the breasts.
Past contraceptive use.	Assessment of the anatomy of the clitoris, hymenal ring, vagina, and cervix. Assess for a vaginal septum. Assess for cervical stenosis or displacement of cervix from midline.
Coital frequency and sexual function.	Examination of position of the uterus and uterine size and mobility.
Relevant medical history of male partner.	Examination of adnexae for a mass or tenderness.
Current medications and allergies.	Examination of the uterosacral ligaments and cul de sac.
History of hirsutism, pelvic or abdominal pain, dyspareunia, thyroid disease, galactorrhea.	
Current occupation and exposure to environmental toxins.	
Use of tobacco, alcohol, and drugs.	
Exercise history and current pattern of exercise.	
History of stress, anxiety, depression.	

Abnormalities in Oocyte Production

Disorders of oocyte production are the most common cause of female infertility. The most common disorders of oocyte production are: anovulation, oligo-ovulation, depletion of the follicle pool, and aging of the ovarian follicle resulting in poor oocyte quality. Anovulation is typically associated with amenorrhea or severe oligomenorrhea. Oligo-ovulation is typically associated with oligomenorrhea (cycle lengths greater than 35 days). A depleted ovarian follicle pool is usually detected by a menstrual cycle day 3 FSH, antral follicle count or antimüllerian hormone measurement. Oocyte quality is difficult to assess. Poor oocyte quality should be suspected when the age of the female partner is older than 40 years of age, even if tests of the ovarian follicle pool indicate good ovarian reserve.[25]

Women who have monthly menses and report moliminal symptoms—such as breast tenderness and dysmenorrhea—are typically ovulatory.[26] The least expensive laboratory method for detecting ovulation is the measurement of the basal body temperature. For most women, the morning basal temperature obtained prior to rising from bed is less than 98°F before ovulation and over 98°F after ovulation. Progesterone production from the ovary appears to raise the hypothalamic set-point for basal temperature by approximately 0.6°F. The normal luteal phase is typically associated with a temperature rise, above 98°F, for at least 10 days in length. Occasionally basal body temperature recordings may appear monophasic even in the presence of ovulation. A biphasic pattern is almost always associated with ovulation. If the pattern is biphasic, coitus can be recommended every other day for a period including the 5 days prior to and the day of ovulation (Fig. 23.3).[27] In most developed countries the basal body temperature method is seldom used to detect or document ovulation in infertile women, with the sequential measurement of urine LH at the appropriate time in the menstrual cycle being the most common approach to detecting impending ovulation.

A serum progesterone level greater than 3 ng/mL is diagnostic of ovulation. In the midluteal and late-luteal phase, progesterone secretion is pulsatile due to the pulsatile nature of luteinizing (LH) secretion.[28] At a conceptual level, the pulsatile nature of progesterone secretion may make it difficult to reliably use a single progesterone measurement as a marker for the adequacy of ovulation. However, in most clinical situations, a single midluteal progesterone measurement appears to be a useful marker of the adequacy of ovulation. Hull and colleagues have suggested that a midluteal progesterone concentration less than 10 ng/mL is associated with a lower per-cycle pregnancy rate than progesterone levels above 10 ng/mL.[29]

An endometrial biopsy with histological secretory changes is a definitive test demonstrating ovulation. Although not currently in clinical use, it is likely that the endometrial biopsy could be used to detect endometrial proteins that can serve as useful markers of endometrial receptivity for implantation. Validation of the detection of implantation markers in endometrial biopsy specimens awaits future research.[30] An important point to emphasize is that in women without ovarian function, the administration of two hormones, estradiol and progesterone, are sufficient to prepare the endometrium for implantation.[31]

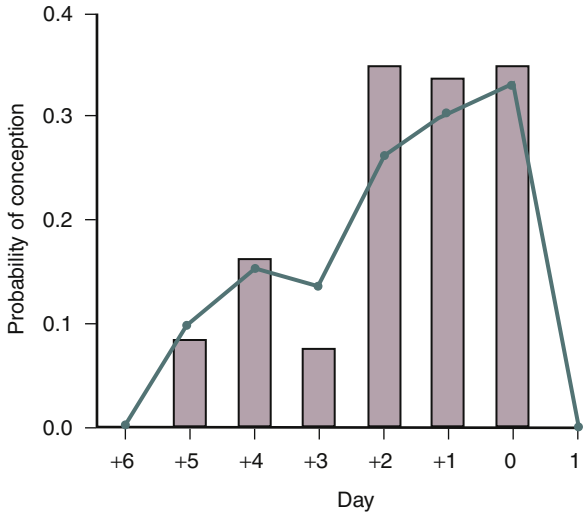


FIGURE 23.3 Probability of conception on specific days near the day of ovulation. The bars represent data reported by 129 women who had sexual intercourse on only 1 day in the 6-day interval ending on the day of ovulation (day 0). The solid line shows the probability of conception based on a statistical analysis of data from 625 cycles. *(From Wilcox AJ, Weinberg CR, Baird DD. Timing of sexual intercourse in relation to ovulation.* N Engl J Med *333:1517–1521, 1995, with permission.)*

Some authorities have suggested that a follicle may luteinize and differentiate into a corpus luteum (secreting progesterone and inducing a secretory endometrium) without rupturing and allowing the oocyte to escape the follicle.[32] The "luteinized unruptured follicle syndrome" may occur occasionally, but it is unlikely to be a major cause of infertility.[33,34] Sonographic examination of the ovary and serial measurement of LH or estrone-3-glucuronide can be used to demonstrate the growth of a dominant follicle, which is a necessary precondition to ovulation.[35] During menses, the follicles in the ovary are approximately 4 to 9 mm in diameter. Prior to ovulation, the dominant follicle reaches a diameter in the range of 20 to 25 mm. Demonstration of follicle growth and rupture of the dominant follicle is presumptive evidence that ovulation has occurred. The demonstration of an LH surge is also presumptive evidence of ovulatory cycles. Ovulation typically occurs about 36 hours after the onset of the LH surge and approximately 24 hours after the LH peak. In one large prospective study, the detection of a urine LH surge by patients using a home detection kit was associated with ovulation as demonstrated by a secretory endometrial biopsy in 93% of cycles.[36] In some infertile women, premature LH surges may occur that are not followed within 36 hours by ovulation. These premature LH surges may make it more difficult to precisely identify ovulation and the fertile part of the menstrual cycle.[37]

Many diseases can cause anovulation and infertility. The most common disorders are hypogonadotropic hypogonadism (WHO I), euestrogenic ovulatory dysfunction (WHO II), and hypergonadotropic hypogonadism (WHO III). The most common causes of adult-onset anovulation are hypothalamic dysfunction (35% of cases), pituitary disease (15%), and ovarian dysfunction (50%).[38,39] The most common causes of hypothalamic dysfunction are abnormalities in

weight and body composition, stress, and strenuous exercise (see Chapter 19). Less common causes of hypothalamic dysfunction are infiltrating diseases of the hypothalamus, such as lymphoma and histiocytosis. The pituitary disorders that cause anovulation (see Chapters 3 and 21) are prolactinoma, empty sella syndrome, Sheehan syndrome, Cushing disease, acromegaly, and other pituitary tumors. The most common ovarian causes of anovulation are ovarian failure (depletion of the oocyte pool) and ovarian hyperandrogenism (e.g., polycystic ovary syndrome [PCOS]). Occasionally thyroid disease can be associated with anovulation.

Evaluation of the various causes of anovulation can be complex. Typically, measurement of body weight and height, and measurement of serum follicle-stimulating hormone (FSH), prolactin, thyroid-stimulating hormone (TSH), and androgens, if indicated, can help identify the cause of the anovulation. A progestin withdrawal test may be helpful to evaluate the degree of hypogonadism present and may help guide treatment choices.

Patients with anovulation have the greatest success with infertility therapy. Treatment of anovulatory disorders can result in fecundability similar to that observed in normal couples (0.15-0.25). The choice of treatment is dependent on the cause of the anovulation. Common treatment choices include:

1. Interventions to modulate weight
2. Reduction in intensity of aerobic exercise
3. Clomiphene citrate
4. Clomiphene plus other hormone adjuvants
5. Gonadotropin treatment
6. Pulsatile gonadotropin-releasing hormone (GnRH)
7. Bromocriptine
8. Glucocorticoids

Weight Abnormalities Associated with Anovulation

Anovulation, oligo-ovulation, and subfertility are commonly observed in women above or below their ideal body weight (see also Chapter 19).[40] In one study of 597 cases of women with anovulatory infertility and 1695 fertile controls, overweight women (body mass index [BMI] greater than 27 kg/m^2) had a relative risk of anovulatory infertility of 3.1 compared with women of BMI 20 to 25 kg/m^2. Excessively thin women with a BMI less than 17 kg/m^2 had a relative risk of anovulatory infertility of 1.6. The investigators concluded that the risk of ovulatory infertility is highest in overweight women, but is also increased in underweight women.[41]

Anovulatory women far below their ideal body weight often have hypogonadotropic hypogonadism. Anovulatory women far above their ideal body weight often have PCOS. For women who are far below (hypogonadotropic hypogonadism) or far above (PCOS) their ideal body weight, appropriate management of dietary intake may be associated with resumption of ovulation.[42] For example, Pasquali and colleagues[43] demonstrated that anovulation in obese women with PCOS could be successfully treated with weight loss. Obese women with anovulation and PCOS were placed on a 1000-calorie to 1500-calorie diet for 6 months. The mean weight loss was 10 kg. After weight loss there was a 45% decrease in basal LH concentration and a 35% decrease in serum testosterone. Many of the women resumed ovulation and became pregnant. Similar results were reported by Clark and colleagues.[44] Thirteen obese anovulatory infertile women were entered into a program of diet and exercise and lost on average 6.3 kg over 6 months. Fasting insulin and testosterone levels decreased, and sex hormone-binding globulin concentrations increased. Twelve of 13 subjects resumed ovulation and 5 became pregnant without any other intervention.

Most studies of the impact of weight loss on reproductive function did not include a control group. Guzick and colleagues reported the results of a randomized, controlled trial of the impact of weight loss on reproductive function.[45] Twelve obese, hyperandrogenic, oligoovulatory women were randomized to either a weight reduction program or a "waiting list" observation control group. The six women randomized to the weight reduction program had a mean decrease in weight of 16 kg, a significant decrease in circulating testosterone, a decrease in fasting insulin, and no change in LH pulse frequency and amplitude. In the women who were randomized to the weight reduction program, four of six resumed ovulation. All of the women in the control group who were anovulatory before the study, remained anovulatory during the period of study observation.

Weight loss is difficult to achieve. Consultation with a nutritionist, encouragement by a physician, a hypocaloric diet, and initiation of an exercise program may be the most effective non-surgical interventions that can help a woman lose weight. Surgical methods of weight reduction can be very effective, especially in women with a BMI over 40 kg/m^2.[46]

Excessively lean women are at increased risk for anovulatory infertility. In experimental models, oligo-ovulation ensues following interventions that result in daily energy expenditure greater than daily calorie intake. In the captive female monkey, regular ovulatory cycles are observed with routine activity and a steady calorie intake at 300 kcal daily. When calorie intake is maintained at 300 kcal daily, but activity is increased to include 6 miles of additional ambulation daily, anovulation and amenorrhea ensue. Increasing calorie intake to up to 600 kcal daily while maintaining 6 miles of exercise daily results in resumption of ovulation and menses. In the exercising monkey, resumption of ovulation can also be initiated without an increase in calorie intake by administering pulses of GnRH.[47]

Women who exercise regularly have more anovulatory cycles than women who are sedentary.[48] In a study of sedentary women assigned to exercise plus calorie restriction or exercise plus a eucaloric diet, the exercise plus calorie restriction group produces greater declines in ovarian steroid production than exercise plus a eucaloric diet.[49] Hormones that help the brain assess the relative levels of calorie intake and energy expenditure include: leptin, insulin, thyroid hormones (thyroxine and triiodothyronine), growth hormone, insulin-like growth factor 1 (IGF1), cholecystokinin, glucagon-like pepetide-1, and ghrelin.

Women with hypothalamic amenorrhea (hypothalamic hypogonadism) often have low levels of leptin. Two clinical trials reported that the administration of exogenous leptin or a decapeptide leptin peptide (metreleptin) to lean women with hypothalamic amenorrhea, resulted in the resumption of ovulatory menses in some of the subjects.[50,51] In

addition, metreleptin administration for 36 weeks increased free triiodothyronine, IGF1, and osteocalcin.[51] Leptin may exert its effect on GnRH secretion by a direct action on kisspeptin releasing neurons.[52]

Lean women with anovulatory infertility are often reluctant to gain weight, alter their diet, or reduce their exercise regimen. However, in one study of 26 underweight women who practiced strict dieting and were infertile, the subjects were counselled by a dietician and given physician-directed advice to increase their BMI. After the intervention, the women gained a mean of 3.7 kg and 73% of the women became pregnant.[53] Interpersonal psychodynamic psychotherapy or cognitive behavior therapy may help WHO I anovulatory women resume ovulating.[54] It is important to try to achieve a normal BMI prior to initiating ovulation induction in excessively lean anovulatory women because pregnancy in women with a low BMI is associated with an increased risk of delivering infants with low birth weight, small head circumference, and microcephaly.[55]

Specific dietary factors may influence the risk of anovulatory infertility. For example, in one prospective study, women who consumed iron supplements were reported to have a 40% lower risk of anovulatory infertility.[56] In another prospective study, dietary patterns characterized by high consumption of monounsaturated rather than transfats, vegetable rather than animal protein, low glycemic carbohydrates, high fat dairy, and multivitamins was associated with a reduced risk of ovulatory infertility.[57] Myo-inositol, a new dietary supplement, is a precursor of D-chiro-inositol which is involved in the control of glucose metabolism and cell response to insulin stimulation. In one small observational study without a control group, oligo-ovulatory women with PCOS who took myo-inositol, 2 g twice daily, reported an increase in spontaneous menstrual cycles. Forty percent of the subjects became pregnant during 6 months of myo-inositol treatment.[58]

Clomiphene

Clomiphene, a non-steroidal triphenylethylene derivative estrogen agonist-antagonist related to tamoxifen and diethylstilbestrol, was first synthesized in 1956. In 1961, Greenblatt and colleagues[59] reported clomiphene to be effective in the induction of ovulation, and the drug was approved by the Food and Drug Administration (FDA) in 1967 (see also Chapter 30). Clomiphene citrate is marketed as a racemic mixture of enclomiphene (E, trans) and zuclomiphene (Z, cis) in a ratio of approximately 3 to 2. The Z-isomer may have greater ovulation-inducing properties than the trans isomer. An important recent observation is that nonsteroidal triphenylethylene compounds like tamoxifen and clomiphene may require bioactivation by the liver cytochrome P450 enzyme 2D6. In one study, liver microsomes containing active *CYP2D6*, metabolized clomiphene to two potent estrogen antagonist compounds, (E)-4-hydroxyclomiphene and (E)-4-hydroxy-N-desethylclomiphene.[60] These two compounds demonstrated 50% inhibition of estrogen receptor function at concentrations of 2.5 and 1.4 nM, respectively. The activity of *CYP2D6* shows significant variation among women, suggesting that allelic variation in this enzyme may contribute to the variability in the response to clomiphene.

Clomiphene has a half-life of approximately 5 days. It is metabolized by the liver and excreted in the feces. Fecal clomiphene can be detected up to 6 weeks after discontinuing the drug. In *normally cycling women*, the administration of clomiphene citrate, 150 mg daily for 3 days, resulted in an increase of serum concentration of LH and FSH of 40% and 50%, respectively.[61] In addition, LH pulse frequency increased from 3.3 to 6.8 pulses per 8 hours. The clomiphene-induced increase in LH pulse frequency indicates that clomiphene has an action at the hypothalamus. In women with PCOS who already have a high LH pulse frequency, clomiphene does not further increase LH pulse frequency, but it does increase LH pulse amplitude and serum levels of LH and FSH.[62] Successful induction of ovulation with clomiphene requires an intact hypothalamic-pituitary-ovarian axis. In contrast, exogenous gonadotropin treatment is effective even in the absence of a functional hypothalamus or pituitary.

Evidence that clomiphene has central nervous system effects includes the observation that clomiphene induces vasomotor symptoms,[63] increases LH pulse frequency,[61] and partially blocks the contraceptive potency of estrogen.[64] Studies in laboratory animals demonstrate that clomiphene can decrease estrogen stimulated hypothalamic tyrosine hydroxylase[65] and that clomiphene increases GnRH secretion from the rat medial basal hypothalamus.[66]

In addition to a hypothalamic site of action, clomiphene also has biological effects on the pituitary, ovary, endometrium, and cervix. In incubations of rat pituitary cells, both estradiol and clomiphene augmented GnRH-induced release of FSH and LH. Zhuang and colleagues[67] demonstrated that clomiphene, estradiol, and diethylstilbestrol all augmented gonadotropin induction of aromatase activity in rat granulosa cells. In hypoestrogenic women receiving exogenous estrogen, clomiphene can cause endometrial atrophy.[68] Clomiphene can diminish estrogen-induced cervical mucus quantity and quality, as demonstrated by decreased ferning and spinbarkeit formation.[69,70]

The WHO classification divides women with anovulation into three major groups: WHO Group I consists of those women with anovulation, low levels of endogenous gonadotropins, and very little endogenous estrogen production (hypogonadotropic hypogonadism). WHO Group II consists of those women with anovulation or oligo-ovulation, a wide variety of menstrual disorders, relatively normal (or elevated) gonadotropin levels, and evidence for significant endogenous estrogen production.[71] Many women in WHO Group II have PCOS. WHO Group III are women with ovarian failure (hypergonadotropic hypogonadism).

Clomiphene is most effective in inducing ovulation in women in WHO Group II. In women with severe hypoestrogenism and hypogonadotropic hypogonadism (WHO Group I), clomiphene is typically ineffective in the induction of ovulation. In contrast, women in WHO Group I respond very well to gonadotropin injections or pulsatile GnRH treatment. Failure to have a withdrawal uterine bleed following the administration of progesterone is presumptive evidence of severe hypoestrogenism in women with anovulation and an anatomically normal uterus.[72] Clomiphene is unlikely to effectively induce ovulation in this setting.[72] The mechanisms responsible for the low rate of ovulation in response to clomiphene in women with hypogonadotropic hypogonadism and low endogenous production of estrogen

are not fully characterized. Maruo and colleagues[73] have reported that clomiphene induction of ovulation has a low chance of success in women with triiodothyronine levels below 80 ng/mL. In preliminary studies, thyroid hormone supplementation appeared to increase the efficacy of clomiphene induction of ovulation in these women.

Clomiphene citrate is also unlikely to be effective in women with a consistently elevated FSH concentration (depletion of oocyte pool). Although clomiphene is relatively contraindicated in women with pituitary tumors, it has been reported to be effective in the induction of ovulation in women with prolactinomas who did not ovulate with bromocriptine treatment alone.[74] Many physicians prefer to document tubal patency and evaluate semen parameters before initiating ovulation induction.

The FDA-approved dosages for clomiphene are 50 or 100 mg daily for a maximum of 5 days per cycle. After spontaneous menses, or the induction of menses with a progestin withdrawal, clomiphene is started on cycle day 3, 4, or 5 at 50 mg daily for 5 days. Starting clomiphene on cycle day 3 or 5 does not appear to influence the per-cycle pregnancy rate.[75] In properly chosen women, approximately 50% will ovulate at the 50 mg daily dosage; another 25% will ovulate if the dose is increased to 100 mg daily.[76] During each cycle, determination of ovulation should be attempted. In most patients, ovulation occurs approximately 5 to 12 days after the last dose of clomiphene. Measurement of the urinary LH surge is often recommended to assist the couple in prospectively determining the periovulatory interval.

Although the FDA has approved maximal clomiphene doses of 100 mg daily, many clinicians have experience prescribing clomiphene at doses of up to 250 mg daily. A woman who does not ovulate with a clomiphene dose below 100 mg daily for 5 days may ovulate if her dose is increased up to 250 mg daily for up to 14 days. Of the women who do not ovulate at doses below 100 mg daily, up to 70% will ovulate at higher doses, but less that 30% become pregnant.[77]

Anovulatory women in WHO Group II have a fecundability of 0.00 without treatment. Over the first three to six cycles of clomiphene treatment, the fecundability is in the range of 0.08 to 0.25. In cases where the only fertility factor is anovulation in the female partner, fecundability with clomiphene treatment is in the range of 0.20 to 0.25 (Fig. 23.4).[78] Women with hyperandrogenemia, markedly elevated BMI, amenorrhea, or advanced age are less likely to ovulate with clomiphene.[79] A unique advantage of clomiphene is that few fertility treatments are available that increase fecundability from 0.00 to 0.20 at a cost in the range of $100. After 3 to 6 months of clomiphene treatment, fecundability appears to decline.

Prior to initiating a clomiphene cycle, many experts obtain a pregnancy test to rule-out an ongoing pregnancy, then prescribe a progestin withdrawal. A commonly used progestin withdrawal is medroxyprogesterone acetate (Provera) 10 mg daily for 5 days. The first day of full withdrawal flow following the cessation of the progestin treatment is Day 1 of the cycle.

During the clomiphene plus dexamethasone treatment cycle, urine LH measurements can be measured by the patient at home to identify the preovulatory LH surge. The LH surge typically occurs 5 to 12 days after the last day of clomiphene medication. The woman's maximal fertile time

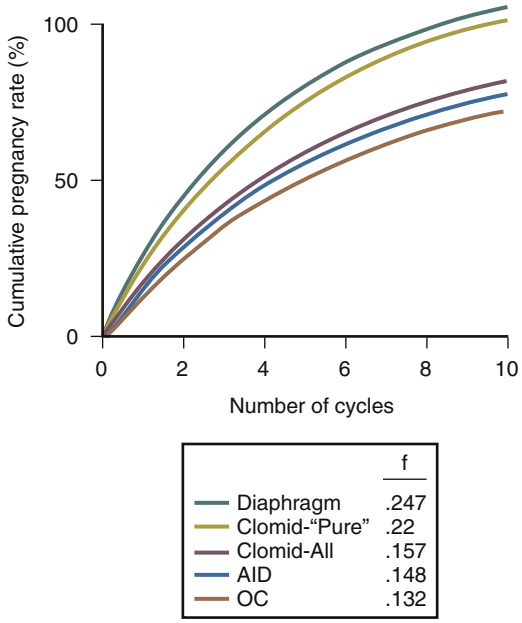


FIGURE 23.4 Cumulative pregnancy rates in women treated with clomiphene for infertility, women discontinuing contraception with the diaphragm or oral contraceptives (OC), and women treated with donor insemination (AID). *(From Hammond MC. Monitoring techniques for improved pregnancy rates during clomiphene ovulation induction.* Fertil Steril *42:503, 1984, with permission.)*

is the day before the urine LH surge, the day of the urine LH surge, and the day following the urine LH surge. Coitus should occur on at least 2 of these 3 days. Alternatively, if the woman prefers not to measure urine LH she can have coitus with her partner every other day for 8 days beginning 5 days after the last clomiphene tablet. Evidence for successful clomiphene induced ovulation is an appropriately drawn serum progesterone level greater than 8 ng/mL. In most clomiphene cycles where successful ovulation has occurred the serum progesterone level is greater than 20 ng/mL. If a menses does not occur within 17 days following the LH surge, a pregnancy test can be obtained.

Preliminary reports suggest that ovulation-inducing medications may be associated with ovarian tumors[80,81] and that the risk may increase with extended use of ovulation-inducing agents. My current view of this data is that nulliparity and infertility are far more important risk factors for ovarian tumors than treatment with an ovulation-inducing medication.[82-84] However, given the reduced fecundability observed in women who do not achieve pregnancy after 6 clomiphene cycles and the risk of ovarian tumors associated with prolonged exposure to clomiphene, limiting clomiphene treatment to fewer than 12 cycles is reasonable. Failure to achieve pregnancy after six clomiphene treatment cycles should prompt a thorough review of the potential causes of the failure and consideration of a new approach to treatment, such as gonadotropin therapy. After four ovulatory cycles induced by clomiphene without achieving a pregnancy, a diagnostic laparoscopy may reveal endometriosis or pelvic adhesions in over 50% of women.[85]

The role of hCG administration in enhancing the pregnancy rate associated with clomiphene treatment is controversial. Some authorities believe that the combination

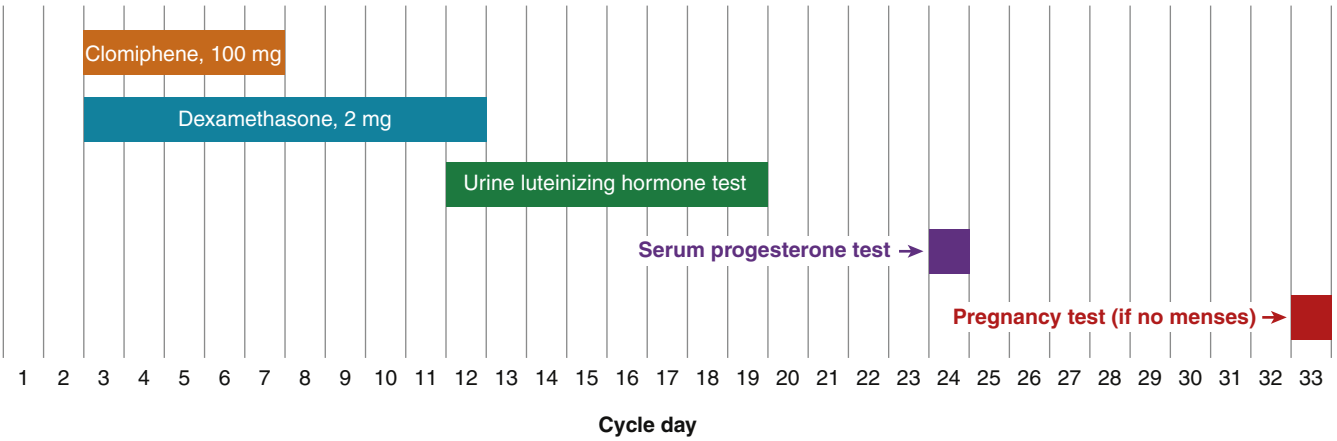


FIGURE 23.5 Schematic outline of a combined clomiphene plus dexamethasone cycle for women who are resistant to ovulation with clomiphene monotherapy.

of clomiphene and a single dose of hCG may increase the efficacy of clomiphene induction of ovulation when women do not ovulate on standard doses of clomiphene.[86] However, in most trials, hCG administration does not consistently improve pregnancy rate compared to women who spontaneously ovulate.[87] After administering clomiphene, sonography can be utilized to monitor follicle size. When mean follicle diameter reaches 18 to 22 mm, hCG can be administered.[88] However, other authorities believe that there is little evidence to support the use of hCG in combination with clomiphene. In one small clinical trial, women receiving clomiphene from cycle days 3 to 7 to induce ovulation were randomized to receive hCG (10,000 IU) to time ovulation, or to monitor the endogenous LH surge using urinary LH testing kits. The pregnancy rate was similar in both groups.[89]

Clomiphene treatment can be associated with adverse changes in the reproductive tract, including induction of a luteal phase defect and the creation of a hostile cervical environment due to low quantity and quality of cervical mucus.[90] Some clinicians recommend endometrial biopsy in a test cycle of clomiphene treatment to assess whether or not clomiphene induces luteal phase deficiency, and many recommend that a postcoital test be performed during the first clomiphene cycle.

In one study of 2369 clomiphene-induced pregnancies, 7% were twins, 0.5% were triplets, 0.3% were quadruplets, and 0.13% were quintuplets.[91] This finding demonstrates that the absolute risk of high-order multiple gestation with clomiphene treatment is quite low. Because clomiphene is a heavily prescribed medication, however, the number of triplets resulting from clomiphene treatment is substantial. In one study that reviewed all the high-order multiple gestations at one tertiary care center, more triplets were conceived after clomiphene treatment than were conceived after treatment with exogenous gonadotropin therapy.[92] The rate of spontaneous abortion after clomiphene-induced ovulation and pregnancy is approximately 15%. The most common symptoms experienced by women taking clomiphene include: vasomotor symptoms (20%), adnexal tenderness (5%), nausea (3%), headache (1%), and, rarely, blurring of vision or scotomata. Most clinicians permanently discontinue clomiphene treatment in women with clomiphene-induced visual changes.

Clomiphene Plus Glucocorticoid Induction of Ovulation

Women with WHO II anovulation who have failed to ovulate using standard doses of clomiphene are referred to as being "clomiphene resistant." A consensus panel of expert fertility specialists recommended that for women with PCOS who are clomiphene resistant the most appropriate next steps in treatment include FSH injections or laparoscopic ovarian drilling.[93] However, for many women these options may be prohibitively expensive. For the clomiphene resistant woman with PCOS, what treatment can be prescribed that is affordable?

Many clomiphene resistant women will ovulate if they are treated with a combination of both clomiphene and dexamethasone. Before initiating combination therapy with clomiphene plus dexamethasone includes the results of the infertility work up should be reviewed to be sure that tubal and male factors are not contributing to the fertility problem. Two randomized clinical trials have reported that, in clomiphene-resistant women, dexamethasone plus clomiphene treatment results in an increase in ovulation and pregnancy rates compared to clomiphene alone.[94,95] One regimen that has been reported to be successful is to treat the clomiphene-resistant woman with clomiphene 100 mg daily for cycle days 3 to 7, and *simultaneously* treat her with dexamethasone 2 mg daily for cycle days 3 to 12 (Fig. 23.5).[94] Treatment with dexamethasone reduces the serum concentration of androgens, thereby increasing the efficacy of the clomiphene. In the randomized trial that used this regimen to treat clomiphene resistant women, the ovulation rate was 75% in the clomiphene plus dexamethasone group and 15% in the clomiphene alone group ($P < 0.001$). The pregnancy rate was 40% in the clomiphene plus dexamethasone group and 5% in the clomiphene alone group ($P < 0.05$). Other investigators have also reported that a glucocorticoid is an effective adjuvant to clomiphene treatment for certain women.[96-98]

Many clinicians instruct their patients to take the dexamethasone at night in order to maximally blunt the early morning corticotropin (ACTH) surge, which stimulates adrenal androgen production. However, experienced clinicians have found that for many women a nighttime dose

of dexamethasone energizes them and causes difficulty in falling and remaining asleep. Some experts recommend that patients take the dexamethasone in the morning. If the combination of clomiphene (100 mg daily for 5 days) plus dexamethasone does not cause ovulation, a cycle with a clomiphene dose of 150 mg daily for cycle days 3 to 7 plus dexamethasone can be prescribed. If this regimen does not cause ovulation, the patient should consider other options for ovulation induction such as weight loss, FSH injections, laparoscopic ovarian drilling, or *in vitro fertilization (IVF)*.

Clomiphene and Estrogen-Progestin Pretreatment

A risk factor for failure to ovulate with clomiphene is an elevated circulating testosterone level. Estrogen-progestin pretreatment prior to a cycle of clomiphene may improve ovulation rates by suppressing circulating testosterone prior to initiation of a clomiphene cycle. In a small case series and a randomized trial, 2 months of continuous estrogen-progestin contraceptive pill prior to treatment with clomiphene was reported to decrease circulating testosterone levels and improve ovulation and pregnancy rates in women with PCOS who had failed to ovulate with clomiphene 150 mg daily for 5 days.[99] In the randomized trial, 48 women who had failed to ovulate with clomiphene 150 mg daily for 5 days were randomized to 42 to 50 days of pretreatment with an estrogen-progestin contraceptive (ethinylestradiol 0.03 mg plus desogestrel 0.15 mg daily with no break) followed by clomiphene 100 mg daily for 5 days, or clomiphene alone.[100] The oral contraceptive regimen (OCP) significantly reduced circulating testosterone prior to initiation of clomiphene. The ovulation rate was 65% and 11% respectively in the OCP-clomiphene citrate (CC) group versus the CC group. Per cycle pregnancy rates were 54% and 4% respectively. A regimen of OCPs followed by clomiphene may be clinically indicated in women who have failed to ovulate with clomiphene or in women known to have an elevated total testosterone. A large-scale randomized trial of this sequential regimen is warranted.

Clomiphene Plus Estrogen and Progesterone Treatment of the Endometrium

In some cases, clomiphene has been reported to adversely affect cervical mucus quality and endometrial morphology.[101] This effect is probably mediated through the anti-estrogenic properties of clomiphene and its metabolites. The anti-estrogenic effects of clomiphene on the cervix and endometrium possibly reduces the pregnancy rate during clomiphene treatment. A number of investigators have reported that the addition of estrogen to clomiphene treatment can increase endometrial growth compared with that observed with clomiphene alone. In a well-designed study, vaginal estradiol (0.1 mg daily) from day 8 to the LH surge and progesterone gel (90 mg daily) starting 3 days after ovulation were demonstrated to improve the number of "in-phase" endometrial biopsies in women taking clomiphene.[102] As of yet, no clinical trial has demonstrated that the combination of clomiphene plus estrogen/progesterone increases pregnancy rates compared with clomiphene without estrogen/progesterone.

An alternative approach to dealing with the anti-estrogenic effects of clomiphene is to start treatment on cycle day 1 rather than cycle days 3, 4, or 5. Conceptually, by treating with clomiphene from cycle days 1 to 4, there are more days for clomiphene to "wash out" of the body prior to ovulation. In one randomized study, 23 women with unexplained infertility were randomized to receive clomiphene (plus intrauterine insemination [IUI]) with treatment from cycle days 1 to 5 or cycle days 5 to 9. The pregnancy rate was reported to be higher in the group that received the clomiphene treatment from cycle days 1 to 4.[103]

Clomiphene and Non-Classical Adrenal Hyperplasia

Many authorities recommend that infertile anovulatory women with non-classical adrenal hyperplasia (NCAH) receive glucocorticoids for induction of ovulation. However, some women with long-standing NCAH also have evidence of ovarian hyperandrogenism and polycystic ovaries by sonographic imaging. Clomiphene alone[104] or clomiphene plus glucocorticoids can be used to induce ovulation and achieve pregnancy in infertile women with NCAH.

Clomiphene Plus Gonadotropin Induction of Ovulation

In women who do not ovulate with standard doses of clomiphene citrate, gonadotropin injections can be added to clomiphene treatment to induce ovulation.[105] The main benefit of this approach to ovulation induction is that it tends to reduce the quantity of gonadotropins needed to induce ovulation during each cycle. The initial rise in LH and FSH induced by clomiphene increases the sensitivity of the follicles to respond to the gonadotropin injections. Typically, clomiphene at doses of 100 to 200 mg daily is administered for 5 days, followed by the initiation of FSH or LH-FSH injections. Investigators have reported that this regimen is associated with a 50% decrease in the dose of gonadotropin required to induce ovulation.[106]

Clomiphene and Metformin

Hyperinsulinemia is a common endocrine abnormality observed in women with PCOS (see Chapter 22). The elevated insulin levels contribute to the reproductive dysfunction by suppressing hepatic sex-hormone-binding globulin production and possibly by acting as a co-gonadotropin with LH in stimulating thecal cell androgen synthesis. Thus, reducing insulin levels is a therapeutic goal in women with PCOS.

Metformin is an oral biguanide antihyperglycemic agent approved for the treatment of type 2 diabetes mellitus. Metformin decreases blood glucose by inhibiting hepatic glucose production and by enhancing peripheral glucose uptake, possibly by interacting with the Peutz-Jegher syndrome tumor suppressor gene *(LKB1)* which activates adenosine-monophosphate-activated protein kinase.[107] It increases insulin sensitivity at the postreceptor level and stimulates insulin-mediated glucose disposal. Generic extended release metformin is available in doses of 500-, 750-, and 1000-mg tablets. The target metformin dose is in the range of 1500 to 2550 mg daily. When using metformin extended-release tablets, the entire daily dose is given at dinner time. To minimize gastrointestinal adverse effects like nausea, many clinicians recommend that metformin should be started at 500 or 750 mg daily for 1 week, followed by an increase in the dose to the target range. Progesterone levels can be measured periodically at appropriate days to determine if

ovulation has occurred, or the patient can keep a basal body temperature record. If ovulation has not occurred after 5 to 10 weeks of metformin treatment, then clomiphene, 50 mg daily for 5 days, can be administered in conjunction with metformin. If the patient becomes pregnant, the metformin therapy can be discontinued. Metformin is a category B drug for pregnant women and has been used by some clinicians to treat diabetes in pregnant women.

The most common adverse effects associated with metformin are gastrointestinal disturbances, including diarrhea, nausea, vomiting, and abdominal bloating. In rare cases, metformin treatment has caused fatal lactic acidosis. In most of these cases, some degree of renal insufficiency was present. Prior to initiating treatment with metformin, the patient's serum creatinine concentration should be measured and demonstrated to be less than 1.4 mg/dL. Other insulin sensitizers may also be effective in induction of ovulation, either alone, or in combination with clomiphene or FSH.

Clinical trials have reported conflicting results concerning the relative efficacy of metformin versus clomiphene. In general, the majority of large-scale clinical trials have reported that both metformin and clomiphene monotherapy are effective at inducing ovulation in women with PCOS, but clomiphene results in a greater per cycle ovulation, conception, and birth rates than metformin.[108] In this study, 626 women with anovulatory infertility caused by PCOS were randomized to receive clomiphene alone, metformin alone, or clomiphene plus metformin. The live birth rate was 27% in the clomiphene-metformin group, 23% in the clomiphene group, and 7% in the metformin group. In contrast, other investigators have reported that single-agent treatment with clomiphene or metformin results in similar pregnancy rates.[109] In some studies, metformin appears to be more effective in inducing ovulation in women with an above average waist to hip ratio, a marker of increased visceral fat.[110]

Clomiphene versus Tamoxifen or Raloxifene for Ovulation Induction

Clomiphene, tamoxifen, and raloxifene are mixed estrogen agonists-antagonists with varying degrees of agonist or antagonist activity in different end organs. In a randomized trial, 371 with anovulatory infertile women with PCOS were randomized to receive clomiphene citrate 100 mg daily or tamoxifen 20 mg daily for 5 days. The ovulation rate was 64% in the clomiphene group and 52% in the tamoxifen group ($P = 0.01$) and the pregnancy rate was 19% and 11% in the clomiphene and tamoxifen groups respectively ($P = 0.04$).[111] Clomiphene at a dose of 100 mg daily appears to be superior to tamoxifen at a dose of 20 mg daily for induction of ovulation in women with PCOS. In a small clinical trial, 5 days of treatment with clomiphene 100 mg daily or raloxifene 100 mg daily resulted in a similar rate of ovulation, but pregnancy rate was not studied in this trial.[112]

Aromatase Inhibitors

Clomiphene is effective in inducing ovulation because of its anti-estrogenic properties. During clomiphene treatment, the hypothalamus and pituitary register a decrease in estradiol feedback, and consequently there is an increase in FSH and LH secretion. Aromatase inhibitors including letrozole and anastrozole block estradiol synthesis, reduce estradiol feedback and increase production of FSH in premenopausal women. Letrozole at doses of 2.5 mg to 7.5 mg daily for 5 days, and anastrozole at a dose of 1 mg daily for 5 days have been demonstrated to induce ovulation in WHO II women. In a clinical trial comparing clomiphene 100 mg daily for 5 days versus letrozole 7.5 mg daily for 5 days in infertile couples undergoing ovarian stimulation plus intrauterine insemination (IUI), the pregnancy rates per cycle were 12% in the letrozole group and 9% per cycle in the clomiphene group.[113] In a clinical trial of letrozole 2.5 mg for 5 days versus anastrozole 1 mg daily for 5 days for ovulation induction in women with PCOS, the per cycle pregnancy rate was 19% in the women treated with letrozole and 10% in the women treated with anastrozole.

In one large randomized clinical trial, clomiphene was demonstrated to be superior to anastrozole for the induction of ovulation in women with WHO II anovulation. In this trial, 271 women with WHO II anovulatory infertility were randomized to receive for 5 days: clomiphene 50 mg, anastrozole 1 mg or 5 mg or 10 mg. The ovulation rates were 65% for clomiphene and 30%, 37%, and 36% for anastrozole 1 mg, 5 mg, and 10 mg, respectively. This study suggests that clomiphene is superior to anastrozole for ovulation induction.[114]

Aromatase inhibitors are not approved by the FDA for ovulation induction. Pregnancy and birth registries indicate that pregnancy outcome following ovulation induction with aromatase inhibitors is good.[115] In one registry, the risk of congenital cardiac malformations was greater with clomiphene induced pregnancy than with letrozole induced pregnancy.[116] However, concern remains about the potential adverse effects of these agents on pregnancy, especially given known adverse effects on rabbit and rodent pregnancy.

Ovulation induction and ovarian stimulation in women with estrogen-sensitive tumors, such as breast cancer may be a special situation that warrants the use of aromatase inhibitors.[117] Aromatase inhibitors increase FSH levels, but block estradiol production resulting in folliculogenesis with relatively reduced levels of circulating estradiol. In women with a history of estrogen-sensitive tumors, inducing folliculogenesis and ovulation while maintaining relatively low levels of circulating estradiol has a theoretical advantage.[118] This effect may be especially advantageous in women with a history of breast cancer planning on undergoing an IVF cycle.[119]

Aromatase inhibitors may be an effective monotherapy option for women who are clomiphene resistant. In one trial, 250 anovulatory women with PCOS who did not ovulate with standard doses of clomiphene were randomized to treatment with letrozole 2.5 mg daily for 5 days or metformin 1500 mg plus clomiphene 150 mg daily for 5 days. The ovulation rate was 65% in the letrozole group and 70% in the metformin-clomiphene group. The pregnancy rate was 15% and 14% in the letrozole and clomiphene groups respectively.[120] Some clinicians believe that aromatase inhibitors are especially effective in inducing ovulation in obese and morbidly obese women who are resistant to clomiphene.[121] In obese and morbidly obese women, aromatase inhibitors may be able to reduce peripheral aromatization of androgens, thereby reducing adipose-dependent estrogen negative feedback on the hypothalamus and pituitary and increasing the rate of ovulation.

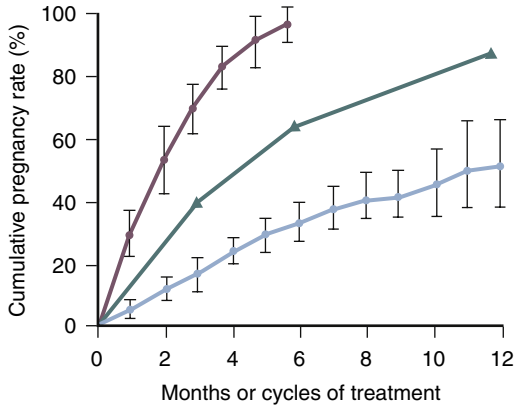


FIGURE 23.6 Cumulative pregnancy rates for infertile anovulatory women treated with gonadotropins for ovulation induction. Solid circles represent the cumulative pregnancy rate in women in World Health Organization WHO Group I. Open circles represent the cumulative pregnancy rate in women in WHO Group II who have failed induction of ovulation with clomiphene. For comparison, the triangles represent cumulative pregnancy rate in normal women. *(From Dor I, Itzkowic D, Mashiach S. Cumulative pregnancy rates following gonadotropin therapy.* Am J Obstet Gynecol *136:102–105, 1980, with permission.)*

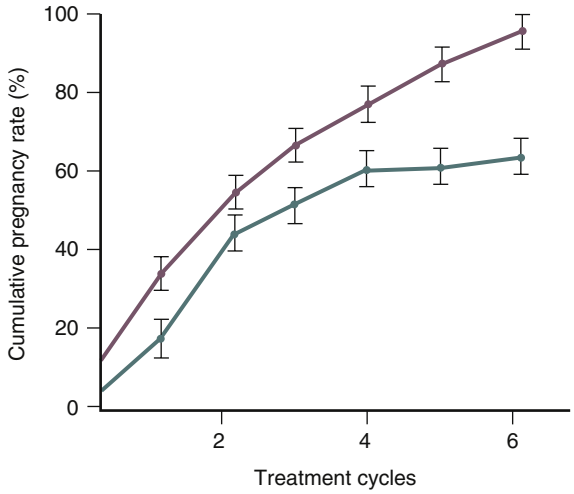


FIGURE 23.7 Cumulative pregnancy rates for hypogonadotropic anovulatory women (World Health Organization WHO Croup I) treated with gonadotropins. Solid circles represent the cumulative pregnancy rate in women less than 35 years old. Open circles represent the cumulative pregnancy rate in women more than 35 years old. *(From Lunenfeld B, Insler V.* Human gonadotropins. *In Wallach EE, Zacur HA [eds].* Reproductive Medicine and Surgery. *St. Louis: Mosby, 1995, p 617.)*

Gonadotropin Induction of Ovulation

The use of gonadotropins, gonadotropins plus GnRH antagonists, and gonadotropins plus growth hormone to treat infertility; and the prevention and treatment ovarian hyperstimulation syndrome are discussed in detail in Chapter 30. Important concepts related to the use of gonadotropins to treat anovulatory infertility is that women with the best prognosis for success have hypogonadotropic hypogonadism (WHO I) (Fig. 23.6) and are younger than 35 years of age (Fig. 23.7 and Fig. 23.8).

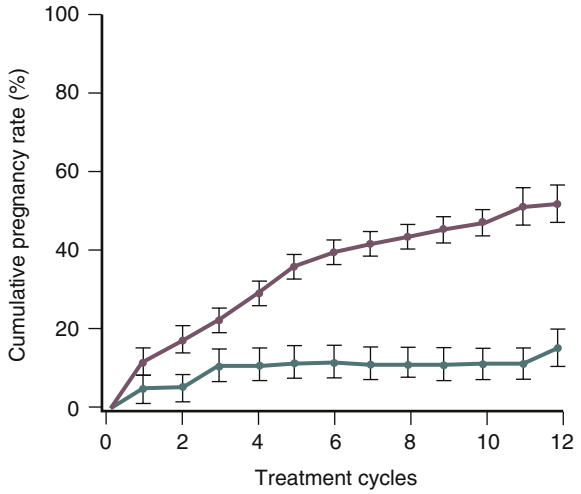


FIGURE 23.8 Cumulative pregnancy rates following gonadotropin treatment for anovulatory women who did not respond to clomiphene induction of ovulation (WHO Croup II). Solid circles represent the cumulative pregnancy rate in women less than 35 years old. Open circles represent the cumulative pregnancy rate in women more than 35 years old. *(From Lunenfeld B, Insler V.* Human gonadotropins. *In Wallach EE, Zacur HA [eds].* Reproductive Medicine and Surgery. *St. Louis: Mosby, 1995, p 617.)*

Ovarian Surgery for Ovulation Induction in PCOS

Ovarian wedge resection was one of the first treatments used to induce ovulation in women with PCOS. However, classical ovarian wedge resection was associated with ovarian and tubal adhesions. Laparoscopic ovarian drilling using insulated needle cautery has been reported to be associated with a 70% rate of ovulation and a 50% pregnancy rate.[122] The procedure can be performed by immobilizing the ovary with a probe and inserting an insulated needle electrode into the ovary. A cutting current of 100 watts can be used to ease the needle into the ovarian cortex. After the needle is inserted into the ovary, a 40-watt coagulating current is applied for 2 seconds at each point of puncture. Each ovary can be treated with 5 to 10 punctures; smaller ovaries should be treated with fewer punctures. In general, one or two punctures are not adequate to ensure ovulation postoperatively.[123] At the completion of the procedure, 1000 mL of crystalloid solution can be left in the pelvis.

In one clinical trial, 88 anovulatory, infertile women with PCOS who had not ovulated with clomiphene were randomized to ovarian surgery, FSH injections, or combination LH-FSH injections. The ovulation rate (70%) and the pregnancy rate (50%) were similar in all three groups. The spontaneous abortion rate was higher in the two groups that received gonadotropins. The investigators concluded that ovarian surgery was as efficacious as gonadotropin injections for ovulation induction in women with PCOS who did not ovulate with clomiphene.[124] In another clinical trial, women with PCOS who failed to ovulate with clomiphene 150 mg daily for 5 days were randomized to gonadotropin injections or laparoscopic ovarian diathermy. Six months after surgery, cumulative spontaneous pregnancy rate was 28%. After three cycles of gonadotropin injections, cumulative pregnancy rate was 33%.[125]

The risks of ovarian surgery for women with hirsutism are greater than the potential benefits. However, for women with PCOS and infertility who have been unable to become pregnant with weight loss, clomiphene and metformin treatment; the option of ovarian surgery for ovulation induction may be warranted prior to induction of ovulation with FSH. Survey data indicate that many patients prefer one surgical intervention compared to ovulation with FSH injections, if the two treatments have comparable success rates.[126] Cost-benefit analyses suggest that laparoscopic surgery for ovulation induction is associated with less healthcare costs than FSH injections.[127] Other authorities believe that the rare serious risks associated with surgery makes FSH injections the preferable option.[128]

Gonadotropin-Releasing Hormone (GnRH) Induction of Ovulation

A key feature of hypothalamic biology is the pulsatile release of the decapeptide GnRH from the arcuate nucleus into the pituitary portal circulation. The pulsatile release of GnRH stimulates the pituitary to produce LH and FSH in a pulsatile manner. In turn, pituitary gonadotropin secretion stimulates follicular development, ovulation, and progesterone secretion in the luteal phase. In women with WHO Group I anovulation (low levels of endogenous gonadotropins and decreased endogenous estrogen production), the pulsatile administration of GnRH is effective in inducing ovulation (Fig. 23.9). The advantages of GnRH induction of ovulation include a reduced need for cycle monitoring and a reduced risk of multiple gestation due, in part, to an intact pituitary feedback system. Unfortunately, GnRH for ovulation induction is currently not available in the United States.

Santoro and colleagues[129] proposed eight criteria for identifying women most likely to safely achieve ovulation with pulsatile GnRH:

1. Primary or secondary amenorrhea for at least 6 months
2. Absence of hirsutism, galactorrhea, or ovarian enlargement
3. Weight not below 90% of ideal body weight
4. No excessive exercise or stress
5. Normal serum prolactin, TSH, dehydroepiandrosterone sulfate (DHEAS), and testosterone concentrations
6. Low gonadotropin concentrations
7. No evidence for a structural central nervous system lesion
8. No recent hormone treatment

GnRH is administered using a computerized pump that delivers one pulse of GnRH every 90 minutes at a dose of 75 to 100 ng/kg per pulse. Interpulse intervals as short as 1 hour[130] or as long as 2 hours[131] have been successfully utilized. GnRH doses as low as 25 ng/kg per pulse can successfully induce ovulation, but are associated with subnormal luteal phase progesterone secretion.[130] Both intravenous and subcutaneous administration of GnRH have been successfully used to induce ovulation. Intravenous administration probably results in more reliable induction of ovulation, but this route is associated with more technical problems (restarting the intravenous catheter) and risk of infection than subcutaneous administration.

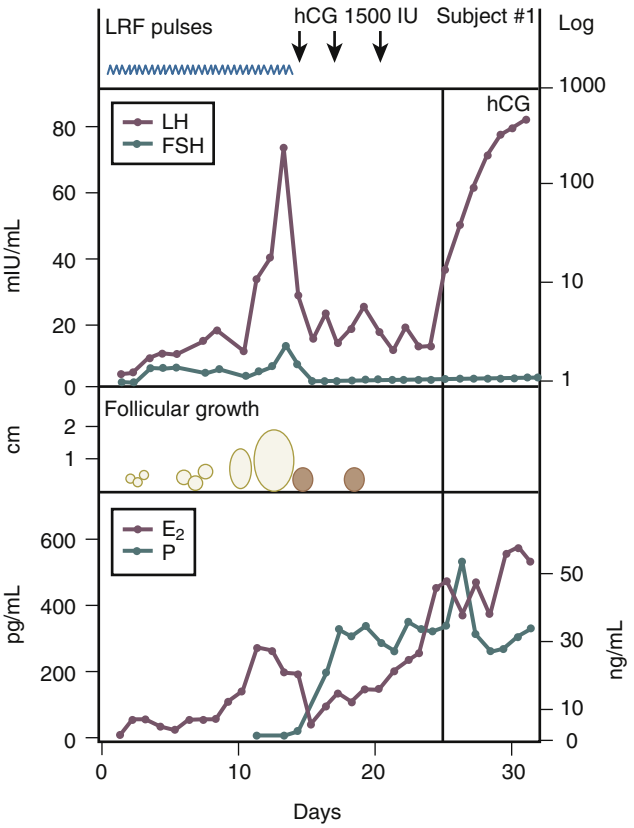


FIGURE 23.9 Endocrine and ovarian follicular response to induction of ovulation with gonadotropin releasing hormone, 5 μg intravenous bolus over 2 hours. LRF, Luteinizing hormone releasing factor, more commonly known as gonadotropin releasing hormone; hCG, human chorionic gonadotropin; LH, luteinizing hormone; FSH, follicle stimulating hormone; P, progesterone; E_2, estradiol; IU, international units; pg, pictograms; ng, nanograms; mIU, milli-international units; mL, milliliters. *(From Reid RL, Leopold GR, Yen SSC. Induction of ovulation and pregnancy with pulsatile luteinizing hormone releasing factor: dosage and mode of delivery.* Fertil Steril *36:565, 1981, with permission.)*

The intensity of clinical monitoring can range from regular follicle monitoring with sonography and serum estradiol measurements to basal body temperature measurement with use of an ovulation predictor kit. Low-intensity monitoring is acceptable because the risk of multiple pregnancy or ovarian hyperstimulation is low with pulsatile GnRH therapy. If no response is observed after 2 to 3 weeks of treatment, the GnRH dose per pulse can be increased to the range of 10 μg to 20 μg per pulse.[131]

Studies that directly compare the efficacy of gonadotropin versus pulsatile GnRH induction of ovulation report equivalent rates of ovulation and pregnancy. However, the risk of multiple gestation is probably higher with gonadotropin treatment (14%) than with pulsatile GnRH treatment (8%).[132] This increased risk is due to a higher rate of multifollicular development with gonadotropin treatment (48% of cycles) than with pulsatile GnRH treatment (19% of cycles). Pulsatile GnRH results in a decreased risk of high-order multiple gestation when compared with gonadotropin treatment.[133]

Hyperprolactinemia

Infertile women with hyperprolactinemia and anovulation often achieve pregnancy after treatment with a dopamine agonist such as bromocriptine or cabergoline. The treatment of hyperprolactinemia is discussed in Chapter 3.

Luteal Phase Deficiency

Historically, *luteal phase deficiency* has been defined as the delayed maturation of the endometrium determined by histological dating of tissue obtained by endometrial biopsy, which lags appropriate development by at least 2 days (see Chapter 10). However, most recent research indicates that delayed maturation of the endometrium is commonly observed at a similar rate in both fertile and infertile women.[134] In addition, histological "dating the endometrium" probably does not have the accuracy or precision necessary to use it as a guide to infertility treatment.[135] Currently it is believed that competent endometrial maturation occurs over a wide range of progesterone concentrations. In human experiments, a progesterone concentration in the range of 5 ng/mL, approximately 75% below normal luteal progesterone concentration, supports adequate histological and biochemical secretory changes consistent with a competent secretory endometrium.[136] Consequently, the role of luteal phase deficiency in female infertility has diminished.

From an endocrine perspective, follicular or luteal function that is far outside the normative range is likely associated with reduced fertility. In the most extreme example, it is known that resection of the corpus luteum and the associated reduction in progesterone secretion, reduces fertility and causes abortion in women prior to about 49 days of pregnancy.[137] In addition, numerous clinical trials have observed that luteal phase progesterone support improves pregnancy rates following IVF cycles involving controlled ovarian hyperstimulation and ovarian follicle puncture to harvest oocytes.[138] It is probable that an occasional case of infertility may be caused by poor follicle development and inadequate corpus luteal secretion of progesterone or a relative resistance to progesterone effect in the endometrium. It is unlikely that luteal phase deficiency is a common cause of infertility.

The Aging Ovary, the Aging Follicle

An immutable feature of ovarian physiology is that the number of oocytes and follicles, is fixed *in utero* and declines following an exponential curve (mathematically similar to the curve for the decay of radioactive material) from the second trimester, as shown in Figure 23.10 (also see Chapters 9 and 15). At birth, estimates of the number of oocytes and follicles in a pair of human ovaries is approximately 2 million. At the completion of puberty, the number of eggs in a pair of human ovaries is in the range of 250,000. After 35 to 37 years of age, the rate of loss of oocytes and follicles appears to accelerate.[139,140] During adult reproductive life, follicles most sensitive to the growth promoting effects of FSH are apparently first selected to become the dominant follicle. As the ovary and the residual follicular pool ages, the remaining follicles appear to be relatively resistant to FSH. The aging follicles contain oocytes that are less likely to result in a successful pregnancy. The decrease in fecundability

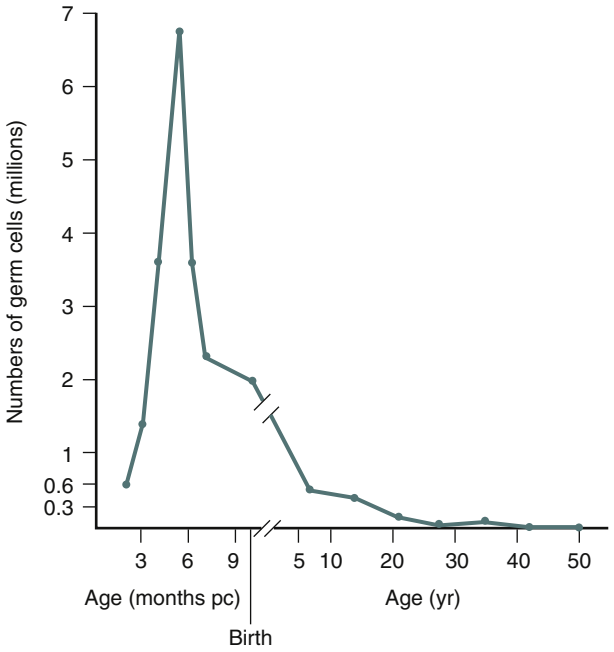


FIGURE 23.10 Changes in the total number of oocytes (and follicles) in human ovaries before and after birth. The number of germ cells in the ovary peaks *in utero* during the second trimester. *(From Baker TC. Radiosensitivity of mammalian oocytes with particular reference to the human female.* Am J Obstet Gynecol *110:746–761, 1971.)*

associated with aging is probably due to a decline in both the quantity and quality of the oocytes. Data to support the concept that the aging oocyte is less likely to result in a successful pregnancy comes from multiple sources (see Fig. 23.7 and Fig. 23.8).

Recent research has called into question some classical tenets of ovarian aging including the potential for renewal of follicles and oocytes in the adult ovary and the mathematical characteristics of the rate of decline of small follicles. Most mammalian species do not have histologically detectable mitotic oogonia or stem-like germ cells in the adult ovary. However, recent studies in rodents and humans have reported that there are rare stem cells in the adult ovary that can be harvested, stimulated to undergo mitotic replication and then be triggered to undergo meiosis to form competent oocytes.[141] The methods used in these experiments are complex, and support of the concept using alternative experimental techniques would be valuable. However, if oogonial stem cells are present in the adult ovary, the discovery opens the possibility of identifying treatment options to premature ovarian failure and menopause. It is clear from multiple studies that the number of small follicles in the ovary decline as a woman ages. Mathematical descriptions of the decline favor one of three models: (1) an exponential rate of decline with a fixed "half-life," similar to models describing the decay of radioactive material; (2) an exponential rate of decline with two distinct rates of decline, a slow rate of decline early in life, and a more rapid rate of decline later in reproductive life; and (3) a power model where the rate of decline accelerates throughout a woman's life.[142] The rate of decline of antimüllerian hormone (AMH), a biochemical marker of ovarian follicle number, and the antral follicle count assessed

by sonography also appear to accelerate with female aging, supporting the power model of decline.[143]

The discovery of interventions that could prevent the decline of oocytes and follicles in the adult ovary is an important priority. In the mouse model, two interventions of caloric restriction or knock-out of the peroxisome proliferator-actived receptor γ coactivator-1α , a component of a metabolical regulatory pathway, were reported to delay the rate of oocyte loss.[144]

For infertile couples where the cause of the infertility is azoospermia in the male partner, the success of donor sperm insemination is directly related to the age of the female partner. In women under 30 years of age, the first three cycles of insemination resulted in a per-cycle pregnancy rate of 0.10. In women older than 35 years of age, the first three cycles of insemination was associated with a per-cycle pregnancy rate of 0.06.148. Data from IVF programs also suggests that the age of the female partner is an important determinant of pregnancy rates. In women younger than 30 years of age, the clinical pregnancy rate per cycle is in the range of 0.25. In women over 40 years of age, the pregnancy rate per cycle is in the range of 0.12 with a high rate of spontaneous abortion.[145]

Changes in both oocyte quantity and oocyte quality (function) account for the relationship between the age of the female partner and fecundability. The poor functional quality of oocytes in the terminal follicle pool is a major cause of the relationship between female aging and diminishing fecundability. The poor functional quality of these oocytes is supported by the observed high rate of trisomic pregnancy that occurs following their fertilization[146] and the poor performance of these oocytes in the process of IVF, including poor fertilization, embryonic growth, low implantation, and pregnancy rates.

Five major methods are in use to assess the size of the ovarian follicle and oocyte pool in infertile women: (1) cycle day 3 FSH concentration, (2) cycle day 3 inhibin B, (3) antimüllerian hormone (AMH) measured at any time in the menstrual cycle,[147] (4) antral follicle count by transvaginal sonography, and (5) the clomiphene challenge test. In the normal menstrual cycle, decreases in estradiol and inhibin A during menses are associated with an increase in FSH production during the first 5 days of the menstrual cycle (see Chapters 8 and 9). The magnitude of the rise in FSH during menses is dependent on the magnitude of negative feedback provided by the circulating inhibin B concentration. During menses, inhibin B is secreted from small follicles. The greater the number of small follicles, the greater the inhibin B concentration during menses and the smaller the rise in FSH during menses because inhibin B has a negative feedback effect on FSH. The increase in FSH during menses stimulates the growth of an ovarian follicle that will be selected to achieve dominance during the cycle. As the selected follicle grows, it secretes increasing quantities of estradiol, inhibin B and inhibin A, thereby suppressing FSH production to low levels. As the follicular pool declines, inhibin B levels during menses decline and the magnitude of the FSH increase during menses increases, resulting in elevated serum FSH concentrations on cycle days 2, 3, and 4. AMH is expressed by small follicles, so as the follicle pool declines, serum AMH decreases. AMH levels are relatively constant throughout the menstrual cycle, increasing the convenience of obtaining a clinical useful AMH measurement compared with the timed measurements required for the interpretation of FSH and inhibin B values. An elevated serum FSH on cycle day 3 or a decreased cycle day 3 inhibin B or decreased AMH at any time during the cycle are good biochemical markers of a depleted follicular pool. A depleted follicle and oocyte pool is associated with decreased fecundability, an increased rate of pregnancy loss and an increased rate of aneuploid pregnancy. Particularly in infertile women older than 35 years of age, measurement of cycle day 3 FSH, inhibin B, or AMH are very helpful in identifying women with a reduced follicular pool. Antral follicle count also provides a clinically useful assessment of the ovarian follicle and oocyte pool.

Antral follicle count (AFC) is the sum of the number of antral follicles 2 to 10 mm in diameter in each ovary assessed in the 2-dimensional ovarian slice with the greatest area by high-resolution transvaginal sonography. AFC is commonly assessed during the early follicular phase of the menstrual cycles, but some authorities report it can be performed at any time in the menstrual cycle.[148] An antral follicle count ≤4 is associated with a poor follicular response to ovarian hyperstimulation, resulting in the development of a low number of large antral follicles and in IVF cycles, the retrieval of a low number of oocytes. However, AFC ≤ 10 are also associated with reduced ovarian response compared to an AFC > 10.[149,150] The AFC count has good inter-observer reliability and the AFC count shows much more variation between women than within the same woman measured in multiple tests.[151]

During menses, negative feedback control of FSH is largely from inhibin B, and to a lesser degree from estradiol. Using an estrogen antagonist such as clomiphene, the estradiol negative feedback is blocked and FSH negative feedback is only from inhibin B, resulting in a greater rise in FSH. This may increase the sensitivity of the measurement of FSH to detect a depleted follicle pool. The clomiphene challenge test is performed by administering clomiphene, 100 mg daily from cycle day 5 to 9. FSH levels are drawn on cycle days 3 and 10. An elevated FSH level on either cycle day 3 or 10 is associated with a diminished ovarian follicular pool and reduced fecundability. In some series, for every 100 women with an elevated day 10 FSH (post-clomiphene challenge), only 40 have an elevated day 3 FSH (pre-clomiphene challenge). Women who may be candidates for the clomiphene challenge test include those over 35 years of age, cigarette smokers, and women with unexplained infertility, stage III or IV endometriosis, previous bilateral ovarian surgery, or history of poor response to FSH stimulation. The clomiphene test is probably effective in detecting women with diminished follicular reserve because it blocks the negative feedback of estrogen, leaving only inhibin B to suppress FSH production. In women with diminished ovarian reserve, inhibin B levels appear to be very low and are incapable, on their own, of suppressing FSH production. Some investigators have reported that the measurement of FSH after a course of clomiphene citrate is more sensitive for identifying women with diminished ovarian reserve than is the cycle day 3 FSH test.[152,153] Other investigators have reported that basal FSH and the clomiphene challenge test have similar performance in identifying women with decreased fecundity due to a depleted follicle pool.[154]

Dozens of studies in IVF have reported that the number of mature follicles stimulated and the number of oocytes retrieved is related to basal FSH, inhibin B, AMH, and antral follicle count.[155] In a classic study of the relationship between cycle day 3 FSH and pregnancy rate in an IVF program, women with a day 3 FSH concentration of less than 10 mIU/mL had an ongoing pregnancy rate of 0.18. In contrast, women with a cycle day 3 FSH level greater than 25 mIU/mL had an ongoing pregnancy rate of 0.00.[156] The cycle day 3 FSH predicts the magnitude of the ovarian response to exogenous gonadotropin stimulation—including the peak estradiol concentration, the number of follicles, and the number of oocytes that are obtained at follicular aspiration. Since measures of the size of the ovarian follicular pool predict the magnitude of the ovarian response to stimulation, these measures can be used to guide the choice of stimulation regimens. For example, using a basal AMH test to guide selection of stimulation regimens, women with low AMH levels (reduced follicle pool) would be stimulated with higher doses of gonadotropins and women with higher levels of AMH (large follicle pool) would be stimulated with lower doses of gonadotropins.[157] Measures of the size of the follicle pool, such as AMH can also be used to assess the magnitude of the decrease in the ovarian follicle pool following ovarian cystectomy for diseases such as endometriosis.[158] It should be noted that although measures of the ovarian follicle pool are good predictors of the number of mature follicles stimulated and the number of oocytes retrieved during ovarian stimulation, they are not as good predictors of pregnancy because they may not adequately assess oocyte *quality* especially in situations where the female partner is older than 40 years of age.[159]

Many genetic and lifestyle factors determine the rate of follicular loss and the age at which serum FSH measured during menses begins to rise. For example, cigarette smoking appears to hasten the pace at which the follicular pool is depleted. Menopause occurs significantly earlier in women who smoke.[160] In women in their mid 30s, cycle day 3 FSH also appears to be approximately 25% higher in cigarette smokers than in non-smokers.[161] In cigarette smokers, the number of oocytes per pair of ovaries appears to be reduced.[162]

Chemotherapy with alkylating agents or pelvic radiation are two important exposures that are associated with a diminished follicular pool. Women who are older than 30 years of age and have completed six courses of chemotherapy for Hodgkin disease typically lose more than 90% of their follicles and many enter menopause immediately after the chemotherapy.[163] Radiation doses as low as 400 rads to the ovary will induce menopause in women over 35 years of age. Girls are much more resistant to the induction of menopause with chemotherapy or pelvic radiation, probably due to their large follicular pool.

Once a diminished ovarian follicular pool has been identified it is often "too late." Fertility treatments at this point have lower success than the same treatments in women with a normal follicular pool.[164] Women with diminished ovarian follicular reserve are probably best counselled to consider oocyte donation or adoption. Consequently, many authorities are recommending that infertility evaluation and treatment be initiated in women older than 35 years of age after only 6 months of failure to conceive. Evaluation of the size of the ovarian follicle pool is probably warranted in infertile women older than 35 years of age. As a simple rule to guide clinicians, advancing age of the female partner is associated with poor oocyte quality, and increasing levels of basal FSH is associated with a reduced ovarian follicle pool and reduced ovarian responsiveness to stimulation.[165]

Cancer Treatment and Infertility

Issues related to fertility preservation and the treatment of infertility in cancer patients is discussed in Chapter 33, Fertility Preservation.

Anatomical Factors in the Female

Tubal Factor Infertility

Tubal or peritoneal disease is identified in approximately 20% of the female partners of infertile couples. Pelvic inflammatory disease (PID), appendicitis, septic abortion, and previous pelvic or tubal surgery are major contributors to tubal disease. Endometriosis commonly causes peritoneal and ovarian adhesions and distorts tubal anatomy, but in almost all women with endometriosis the fallopian tubes are patent. The rate of tubal infertility has been reported to be 12%, 23%, and 54% after one, two, and three episodes of PID, respectively.[166] Subclinical pelvic infections with chlamydia trachomatis is a major cause of tubal disease associated with infertility. Patton and colleagues[167] studied tubal biopsy specimens from 25 women with PID and tubal infertility. *Chlamydia trachomatis* was detected in 3 out of 25 specimens by culture, 12 out of 24 specimens by *in situ* hybridization, 15 out of 22 specimens by immunoperoxidase staining, and 2 out of 10 specimens by transmission electron microscopy. Serum antibodies against chlamydia were detected in 15 out of 21 subjects. In this cohort, chlamydia was identified in 19 out of 24 women with PID and infertility.[167] Many subsequent studies have demonstrated that circulating high-titre chlamydia antibodies are associated with tubal disease as detected at the time of laparoscopy.[168] In one large population study, the presence of chlamydia antibodies was strongly associated with tubal factor infertility.[169] Approximately 1% to 4% of women of 18 to 26 years of age are infected with Chlamydia.[170] A history of a ruptured appendix increases the risk of developing tubal factor infertility. In one case-control study, a history of ruptured appendix was associated with a 4.8-fold increase in the risk of tubal infertility. Appendicitis without rupture was not associated with an increased risk of tubal infertility.[171]

Following pelvic surgery, adhesions develop in approximately 75% of women.[172] The mechanism of postoperative adhesion formation is not fully understood, but it involves invasion of fibroblasts into the postsurgical fibrinous bridges. As a result, adhesive tissue develops, connecting two normally unconnected structures or covering the surface of a structure with *de novo* adhesions (Fig. 23.11). In the normal peritoneal healing process, a serosanginous proteinaceous fluid exudes from the site of injury and coagulates into fibrin bands. In the normal healing process,

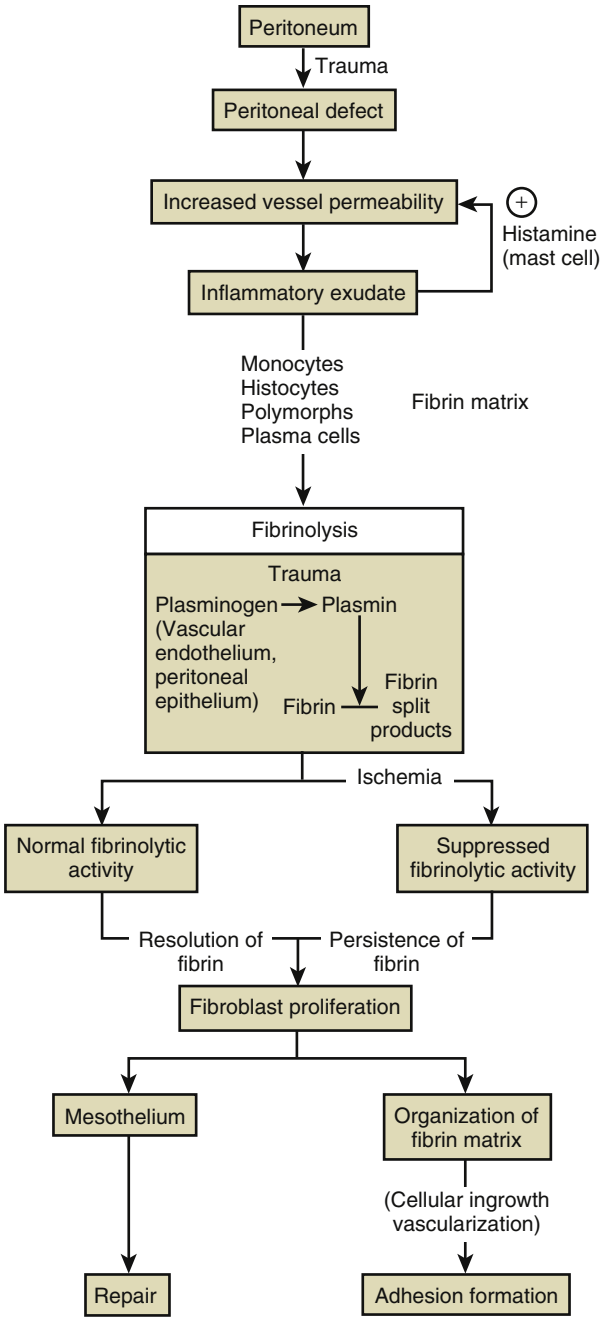


FIGURE 23.11 Schematic of the normal healing response to a surgical injury in the pelvic peritoneum. *(From Montz FJ, Shimanuki J, DiZerega CS. Postsurgical mesothelial re-epitheliazation. In DeCherney AH, Polan ML [eds], Reproductive Surgery. Chicago: Mosby Year Book, 1987.)*

endogenous fibrinolytic activity lyses these fibrin bands within 4 days. If the fibrinous bands are invaded by fibroblasts, angiogenesis occurs and a permanent bridge of tissue (an adhesion) is created. Factors that decrease fibrinolytic activity (ischemia, infection, drying of peritoneal surfaces) or increase fibroblast infiltration of the fibrin clot will increase the chance of developing adhesions.[173] Factors that increase fibrinolytic activity, such as plasmin preparations (plasmin, actase, and fibrinolysin) or plasmin activators (streptokinase, urokinase, and tissue-type plasminogen activator), are efficacious in preventing postsurgical adhesion formation.[174]

Methods reported to minimize postoperative adhesions include the use of dextran, adhesion-prevention barriers like regenerated oxidized cellulose (Interceed) and expanded polytetrafluoroethylene (PTFE, Gore-Tex), heparin, glucocorticoids, fibrinolytic agents, hyaluronic acid-based fluid agents, polyethylene glycol hydrogel, and nonsteroidal anti-inflammatory agents. Most of these agents have been demonstrated to be effective in laboratory models of adhesion formation.[175-177] Little clinical data is available to help guide the clinical use of adhesion prevention agents

Oxidized regenerated cellulose (Interceed), one of the most widely studied adhesion prevention adjuvant agents, becomes a gel shortly after placement on a surgically traumatized pelvic surface. The gel reduces the chance of formation of fibrin bridges between two opposing structures and thereby reduces the chance of adhesion formation. The material is resorbed within 1 week as it is metabolized into glucose and glucuronic acid. Oxidized regenerated cellulose has been demonstrated to reduce adhesion formation in women in well-controlled randomized prospective studies. In one study of 66 women with bilateral adnexal adhesive disease, the use of Interceed on one adnexa resulted in a 39% reduction of postoperative adhesion scores compared with the adnexa that did not receive the Interceed barrier. The use of Interceed resulted in a twofold increase in the number of adnexae without adhesions at the second-look laparoscopy.[178] Similar findings were obtained when oxidized regenerated cellulose was utilized to reduce adhesions after ovarian surgery.[179] A disadvantage of Interceed is that the surgeon must place the material over the area that is thought to be at high risk of adhesion formation. In many surgical cases, adhesions may occur at sites distant from the area where the Interceed is placed.

As noted above, the three most commonly utilized tests of tubal patency are the HSG, the hysterosalpingo contrast sonography (-), and laparoscopy. The advantages of HSG are that it utilizes few resources, produces data concerning the shape of the uterine cavity, and may increase fecundability by altering the peritoneal environment. The major disadvantages of HSG are that it requires a fluoroscopy imaging device, causes pain during the performance of the procedure, and provides no information concerning the presence of peritoneal diseases like endometriosis and ovarian adhesions. In addition, if the HSG demonstrates proximal tubal occlusion, a confirmatory test (selective tubal catheterization or laparoscopy) is required. This additional testing is necessary because about 15% of the cases where HSG demonstrates proximal tubal occlusion are actually due to "tubal spasm" that is spontaneously reversible. HSG is usually performed between cycle days 5 and 12. Many centers prepare women for the procedure with a short course of an antibiotic and an antiprostaglandin agent like ibuprofen immediately before the procedure. The risk of infection following HSG is in the range of 1%.[180]

Many studies report that following an HSG, fecundity is increased in the 12 to 24 months following the procedure. For example, in a clinical trial, women with infertility were randomized to HSG with an oil-based contrast media or with no intervention. After 24 months, pregnancy rates were 58% and 41% in the HSG and no intervention groups,

respectively ($P = 0.03$).[181] The fertility enhancing effects of an HSG appear to occur both with oil-based and water-based contrast agents. In one trial, 175 women were randomized to undergo HSG with either oil-based or water-soluble contrast agents. The oil-based contrast agent gave better resolution of the uterine cavity and the aqueous agent gave better resolution of the tubal mucosa. The post-procedure pregnancy rates were similar in the two groups.[182]

The main diagnostic advantage of laparoscopy over HSG is that it has better sensitivity and specificity for diagnosing tubal and peritoneal disease than HSG. In addition, laparoscopy can diagnose endometriosis and can be used to treat abnormalities observed at the time of the procedure.

HyCoSy is performed by cannulating the cervical os, and under ultrasound imaging injecting an ultrasound contrast media into the uterus. Specialized ultrasound devices that utilize contrast-tuned imaging are available to enhance the interpretation of the finding, but standard ultrasound devices can be used for the procedure.[183] Ultrasound contrast media are microbubbles suspended in a solution. The shell of the microbubble is made of albumin, galactose, lipids, or polymers. The bubble contains a gas that has low solubility in aqueous fluids, such as sulphur hexafluoride or octafluoropropane. The gas in the bubble generates a strong unique signature during ultrasound imaging. The media fills the uterine cavity providing an image of the shape of the cavity and detecting small intracavity filling defects caused by polyps or submucus myomas. If the proximal and distal fallopian tubes are patent, the contrast media flows into the proximal tube and out the distal tube into the peritoneal cavity. HyCoSy is an office-based procedure that is performed in the early follicular phase. HyCoSy is associated with less pain than HSG.[184] HyCoSy has sensitivity to detect tubal disease similar to HSG, but is superior to HSG for the detection of small intracavitary lesions.[19]

As noted above, Chlamydia infection is a common cause of tubal damage. One novel strategy for triaging infertile women into groups at high and low risk for tubal occlusion is the measurement of circulating anti-Chlamydia antibodies. For infertile women with absent or low-titre antibodies to Chlamydia the rate of tubal damage on laparoscopy or HSG is about 5%. For infertile women with high-titre antibodies to Chlamydia the rate of tubal damage on laparoscopy or HSG is about 35%.[185] In women with high titre antibodies to Chlamydia, more severe tubal damage is observed than in women with low titre or absent antibodies.[186] Testing for anti-Chlamydia antibodies as a screening strategy to identify infertile women with tubal disease is limited by false positive tests due to cross-reactivity with Chlamydia pneumoniae. Therefore, HSG or HyCoSy remains the first line test to evaluate tubal patency.[187]

Although Chlamydia is the most common cause of tubal damage in North America and Europe, tuberculosis is a common cause of distal tubal occlusion for women from Northern India and Nepal.[188] In areas with a high prevalence of tuberculosis, evaluation of the female partner for tuberculosis using a polymerase chain reaction test on an endometrial biopsy specimen identified women who would benefit from antimicrobial therapy.[189]

Surgery for the treatment of infertility due to tubal disease is most successful if disease is localized in the distal portion of the tube. *Fimbrioplasty* is the lysis of fimbrial adhesions or dilatation of fimbrial strictures. *Neosalpingostomy* is the creation of a new tubal opening in a fallopian tube with a distal occlusion. Dlugi and colleagues[190] evaluated the success of unilateral versus bilateral fimbrioplasty or neosalpingostomy in 113 women with tubal factor infertility. Overall, these procedures were associated with a postsurgery monthly pregnancy rate of 0.026. Similar monthly pregnancy rates following fimbrioplasty or neosalpingostomy have been reported by Canis and colleagues.[191] Women with major adhesions who had bilateral procedures had the lowest chance of achieving pregnancy. In contrast, *in vitro* fertilization treatment of tubal disease is associated with a per cycle pregnancy rate of approximately 0.30. After tubal surgery, however, couples can attempt to achieve conception naturally over many menstrual cycles. When viewed from a cumulative perspective, approximately 50% to 70% of the good-prognosis patients treated with distal tubal surgery became pregnant, and approximately 10% of the pregnancies achieved are ectopic pregnancies.

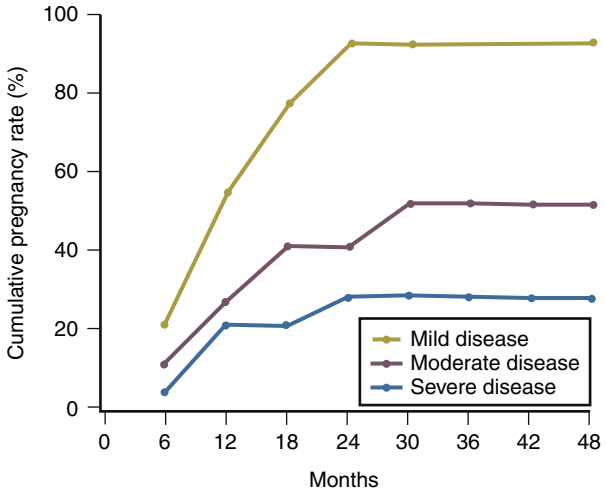


FIGURE 23.12 Life table analysis of pregnancy outcome following neosalpingoneostomy, by extent of disease. *(From Schlaff WE, Dassiakos D, Damewood MD, Rock JA. Neosalpingostomy for distal tubal obstruction: prognostic factors and impact of surgical technique.* Fertil Steril *54:984, 1990, with permission.)*

Poor prognostic factors for successful pregnancy following surgical treatment of distal tubal disease include tubal diameter greater than 30 mm, absence of visible fimbriae, dense pelvic adhesions, ovarian adhesions, advanced age of the female partner, diminished ovarian follicle number, and prolonged duration of the infertility problem (Fig. 23.12).[192] Infertile women with bilateral proximal and distal tubal disease have very low chances of conceiving following surgical treatment.[193] Treatment with IVF is much more successful than surgical treatment in this group of women.

In women with tubal infertility, proximal tubal occlusion accounts for about 20% of cases. Causes of proximal tubal occlusion include mucus debris, spasm at the uterotubal junction, or occlusion. Occlusion is commonly caused by fibrosis associated with salpingitis isthmica nodosa, pelvic inflammatory disease, adenomyosis, fibroids, or endometriosis. A major advance in the treatment of proximal tubal occlusion associated with infertility is the development of flexible tip guidewire techniques to restore patency to the proximal portion of the fallopian tube (Fig. 23.13 and Fig. 23.14).[194]

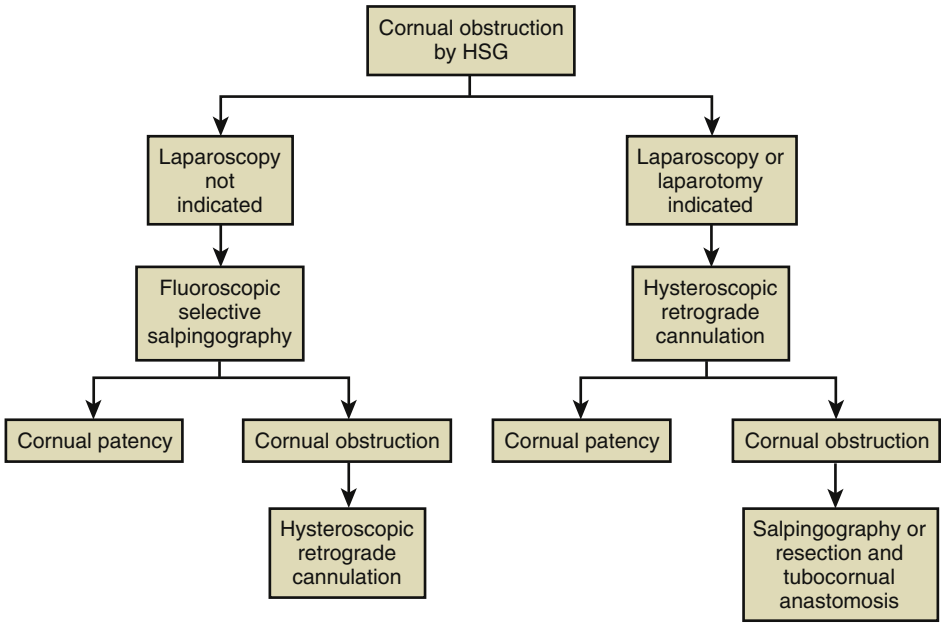


FIGURE 23.13 Schematic algorithm for treatment of women with proximal tubal obstruction. If laparoscopy or laparotomy is planned due to known major pelvic disease, proximal obstruction can be evaluated and treated by a hysteroscopic procedure performed at the same operation. If the patient is not thought to require a surgical procedure for pelvic pathology, proximal tubal obstruction can be evaluated and treated by fluoroscopic selective salpingography and catheter treatment. *(From Novy MI, Thurmond AS, Patton P, et al. Diagnosis of cornual obstruction by transcervical fallopian tube cannulation.* Fertil Steril *50:434, 1988, with permission.)*

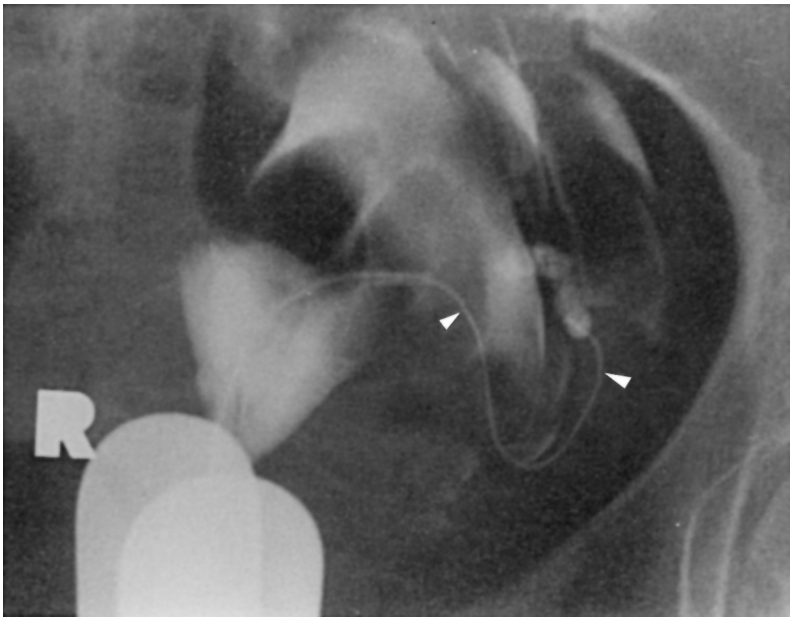


FIGURE 23.14 Transcervical bougie dilatation recanalizes an obstructed right tube. Note residual strictures around arrowheads at the isthmic segment of the tube, suggesting the presence of salpingitis isthmica nodosa. *(From Lang EK, Dunaway HH. Recanalization of obstructed fallopian tube by selective salpingography and transvaginal bougie dilatation: outcome and cost analysis.* Fertil Steril *66:210–215, 1996, with permission.)*

In one study of transcervical fluoroscopic catheter recanalization, successful recanalization was achieved in 47 of 65 tubes treated (72%). Of the 40 women with open tubes following the procedure, 9 achieved live births (23%), 4 had ectopic pregnancies (10%), and 1 woman became pregnant but had an early abortion. In the 11 women in whom tubal recanalization was not successful, there were no pregnancies.[195] In a large meta-analysis, tubal cannulation was successful in 85% of cases and 50% of the successfully treated women conceived.[196] If tubal cannulation cannot be accomplished, IVF is a better treatment option than surgery to reimplant the tubes into the uterus or excise the occlusion and reanastomose the tubes to the uterine cornua.[197]

One of the most successful surgical procedures for infertility is the microsurgical reanastomosis of fallopian tubes that were subjected to surgical sterilization procedures. The clinical characteristics that are associated with a high success rate for surgical reanastomosis include: (1) patient under 40 years of age; (2) tubal length greater than 4 cm; (3) sterilization performed with the Falope ring, clip, or Pomeroy tubal ligation technique; and (4) absence of associated pelvic pathology. Cumulative pregnancy rates in the year following the procedure are in the range of 50% to 80%. In one large case series that studied the impact of the age of the female partner on the postreanastomosis pregnancy rate, the reported cumulative intrauterine pregnancy rates were 81%, 67%, 50%, and 13% for female partners younger than 36, 36 to 40, 40 to 43, and older than 43 years of age.[198] Laparoscopic and robot assisted laparoscopic surgical reanastomosis have largely replaced traditional laparotomy approaches to tubal reanastomosis.[199,200] In a cohort study of the role of IVF or surgical reanastomosis for the

treatment of women with a tubal sterilization who wanted to conceive, for women younger than 37 years of age, surgical reanastomosis and IVF resulted in similar cumulative pregnancy rates, but surgical reanastomosis was reported to be more cost effective than IVF. For women older than or equal to 37 years of age, surgical reanastomosis resulted in fewer pregnancies than IVF.[201]

For infertile women with tubal disease, treatment with IVF is associated with a pregnancy rate of approximately 30% in the first cycle of treatment. Several studies indicate that hydrosalpinges decrease the pregnancy rate in IVF cycles.[202,203] The fluid in the hydrosalpinx may contain toxic factors that decrease the implantation rate of embryos or have direct embryotoxicity.[204] A meta-analysis of three controlled trials reported that laparoscopic salpingectomy for hydrosalpinx prior to IVF increased pregnancy rates by 75% compared with not performing the surgery.[205] In one trial, 204 women were randomized to undergo salpingectomy prior to IVF or to undergo IVF without prior salpingectomy.[206] The live birth rate was 29% in the women who had salpingectomy followed by IVF and 16% in the women who had IVF alone ($P < 0.05$). Laparoscopic salpingectomy should be considerd for all women with hydrosalpinges prior to undergoing IVF. Interestingly, in infertile women with one patent tube of normal caliber, and one blocked tube with a hydrosalpinx, surgical removal of the single blocked tube is associated with a good spontaneous pregnancy rate.[207] An alternative to salpingectomy of hydrosalpinges prior to IVF is to clip the proximal end of the tube to prevent the fluid from the hydrosalpinx to drain into the uterine cavity and disrupt embryo-endometrial interaction.[208,209] Some clinicians advise that if the proximal tube is clipped that the distal end of the hydrosalpinges should be widely fenestrated to prevent fluid from becoming trapped within the tube.

Uterine Factor Infertility

Congenital uterine abnormalities, such as a uterine septum, bicornuate uterus or uterine didelphys are associated with an increased risk of spontaneous abortion and preterm birth.[210,211] These problems are discussed in detail in Chapters 14 and 27.

Cervical Factor Infertility

The cervix is an active participant in shepherding sperm from the vagina to the upper reproductive tract. In the normal cervix, the secreted cervical mucus has physicochemical properties that facilitates the transport of sperm. Congenital malformation and trauma to the cervix may impair the ability of the cervix to produce normal mucus.

The postcoital test is the procedure most frequently utilized to examine the adequacy of the cervical mucus and the sperm-mucus interaction. Late in the follicular phase, the infertile couple is instructed to have sexual intercourse. The female partner is seen after intercourse and a small amount of cervical mucus is obtained using oval forceps with a hollow aperture. The glycoproteins in the mucus support the property of *spinnbarkeit*, or stretchability of the mucus. By allowing the prongs of the forceps to separate the spinnbarkeit, the mucus can be tested. A separate aliquot is dried on a glass slide. Due to the high concentration of salts in the normal mucus in the late follicular phase, the dried mucus will crystallize in a fern-like pattern. A third aliquot is placed on a glass slide overlaid with a cover slip and examined under the high power microscope objective for the presence of sperm.

Two major problems with the postcoital test are that there is little consensus on the normal range, and that the test has poor predictive value for pregnancy. For example, some authorities suggest that a normal test requires more than 20 sperm per high-power field.[212] Other authorities conclude that the presence of a single sperm indicates a normal test.[213] In one study of the relationship between the postcoital test and fecundability, 20% of fertile women were observed to have one sperm or less per high-power field.[212] In another study, fecundability did not seem to be altered by the presence of between 0 and 11 sperm per high-power field.[214] Further reducing the potential validity of the postcoital test is the observation that the inter-observer and intra-observer reproducibility is poor.[215]

Dysplasia of the cervix is a common problem that is often treated with excision of cervical tissue infected with human papilloma virus. Recent epidemiological studies have reported that loop electrosurgical excision (LEEP) of cervical tissue is associated with cervical stenosis,[216] preterm delivery, and low birth weight infants.[217]

Endometriosis

The relationship between endometriosis and infertility and the treatment of infertility caused by endometriosis are reviewed in Chapter 26.

Uterine Leiomyomata

The impact of uterine leiomyomata on fertility is reviewed in Chapter 27.

Immunological Factors and Recurrent Abortion

Recurrent abortion caused by autoimmune diseases, coagulopathies, and infections are discussed in Chapter 14.

Genetic Causes of Infertility

For many decades it has been known that major chromosomal abnormalities are often associated with infertility. For example, women with 45,X (Turner syndrome) have premature depletion of the oocyte pool and are typically sterile. Translocations and interstitial deletions of the X chromosome are associated with premature ovarian failure, although the identity of the genes in these deletions remains to be established. In infertile men, Yq11 microdeletions are observed in about 5% of cases.[218] A major goal of reproductive scientists is to identify individual genes that are associated with infertility. Recently, a few genes in women that influence fecundability have been identified.[219] Genes in which mutations affecting fertility and fecundity have been identified include Galactose-1-phosphate uridyl transferase *(GALT)*,[220] the FSH receptor,[221] the LH receptor,[222] premutations in the *FMR1* (fragile X syndrome) gene,[223] the *FOXL2* transcription factor

gene,[224] the *BMP 15* gene,[225] the *NR5A1* (steroidogenic factor 1) gene,[226] and the ataxia—telangiectasia *(ATM)* gene.[227]

Development of low cost methods for high resolution genetic testing is rapidly changing the approach to genotype-phenotype studies of infertile couples. Comparative genomic hybridization[228] and whole genome multiplex sequencing[229] increase the sensitivity of genetic tests to find "broken genes" that might cause or contribute to infertility.

Unexplained Infertility

Unexplained infertility is diagnosed when a couple has failed to achieve a pregnancy after 12 months of attempting conception and has completed a thorough evaluation without finding a cause for the infertility.[230] The diagnosis of unexplained infertility rests heavily on the definition of what constitutes a thorough fertility evaluation. For many fertility specialists, a thorough evaluation includes documentation of the following normal findings:

- Adequate ovulation, using either a midluteal phase serum progesterone determination of greater than 3 ng/mL
- Tubal patency, as determined by HSG, HyCoSy, or laparoscopy
- Normal semen analysis, demonstrating 15 million sperm per millilitre, greater than 32% motility and greater than 4% normal morphology by strict criteria
- Adequate ovarian oocyte reserve, using either a cycle day 3 FSH determination (below 15 mIU/mL), a clomiphene challenge test (both cycle day 3 and 10 FSH measurements below 15 mIU/mL), or antral follicle count greater than 4.

Many specialists believe that laparoscopy is an important component of a thorough infertility evaluation because it identifies undetected peritoneal adhesions and endometriosis lesions that would otherwise remain undetected. Other specialists believe that laparoscopy is not a necessary component of a thorough infertility evaluation, and should be reserved for use in selected cases.

The benefit of laparoscopy is that it allows a complete evaluation of the pelvis for diseases such as endometriosis and pelvic adhesions and treatment of those lesions at the time of the surgery. On the downside, laparoscopy is an expensive surgical procedure and the presence of endometriosis and pelvic adhesions does not influence the approach to treatment because empiric treatment quickly escalating to IVF is a practical and successful approach.

In one large retrospective study of 495 infertile couples with normal ovulation, normal HSG, and normal semen analysis, laparoscopy was performed on all the female partners and 35% were diagnosed with either major pelvic adhesions and tubal disease (10%) or endometriosis (21%).[231] In a separate study, a cost-benefit analysis concluded that for couples with normal ovulation, HSG and semen analysis, a laparoscopy to detect and treat endometriosis and peritoneal adhesions followed by expectant management was more cost effective than no laparoscopy or no laparoscopy but IUI followed by superovulation IUI followed by IVF.[232] One approach is to limit the use of laparoscopy in the infertility evaluation to those women who have a high likelihood of having endometriosis or pelvic adhesions, such as women with a history of severe dysmenorrhea or dyschezia.

Many cases of unexplained infertility are probably caused by the presence of multiple factors (e.g., female partner 37 years of age or older, male partner with semen parameters in the low end of the normal range). Each of these factors on their own do not substantially reduce fertility; when more than one factor is present, however, the pregnancy rate is reduced. Subtle changes in follicle development, ovulation, oocyte function, the luteal phase, and sperm function have been reported in couples with unexplained infertility.[233] In some couples with unexplained infertility, the male partner has a semen analysis with sperm concentration and motility at the lower end of the normal range.[234]

When couples with unexplained infertility are treated with IVF, they demonstrate reduced oocyte fertilization and embryo cleavage rates compared with couples where tubal factor is the cause of the infertility. For example, in one study, the oocyte fertilization rate for tubal factor infertility and unexplained infertility were 60% and 52%, respectively. When couples with unexplained infertility are treated with IVF, they have a higher rate of complete fertilization failure than couples with tubal factor infertility (6% versus 3%). These results suggest that couples with unexplained infertility probably have subtle functional abnormalities in oocyte and sperm function.[235]

Empirical Treatment of Infertility

The management of couples with unexplained infertility typically starts with treatments that consume few resources (lifestyle changes, timing of the intercourse to the fertile time of the menstrual cycle, expectant management, IUI, clomiphene, clomiphene plus IUI) and moves sequentially to treatments requiring proportionately greater resources (gonadotropin injections plus IUI or IVF), as shown in Figure 23.15.[236] This sequential approach to infertility, utilizing progressively more resource-intensive interventions, can be described as a "staircase" approach to empirical infertility treatment. The rationale underlying this strategy is that treatment is initiated with low-cost, low-risk interventions; and then with each step in the program, interventions are initiated that utilize greater resources and carry more risk. This approach has been demonstrated to be cost-effective in the treatment of unexplained infertility.[237] The pace at which the staircase is climbed is dependent on many factors, including the age of the female partner and the beliefs and values of the clinicians and patients.

Lifestyle changes can enhance the fertility of couples.[238,239] Lifestyle changes that can improve fertility include: normalize body mass index (BMI), stop smoking, reduce the use of caffeine and alcohol, reduce stress, reduce excessive exercise and for some couples, increase the frequency of sexual intercourse at the fertile time of the cycle. Lifestyle changes to enhance fertility have not been tested in high-quality clinical trials. Most of the evidence linking optimizing lifestyle choices and fertility are derived from epidemiological studies. Most epidemiological studies report that the magnitude of the impact of optimizing lifestyle choices on fertility is greater for the female partner than the male partner, but both partners should jointly participate in optimizing their reproductive health. Epidemiological studies

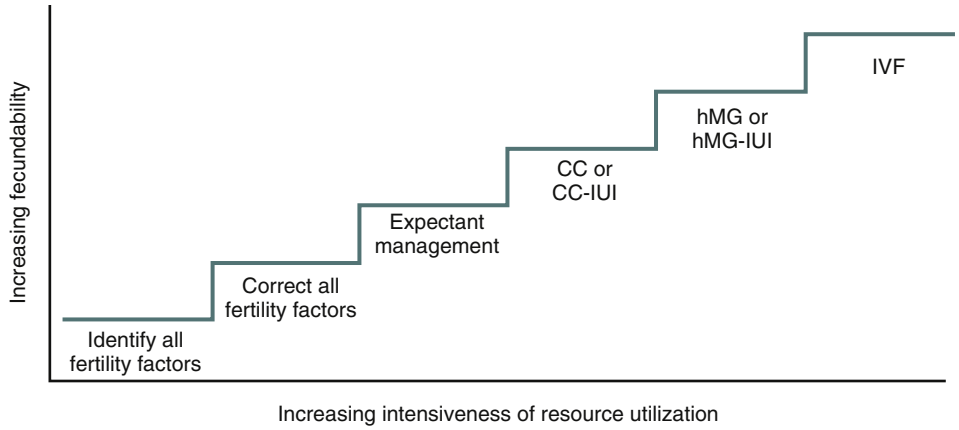


FIGURE 23.15 Staircase approach to empirical infertility treatment. For women over 35 years old, the first three steps in the algorithm should be rapidly completed. In women less than 30 years old, more time can be spent on the first three steps in the staircase. CC, clomiphene; IUI, intrauterine insemination; hMG, human menopausal gonadotropins; IVF, *in vitro* fertilization.

indicate that for the female partner, cigarette smoking and excessive caffeine and alcohol consumption reduce fertility. Couples with unexplained infertility should be counselled to stop smoking cigarettes. The female partner should be counselled to reduce caffeine intake to no more than 250 mg daily (two cups of coffee) and to reduce alcohol intake to no more than four standardized drinks per week. The male and female partner should be guided to try to achieve an optimal body mass index, ranging from 19 to 25 kg/m^2.[240,241] For couples with unexplained infertility and a low frequency of coitus, recommending an increase in coital frequency to at least twice per week may be associated with increased pregnancy rates.[242]

Based on data from observational studies, dietary patterns appear to influence the ovulation rate in women and semen quality in men. In women, an increase in the frequency of normal ovulatory cycles is observed with the following dietary patterns: high intake of low-glycemic index food, low protein intake from animal sources, high protein intake from vegetable sources, high intake of fat from dairy products, and high iron intake.[243] A Mediterranean-type dietary pattern appears to optimize fertility potential.[244] Among men a diet high in saturated fats appears to be associated with a reduction in sperm concentration in an ejaculate and high intake of omega-3 fats was associated with superior sperm morphology.[245]

In the treatment of unexplained infertility, timing intercourse to the portion of the menstrual cycle with the greatest chance for conception is an important and inexpensive intervention that can increase fecundability.[246] Urine LH testing at home is commonly utilized to identify the LH surge which occurs 1 to 2 days before ovulation. The days of the cycle with the greatest probability of pregnancy with one episode of sexual intercourse are the days of ovulation, and the two days before ovulation.[45,247,248] Since the urine LH surge occurs 1 to 2 days before ovulation it identifies in a prospective manner the fertile days of the cycle.

For young couples with unexplained infertility, expectant management is a reasonable and cost-effective approach to treatment.[249-251] When couples with unexplained infertility are followed prospectively without active treatment, approximately 1% to 4% become pregnant each month. For couples with unexplained infertility and a fecundability less than 0.01 (poor prognosis couples), waiting to initiate treatment is not cost effective. For couples with unexplained infertility and a fecundability of 0.04 (good prognosis couples), expectant management with timing of intercourse to the fertile part of the cycle is cost effective. Fertility factors that improve prognosis in couples with unexplained infertility include: female partner younger than 32 years of age, couple with less than 2 years of infertility, couples with a prior pregnancy, and a semen analysis with sperm motility greater than 40%.[252] Effective fertility treatment for unexplained infertility must increase the pregnancy rate above this baseline fecundability of approximately 0.01 to 0.04. The age of the female partner influences the pregnancy rate associated with expectant management.[253] For women over age 37, the pregnancy rate per cycle with expectant management is under 1%. For couples where the female partner is over 37 years of age, the ovarian oocyte pool declines rapidly—inevitably causing ovarian aging to become the cause of the fertility problem. For these women, expectant management is not recommended. Many couples of all ages become frustrated with their inability to conceive. For these couples, active treatment is recommended.

Intrauterine Insemination

The IUI procedure consists of washing an ejaculated semen specimen to remove prostaglandins and other factors and then concentrating the sperm in a small volume of culture media with a high protein concentration to enhance capacitation and the acrosome reaction. The sperm suspension is then injected directly into the upper uterine cavity using a small catheter threaded through the cervix. In one study of various methods used to prepare the sperm for intrauterine insemination, the swim-up and Percoll gradient preparation techniques resulted in superior pregnancy rates than the simple wash, swim-down, or refrigeration/heparin techniques.[253] The intrauterine insemination is timed to take place just prior to ovulation, typically using home urine LH monitoring. In couples reporting male infertility, IUI more than doubles the pregnancy rate compared with intracervical insemination or timed natural cycles (odds ratio 2.2; 95% confidence interval, 1.4 to 3.4).[254] In one study of couples with mild male infertility, the pregnancy rate per cycle was 6.5% for IUI versus 3.1% for intracervical insemination or timed natural intercourse.[255] IUI also appears to be effective for couples with unexplained infertility.

In a large clinical trial sponsored by the National Institutes of Health, 932 infertile couples with unexplained infertility or stage I or II endometriosis were randomized

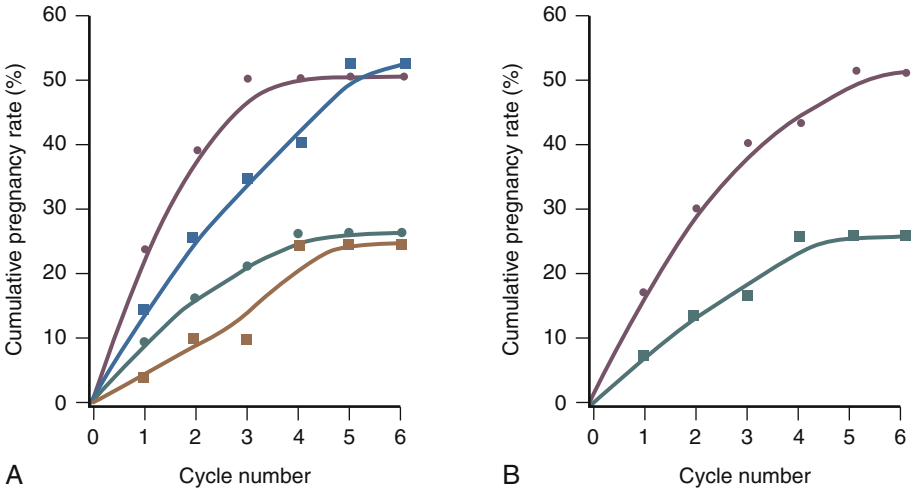


FIGURE 23.16 Cumulative pregnancy rates by Kaplan Meier life-table analysis for 290 infertile couples undergoing clomiphene IUI therapy stratified by the age of the female partner. **A**, Ages of women: purple circles, under 30; blue squares, 31 to 35; green circles, 36 to 40; brown squares, 41 or over. **B**, Ages of women: circles, under 35; squares, over 35. *(From Agarwal SK, Buyalos RP. Clomiphene citrate with intrauterine insemination: is it effective therapy in women above the age of 35 years?* Fertil Steril *65:759–763, 1996, with permission.)*

to one of four treatment groups: intracervical insemination of sperm (ICI); IUI; FSH injections plus ICI; or FSH injections plus IUI.[234] The purpose of the ICI was to be a control treatment that mimicked natural intercourse. The purpose of the IUI was to place a large number of sperm high in the reproductive tract. The purpose of the FSH injections was to stimulate multiple follicular development and ovulation, thereby increasing the number of oocytes available for fertilization in a single cycle (this type of treatment is explained later in this chapter). Most of the women in this study had either unexplained infertility or early stage endometriosis. The investigators reported that the per-cycle pregnancy rate in the group that received the control (ICI treatment) was 2%.[234] This pregnancy rate is similar to expectant management. IUI treatment was associated with a 5% per-cycle pregnancy rate. The per-cycle pregnancy rates in the FSH-ICI and FSH-IUI groups were 4% and 9%, respectively. In this study, IUI was clearly effective for the treatment of unexplained infertility.[234]

Multiple procedure-related factors appear to influence the effectiveness of IUI. Most recent studies report that one IUI per cycle results in a pregnancy rate similar to 2 IUI procedures per cycle.[256,257] In couples with a mild male factor problem, double IUI may increase the pregnancy rate.[258] Resting supine after the IUI procedure may also be associated with higher pregnancy rates than those in patients who ambulated soon afterwards.[259]

Clomiphene Citrate

Clomiphene has been demonstrated to be effective in the treatment of unexplained infertility. In normal ovulatory women, clomiphene treatment often results in the ovulation of two follicles and an increase in luteal phase progesterone production. These treatment-dependent changes probably increase fecundability. A 2000 meta-analysis of 11 prospective trials of clomiphene treatment for women with unexplained infertility demonstrated that clomiphene was superior to placebo or no treatment. The odds ratio for clinical pregnancy per clomiphene treatment cycle was 2.5 (95% confidence interval, 1.35 to 4.62).[260] In one trial where 118 female partners from couples with unexplained infertility were randomized to treatment with placebo or clomiphene citrate (100 mg daily, cycle days 2 to 6), the per-cycle pregnancy rates were 5% and 7%, respectively ($P < 0.05$).[261]

Although the absolute treatment effectiveness is modest, the low cost and low adverse effects of clomiphene make it a useful initial treatment for unexplained infertility. The main complication of clomiphene is an increase in multiple gestation. In one study of 2369 clomiphene-induced pregnancies, 7% were twins, 0.5% were triplets, 0.3% were quadruplets, and 0.13% were quintuplets.[262] The risk of high-order multiple pregnancies with clomiphene treatment is low, but the high volume of clomiphene cycles makes this intervention an important contributor to the total number of high-order pregnancies.[263]

Many normal ovulatory women undergoing empirical treatment of infertility with clomiphene have endometriosis. Many clinicians believe that clomiphene use in this setting may be associated with an increase in estradiol levels and an increase in endometriosis disease activity, including pelvic pain and the growth of ovarian endometriosis cysts.

Clomiphene Plus Intrauterine Insemination

The combination of clomiphene (increases the rate of double ovulation) plus IUI (places a large number of motile sperm high in the female reproductive tract) may simultaneously treat mild abnormalities of ovulation, oocyte function, and sperm function. In one study, 67 couples were randomized to treatment with clomiphene plus IUI or placebo. The pregnancy rate per cycle was 3.3% for control group and 9.5% for clomiphene plus IUI group.[264] For clomiphene-plus-IUI cycles, timing of the IUI is usually based on home urine LH measurement. Timing IUI with home urine LH measurement or exogenously administered hCG is associated with a similar pregnancy rate, but hCG administration is associated with greater cost and an increased risk of OHSS.[265] Advancing age of the female partner is associated with a decrease in success rates for IUI therapy (Fig. 23.16).[266]

Gonadotropin Injections and Gonadotropin Injections plus Intrauterine Insemination

Both gonadotropin injections alone and gonadotropin injections plus IUI increase fecundability in women with unexplained infertility. Gonadotropin injections plus IUI

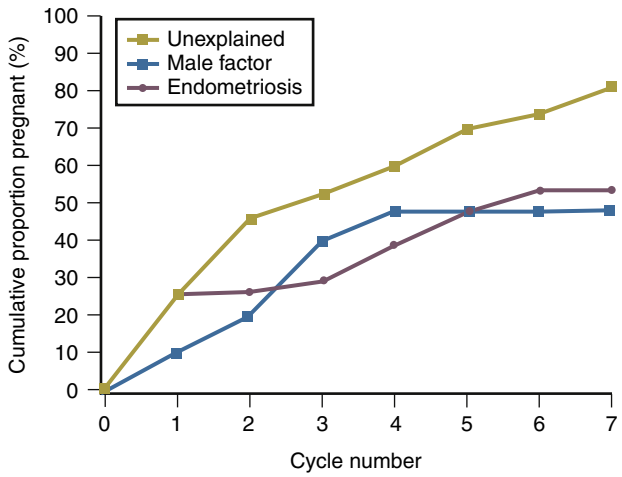


FIGURE 23.17 Cumulative pregnancy rate for gonadotropin-intrauterine insemination treatment for various fertility conditions: Unexplained, early stage endometriosis, and male factor. *(From Nulsen JC, Walsh S, Dumex S, Metzger DA. A randomized and longitudinal study of human menopausal gonadotropin with intrauterine insemination in the treatment of infertility.* Obstet Gynecol *82:780–786, 1993, with permission.)*

also appears to increase fecundability in infertile women with stage I or II endometriosis and in infertile men with semen abnormalities (Fig. 23.17).[267] In one study, 932 infertile couples with unexplained infertility or stage I or II endometriosis were randomized to one of four treatment groups: ICI, IUI, FSH injections plus ICI, or FSH injections plus IUI. The pregnancy rate in the control, the ICI group, was 2% per cycle. In the FSH-plus-ICI and the FSH-plus-IUI groups, the pregnancy rate per cycle was 4% and 9%, respectively.[234] The main complication of the use of FSH injections in the treatment of infertility in women with unexplained infertility is an increase in the rate of multiple gestations and ovarian hyperstimulation. Of the ongoing pregnancies in this study, 3% were quadruplets, 5% were triplets, and 20% were twins.

In another study of gonadotropin injections with or without IUI, Serhal and colleagues[268] randomized 62 couples with unexplained infertility to receive IUI alone, gonadotropin injections alone, or gonadotropin injections plus IUI. The per-cycle pregnancy rate was 2.2% for IUI alone, 6.1% for gonadotropin injections alone, and 26% for gonadotropin injections plus IUI. Similar results have been reported by other investigators.[269,270]

Although there is evidence for the efficacy of gonadotropin with or without IUI for the treatment of unexplained infertility, many authorities highlight the increased risk of multiple gestation with these therapies, and advise that the use of gonadotropin-IUI should be very limited.[271,272] In addition, if good prognosis couples with unexplained infertility can be counselled to continue to try to get pregnant on their own, and not pursue gonadotropin therapy, many will become pregnant spontaneously.[273] In a recent trial comparing the 3 step sequential treatment protocol: clomiphene-IUI, followed by gonadotropin-IUI, followed by IVF versus a 2 step sequential treatment protocol: clomiphene-IUI, followed by IVF, the clinical utility of the gonadotropin-IUI was poor.[274] In this study, the per cycle pregnancy rate for clomiphene-IUI was 7.6%, gonadotropin-IUI 9.8%, and IVF-31%. Since gonadotropin-IUI is expensive, and much less effective than IVF, the most cost-efficient approach was the two-step sequential treatment plan of clomiphene-IUI followed by IVF. The two-step approach was approximately 15% less expensive per live birth than the traditional 3-step approach.

Many authorities strongly caution against the use of FSH-IUI in the empiric treatment of infertility because it increases the risk of high-order multiple gestations and because IVF is a more effective intervention.[275,276] When using FSH-IUI, it is difficult to prevent all high-order multiple pregnancies, but withholding hCG administration in the presence of ≥3 follicles over 15 mm is one intervention that has been reported to reduce the multiple pregnancy rate.[277] Over the past 30 years, the pregnancy rate per cycle of IVF has increased from approximately 5% to 40% per cycle. As the pregnancy rate per cycle of IVF increases, the clinical utility of FSH-IUI decreases because IVF becomes a more cost-effective method of achieving a pregnancy.

In IVF cycles, the addition of a GnRH agonist or antagonist analogue to the gonadotropin injection regimen is known to improve pregnancy rates. However, the addition of a GnRH agonist analogue to a regimen of gonadotropin injections plus IUI does not appear to consistently increase the pregnancy rate in couples with unexplained infertility. In a study of the effect of adding a GnRH agonist to FSH-IUI therapy, 91 couples with unexplained infertility were randomized to receive treatment with gonadotropin injections and IUI or GnRH agonist analogue treatment plus gonadotropin injections and IUI. The pregnancy rate per cycle was 11% and 13%, respectively.[278] In a study of the effect of adding a GnRH antagonist to FSH-IUI treatment, 233 couples with unexplained or male factor infertility were randomized to receive FSH-IUI or a GnRH antagonist plus FSH-IUI. The pregnancy rate per cycle was 12% and 8% in the FSH-IUI and GnRH antagonist-FSH-IUI groups respectively ($P = 0.03$).[279]

In Vitro Fertilization

In Vitro Fertilization (IVF) is effective in the treatment of unexplained infertility.[280] In one randomized trial, 116 couples with unexplained infertility and a poor prognosis for spontaneous pregnancy were randomized to single embryo transfer IVF or FSH-IUI. The live birth rate was 22% and 7% for IVF and one cycle of FSH-IUI respectively. However, when the women in the FSH-IUI group completed 3 cycles of FSH-IUI, the cumulative life birth rate was 21%. The multiple pregnancy rate was 14% in the single embryo transfer IVF group and 25% in the FSH-IUI group.[281] In one cohort study, couples with unexplained infertility were treated for up to 3 cycles with gonadotropin injections plus IUI. The couples who did not become pregnant were then treated with IVF.[282] The per-cycle pregnancy rate was 16% for the couples treated with gonadotropin injections plus IUI, and 37% for the couples treated with IVF. Published cohort studies with no control group report that the treatment of unexplained infertility with IVF results in per-cycle pregnancy rates in the range of 20% to 40%.[283]

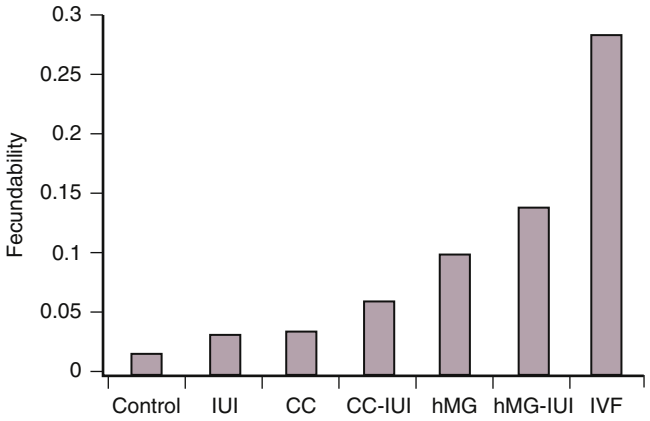


FIGURE 23.18 Fecundability associated with empirical treatment of unexplained infertility. Control, no treatment; IDI, intrauterine insemination; CC, clomiphene citrate; CC-IDI, clomiphene citrate plus intrauterine insemination; hMG, human menopausal gonadotropin; hMG-IUI, human menopausal gonadotropin plus intrauterine insemination; IVF, *in vitro* fertilization and embryo transfer.

Pace of Escalation in the Treatment of Unexplained Infertility

As noted above, couples with unexplained infertility and a good prognosis may be offered expectant management, and if that is not successful proceed slowly from low-resource interventions to resource intensive treatments like FSH-IUI or IVF. For couples with unexplained infertility and a poor prognosis (female age >37 years, >2 years of infertility) it may be preferable to more rapidly move from low-resource interventions to FSH-IUI or IVF. Studies of cohorts of infertility patients report that after 3 cycles of any one treatment, such as IUI, CC-IUI, or FSH-IUI, the pregnancy rate tends to decline. In general, after 3 cycles of any one treatment, it is recommended to consider advancing to the next step in the treatment algorithm (Fig. 23.18).[284]

Environmental Exposures Associated With Infertility

The impact of environmental factors on reproduction is discussed in Chapter 20.

Contraindications to Infertility Treatment

An adage in medicine is "first do no harm." Fertility treatments have the potential to significantly harm women and children born of fertility treatments. Counterbalancing the potential harm caused by fertility treatment is the right of patients to request treatments to achieve an important health goal, even if some risks are involved. There are a limited number of diseases that are an *absolute contraindication* to fertility treatment. In one such disease, Eisenmenger, pregnancy has an approximately 50% likelihood of causing death to the mother.[285] Many authorities strongly caution against fertility treatment in women with Turner syndrome due to an approximately 1% to 2% risk of aortic dissection.[286] However, many high-risk obstetricians will not advise against pregnancy in these women if their aortic root diameter is not dilated. The age of the female partner is a factor that must always be considered when recommending fertility treatment. There must be an age of the female partner where fertility treatment poses too great a risk for mother and child, such as age greater than 55 years. Population studies raise the concern that oocyte donation to older women is associated with an increased maternal mortality rate.[287]

There are many clinical situations where there is a relative contraindication to fertility treatment. Most of these contraindications are due to obstetrical considerations: the pregnancy poses serious risks during the pregnancy or at the time of delivery. For example, a woman with massive pelvic adhesions and a frozen pelvis can achieve a pregnancy with IVF. But at delivery if an emergency caesarean delivery is necessary, a bowel resection may be needed to gain access to the uterus to complete the caesarean delivery. The BMI of the female partner is of particular concern to obstetricians because of the high risk of difficult vaginal delivery. Obstetricians and fertility specialists have not reached a consensus about the wisdom of fertility treatment in women with a BMI greater than 60 kg/m^2. Many fertility specialists believe that delaying fertility treatment in a 40-year-old infertile woman with a BMI greater than 60 kg/m^2 is unethical because her ovarian follicle pool is nearly depleted.[288] Delaying her fertility treatment for additional attempts at weight loss may result in further depletion of her ovarian follicle pool. In contrast, obstetricians counsel that in women with a BMI greater than 60 kg/m^2 the risks of pregnancy, including a high rate of difficult caesarean delivery, make it dangerous to use fertility treatments.[289]

Infertility Treatment and Pregnancy Outcomes

The outcome of pregnancy in infertile women, regardless if the pregnancy arises spontaneously or following fertility treatment, appears to be different than in non-infertile women. For example, in a study from the Danish National Birth Cohort, fetal birth weight was compared in 51,000 singletons born of fertile couples, 5787 born of infertile couples *conceiving naturally* and 4317 born of infertile couples after fertility treatment. Maternal age and body mass index were greater in the infertile couples. After adjusting for these variables and smoking status, both the infertile couples who conceived naturally and those who conceived after fertility treatments had an increased risk of having a baby with a birth weight less than or equal to 5% compared with non-infertile women (6% versus 4.2%).[290] Similar findings have been reported in studies from Finland, subfertile women who conceived spontaneously and those who conceived following fertility treatment had a similar rate of preterm birth, low birth weight, and need for neonatal intensive care.[291] A study from Norway examined the birth outcomes in 2546 women who had 1 child by IVF and 1 child conceived without treatment.[292] Compared to the entire Norwegian population, IVF conceptions were associated with a lower mean birth weight (25 g), shorter duration of gestation (2 days), and increased risk for small for gestational age birth weight (OR 1.26, 95% CI, 1.10 to 1.44). However, when compared to their matched sibling who was conceived without fertility treatment, there was no differences in birth weight, length of gestation, or risk

for small for gestational age birth weight. The biological processes causing infertility may have a larger impact on pregnancy outcome and fetal growth than the infertility therapy itself.[293]

Adoption

A difficult decision in fertility treatments is deciding when to cease active interventions designed to increase fecundability. Throughout the process of fertility treatment, it is prudent to raise the issue of when to cease active intervention and to offer adoption as an alternative method of building a family. It may be useful for couples to simultaneously explore the option of adoption while they are undergoing fertility therapy. If the fertility therapy fails, adoption may help couples cope with the symbolic loss created by their infertility.

Psychosocial Aspects of Infertility

Many observational studies indicate that stress is associated with infertility; in turn, the treatment of infertility can cause stress. Reducing stress prior to initiating intensive fertility treatments may improve the ability of the couple to successfully complete the treatments recommended.[294] For example, in one study, 151 women were given a standardized test to evaluate their moods, sense of optimism, social support networks, self-perceived stress, and methods of coping prior to undergoing IVF. Elevated levels of baseline stress were associated with fewer oocytes retrieved and fertilized and fewer embryos.[295] No definitive clinical trial demonstrates that reducing stress prior to infertility treatment improves pregnancy rates. In one small clinical trial, however, Domar and colleagues reported that treatment of infertile women with a support group or a structured relaxation program was associated with better pregnancy rates with infertility treatment than those observed in a control group.[296]

Infertility and the associated diagnostic and therapeutic procedures can produce substantial stress for both male and female partners and disrupt their relationship with each other. Inherently, diagnostic and therapeutic procedures offer hope for an imminent successful conception, but each subsequent menstrual cycle rekindles the feeling of loss. The repetitive cycles of hope and loss can be very stressful for couples with infertility.[297] Infertility may be perceived by the couple as a loss that is difficult to grieve because the absence of fertility is somewhat intangible. Infertility may be especially stressful for those couples where the cause of the infertility is difficult to identify.[298] The classic progression of emotions related to a loss are often expressed by the infertile couple.[299] These include the feelings of disbelief and surprise, denial, anger, isolation, guilt, grief, and resolution. For many couples, the female partner bears a disproportionate degree of responsibility for the loss symbolically represented by infertility. Although infertility treatment is often perceived as extremely stressful, infertility treatment does not appear to be associated with long-term emotional distress, dysfunction, or *de novo* psychiatric conditions.[300] Recent preliminary findings suggest that effective management of the stress and psychosocial sequelae of infertility might decrease the cost of fertility treatments and increase the fecundability of the couple.

Patients report that it is important that they be treated with respect and dignity and have all treatment options thoroughly and fairly presented. Patients wanted their clinicians to recognize their distress and respond in an empathic manner.[301,302] In most surveys, patients report that they are highly satisfied with the care provided by their fertility clinicians.[303]

Social and Ethical Issues

Medicine is an ethical profession that has long adhered to basic principles of human rights: respect for the dignity of human life, the right of an individual to participate in decisions that affect their health, an unwavering dedication to seek good and to avoid unnecessary harm, and a commitment to treat the patient fairly. Fertility practitioners also have an ethical obligation to protect the security of the human genetic material in their custody. Most ethicists are in agreement that the inviolability of each human precludes any medical intervention without the individual's consent. Free and informed consent is the cornerstone of ethical medical practice. Practices that are deceptive or could have the appearance of being deceptive undermine the credibility of clinicians in the fertility profession. Embryonic development is a continuous biological process. Current law tends to assign gradually increasing rights to the developing fetus. A human does not acquire full legal identity until birth but is offered some legal protection as a fetus *in utero* (e.g., restrictions on legal abortion in the third trimester). Modern society is not unified on the point in the developmental process where the developing embryo or fetus becomes a unique individual with full rights to inviolability and inalienability. This disagreement, which is most obvious in the debate over abortion, will make it difficult to reach consensus on the ethics of certain types of fertility research, such as that performed on discarded embryos. However, most practitioners and ethicists are in agreement that cloning of humans is not ethical.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 24

Male Infertility

Paul J. Turek

Male infertility affects 15% of reproductive age men worldwide and is treatable in many cases. In the United States, 8 million couples are affected.[1] Roughly 50% of couples have causal or associated male factors.[2] In addition, 1% to 10% of male factor infertility is a result of an underlying, often treatable, but possibly life-threatening medical condition.[3] In addition to well-established etiologies, genetic causes of male infertility are now commonly diagnosed. Finally, impaired semen quality has now been linked to an increased risk of later testicular and prostate cancers.[4,5] For these reasons, the male evaluation is conducted systematically to acquire relevant information from the history, physical examination, semen analysis, and hormone assessment. The American Urological Association and the American Society for Reproductive Medicine guidelines recommend that evaluations of male and female partners occur in parallel. Using evidence-based medicine, this chapter outlines diagnostic and treatment algorithms that guide clinical management. Randomized controlled clinical trials, basic scientific studies, meta-analyses, case-controlled cohort studies, best-practice policy recommendations, and reviews from peer-reviewed literature are incorporated into algorithms that provide timely guidelines to the current management of male infertility.

The Male History and Physical Examination

A thorough history reviews past and current attempts at paternity (Fig. 24.1 and Box 24.1). Important medical problems to elucidate include fevers and systemic illnesses such as diabetes, cystic fibrosis, cancer, and infections. Prior surgery, including orchidopexy and herniorraphy; trauma; retroperitoneal, pelvic, and bladder procedures; and prostate surgery may impair fertility. A family history of cryptorchidism, midline defects, or hypogonadism is also important. A developmental history of hypospadias, congenital anomalies, prenatal DES (diethylstilbesterol) exposure, and medication use (Box 24.2) should be reviewed. A social history with the habitual use of the gonadotoxins alcohol, tobacco, recreational drugs, anabolic steroids, and wet heat exposure should be elucidated. Spermicidal lubricants and incorrect patterns of timing intercourse may be noted from a sexual history. Lastly, an occupational history determines exposure to ionizing radiation, chronic heat, benzene-based solvents, dyes, pesticides, herbicides, and heavy minerals.

The physical examination assesses body habitus including obesity, gynecomastia, and secondary sex characteristics. The phallus may reveal hypospadias, chordee, plaques, or venereal lesions. The testes should be evaluated for size, consistency, and contour irregularities suggestive of a mass. At least 80% of testis volume is determined by spermatogenesis; hence, testis atrophy is likely associated with decreased sperm production. The epididymides are palpated for induration, fullness, or nodules, which are indicative of infection or obstruction. Delineation of each vas deferens may reveal agenesis, atresia, or injury. The spermatic cords should be examined for asymmetry suggestive of lipoma or varicocele, lesions that are differentiated by an examination in both the standing and supine positions. Meaningful varicoceles are diagnosed exclusively by physical examination. Lastly, a rectal examination is important in identifying large cysts, infections, or dilated seminal vesicles, all of which can be associated with infertility.

Semen Evaluation

Although not a true measure of fertility, the semen analysis, if abnormal, suggests that the probability of achieving fertility is lower than normal.[6] Two semen analyses, performed after 2 to 3 days of sexual abstinence, are sought owing to the large biological variability in semen quality. Lubricants should be avoided and the specimen kept at body temperature during transport. Normal reference values can be found in Table 24.1 and are based on a recent World Health Organization (WHO) analysis of 1800 new fathers from eight countries and three continents.[7] Recall that human spermatogenesis takes 60 to 80 days to complete (Fig. 24.2), so that an individual semen analysis reflects biological influences occurring 2 to 3 months prior.[8]

The formal evaluation of sperm shape is termed morphological assessment. Several descriptive systems exist to evaluate morphology, and within each system, sperm are designated normal or abnormal based on specific size criteria. It is believed that sperm morphology may correlate with a man's fertility potential as reflected by *in vitro* fertilization (IVF) success, although the reliability of this finding has recently been called into question.[9,10] Specifically, sperm morphology has been correlated with the ability of sperm to successfully penetrate cervical mucus, bind to the zona pellucida, and influence embryo implantation rate. In general, the percentage of sperm with normal morphology has discriminatory power in distinguishing fertile from infertile semen, although no particular value is diagnostic of fertility.[6] However, sperm morphology provides no insight into the chromosomal complement of the cell. Morphology for a given patient can also change depending on stress or toxic insult to the testes. Morphological examination can complement the

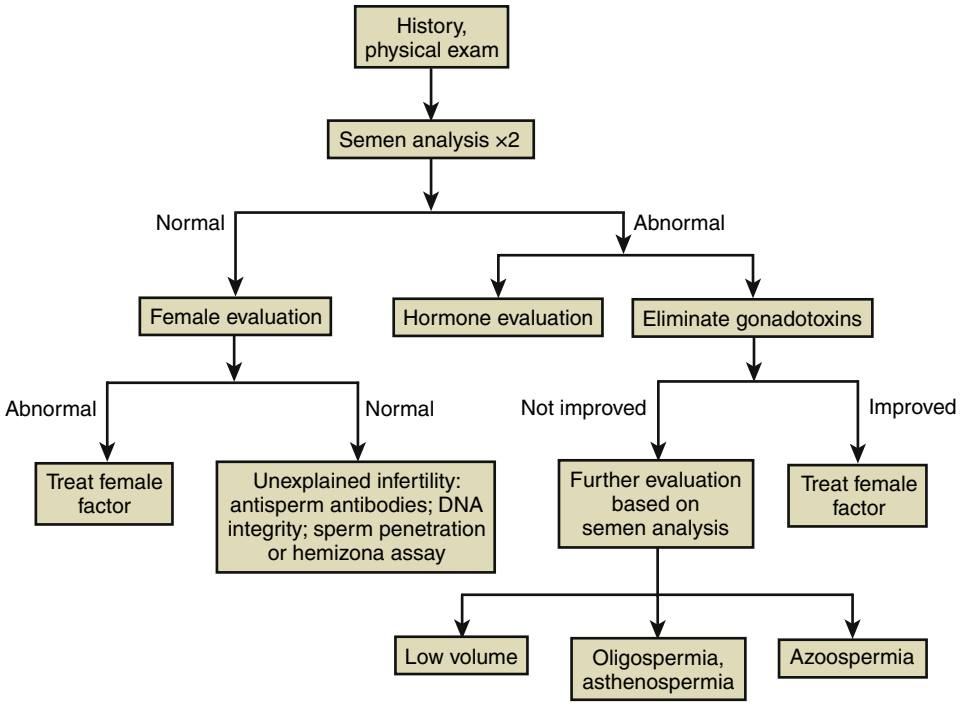


FIGURE 24.1 General algorithm for the diagnostic evaluation of male infertility. *(From Turek PJ. Practical approach to the diagnosis and management of male infertility.* Nature Clin Pract Urol *2:1, 2005.)*

Box 24.1 Components of the Male Infertility History

MEDICAL HISTORY
- Fevers
- Systemic illness—diabetes, cancer, infection, obesity
- Genetic disease—cystic fibrosis, Klinefelter syndrome

SURGICAL HISTORY
- Orchidopexy, cryptorchidism
- Herniorraphy
- Trauma, torsion
- Pelvic, bladder, or retroperitoneal surgery
- Transurethral resection for prostatism
- Pubertal onset

FERTILITY HISTORY
- Previous pregnancies (current and with other partners)
- Duration of infertility
- Previous infertility treatments
- Female evaluation

SEXUAL HISTORY
- Erections
- Timing and frequency
- Lubricants

FAMILY HISTORY
- Cryptorchidism
- Midline defects (Kartagener syndrome)
- Hypospadias
- Exposure to diethylstilbestrol
- Other rare syndromes—prune belly, etc.

MEDICATION HISTORY
- See Table 24.2

SOCIAL HISTORY
- Alcohol
- Smoking/tobacco
- Cocaine
- Anabolical steroids
- Wet heat exposure (hot tubs, baths)

OCCUPATIONAL HISTORY
- Exposure to ionizing radiation
- Chronic heat exposure (cooks, firefighters)
- Aniline dyes
- Pesticides
- Heavy metals

routine semen analysis in the male evaluation but its exact role in the estimation of male fertility is currently unclear.[10]

Results of Initial Male Evaluation

The initial male evaluation may be normal or abnormal (Fig. 24.1). If normal, further consideration should be given to female factor evaluation, including a thorough assessment of ovulation, pelvic anatomy, and age-related fertility issues. If the initial male assessment reveals abnormalities, then further evaluation or treatment is indicated.[11] Clinicians can have a significant impact in reducing male factor infertility by simple counseling regarding coital timing, avoiding wet heat and other exposures, and lifestyle changes. For example, coital lubricants should also be avoided if possible, including Surgilube®, K-Y jelly®, and saliva. If necessary, vegetable oils are safe for sperm.

Likewise, androgenic steroids often taken to increase muscle mass, act as male contraceptives.[12] Excess testosterone inhibits the pituitary-gonadal hormone axis and inhibits sperm production. The routine use of hot tubs, baths, or saunas should be discouraged, as these activities

Box 24.2 Drugs with Potential Adverse Effects on Male Fertility

COMPETITION WITH ANDROGEN RECEPTOR
- Spironolactone
- Cimetidine
- Flutamide
- Nilutamide
- 5 alpha reductase inhibitors

DIRECT TOXIC EFFECT ON LEYDIG CELLS
- Alkylating agents (e.g., cyclophosphamide)
- Alcohol
- Selective serotonin reuptake inhibitors (SSRI)

INHIBITORS OF TESTOSTERONE SYNTHESIS
- Ketoconazole
- Spironolactone
- Cyproterone
- Tetracycline
- Alcohol

STIMULATORS OF ESTRADIOL SYNTHESIS OR ACTIVITY
- DES (diethylstilbestrol)
- Digoxin
- Dibromochloropropane (pesticides)
- Spironolactone
- Medroxyprogesterone
- Bisphenol-A

HEAVY METAL TOXINS
- Lead
- Arsenic
- Cadmium
- Mercury

PITUITARY INHIBITION
- Testosterone
- Marijuana

MITOTIC INHIBITORS
- Allopurinol
- Colchicine
- Sulfasalazine
- Nitrofurantoin

EFFECTS ON FERTILIZATION
- Nicotine
- Calcium channel blockers

EJACULATORY DYSFUNCTION
- Lithium
- Antipsychotics
- Tricyclic antidepressants
- Valproic acid
- Phenytoin

UNCLEAR MECHANISM
- Monoamine oxidase inhibitors
- Cocaine
- Finasteride
- Seritonergic reuptake inhibitors

Table 24.1 Semen Analysis—WHO 5th Centile Reference Values for Fertile Men

Characteristic	5th Centile Value
Ejaculate volume	>1.5 mL
Sperm concentration	>15 × 10^6 sperm/mL
Motility	>40%
Forward progression	2 (scale 1-4)
Morphology	>4% (Strict)
Other features	No agglutination (clumping), white blood cells, or increased viscosity

WHO, World Health Organization.

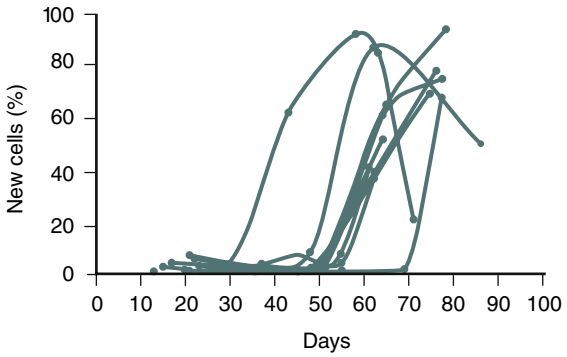


FIGURE 24.2 Timeline to make and ejaculate sperm. Labeling curves for 11 subjects with normal semen analyses. Subjects were given deuterated water daily for 3 weeks and semen was collected regularly for 90 days. Sperm DNA enrichment was measured and percentage of new sperm in the ejaculate was calculated. Overall, the mean time to detection of labeled sperm in the ejaculate was 64 ± 8 days (range 42 to 76), much faster than the 90 days classically ascribed to this process. *(From Misell LM, Holochwost D, Boban D, et al. A stable isotope/mass spectrometric method for measuring the kinetics of human spermatogenesis in vivo.* J Urol *175:242, 2006.)*

elevate intratesticular temperature and may impair sperm production.[13] As obesity is strongly associated with poor semen quality, weight reduction should be considered.[14] Sports such as bicycling are safe unless associated with significant urinary symptoms (prostatitis) or pelvic "numbness" that may predispose to erectile dysfunction.

Further Male Factor Evaluation

If lifestyle changes are unsuccessful, then further evaluation is warranted, following published best-practice policies.[11] This should include an assessment of the pituitary gonadal axis with testosterone and follicle-stimulating hormone (FSH) levels. Hormone testing is indicated in infertile men with sperm densities below 10 × 10^6 sperm/mL, or with evidence of a medical endocrinopathy.[15] The chance of a clinically significant endocrinopathy presenting as infertility is approximately 2%.[15] The more common patterns of hormonal disorders observed in male infertility are found in Table 24.2.

Adjunctive sperm tests are also available to diagnose the cause of male factor infertility and are pursued based upon the predominant semen analysis finding, as discussed later. When the infertility is unexplained, several tests can be considered, the most popular of which is an assessment of sperm deoxyribonucleic acid (DNA) integrity.[16] Evidence suggests that the

Table 24.2 Characteristic Endocrine Profiles in Infertile Men

Condition	T	FSH	LH	PRL
Normal	NL	NL	NL	NL
Primary testis failure	Low	High	NL/high	NL
Hypogonadotropic hypogonadism	Low	Low	Low	NL
Hyperprolactinemia	Low	Low/NL	Low	High
Androgen resistance	High	High	High	NL

FSH, follicle stimulating hormone; LH, luteinizing hormone; NL, normal; PRL, prolactin; T, testosterone.

quality of sperm DNA-chromatin packaging is important for fertility. High levels of reactive oxygen species and oxidative stress are known to cause sperm DNA fragmentation. The structure of sperm chromatin (the DNA-associated proteins) can be measured by several methods, including the single cell gel electrophoresis (comet) and terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assays as well as by flow cytometry after acid exposure and acridine orange staining.[16] These tests measure the degree of DNA fragmentation with chemical stress on the sperm DNA-chromatin complex, and can indirectly reflect the quality of sperm DNA integrity. Abnormally fragmented sperm DNA rarely

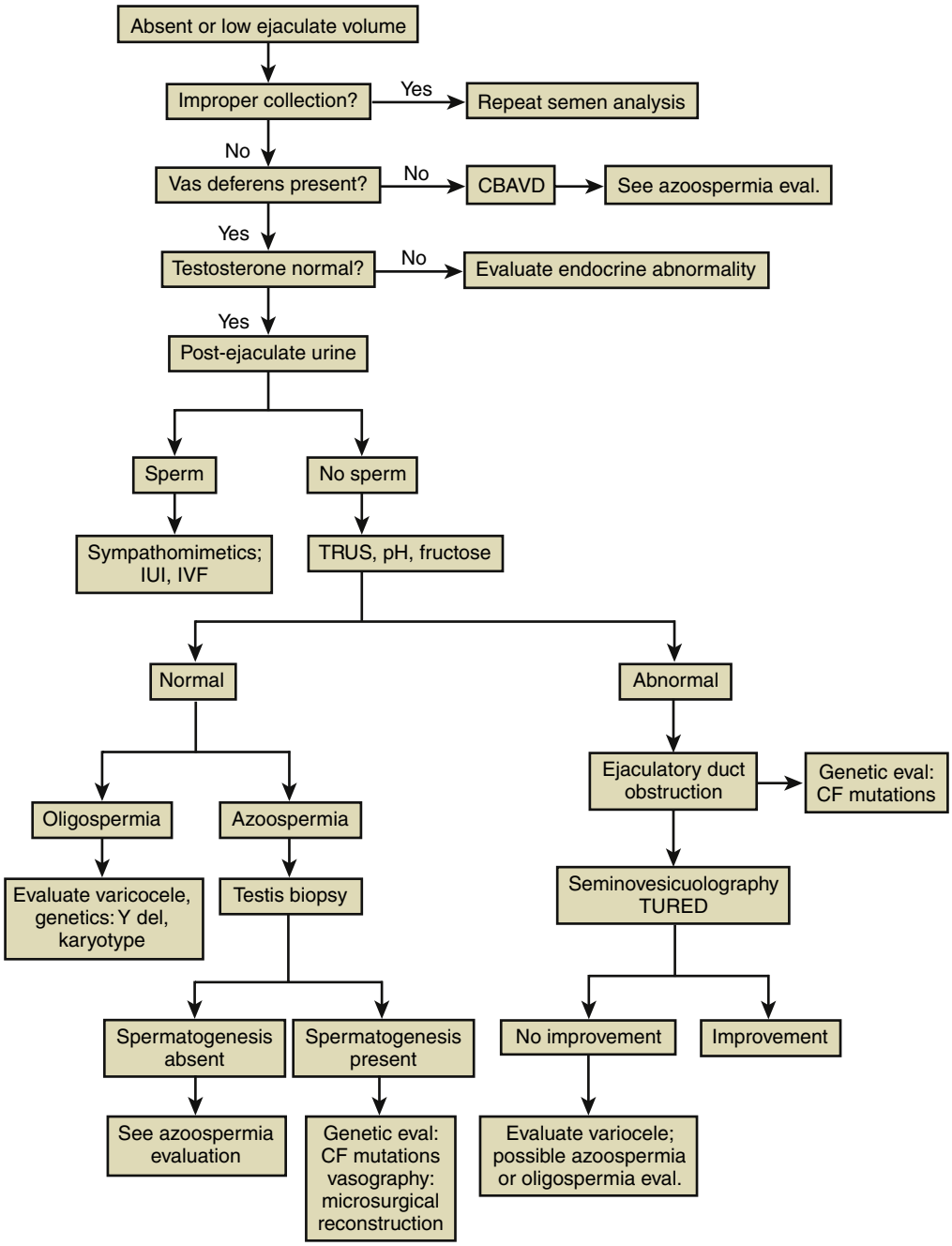


FIGURE 24.3 Algorithm for evaluation of absent or low ejaculate volume. CBAVD, congenital absence of vas deferens; CF, cystic fibrosis; IUI, intrauterine insemination; IVF, *in vitro* fertilization; TRUS, transrectal ultrasound; TURED, transurethral resection of the ejaculatory duct; Y del, Y chromosome deletion. *(From Turek PJ. Practical approach to the diagnosis and management of male infertility.* Nature Clin Pract Urol *2:1, 2005.)*

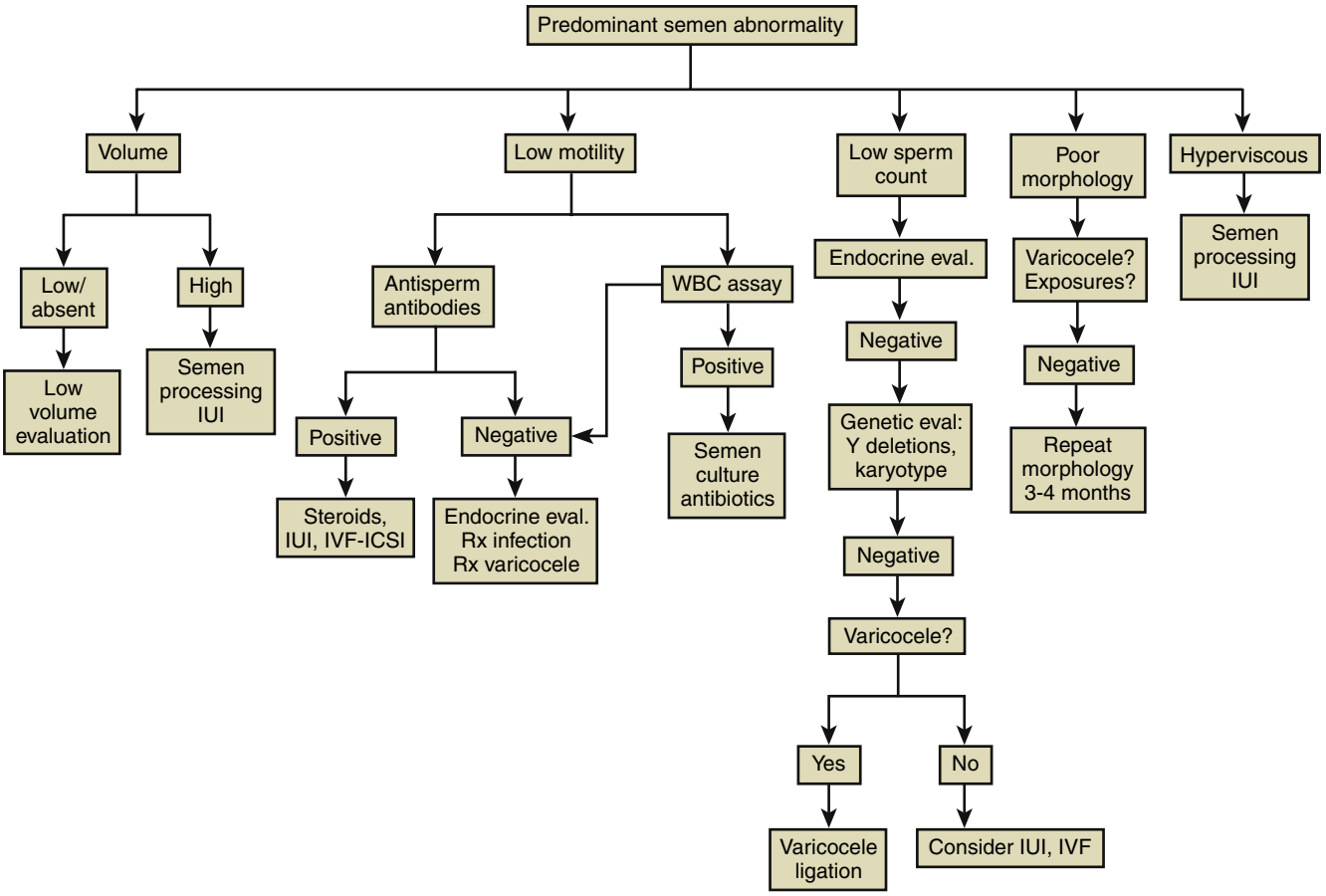


FIGURE 24.4 Algorithm for evaluation of semen analysis abnormalities. ICSI, intracytoplasmic sperm injection; IUI, intrauterine insemination; IVF, *in vitro* fertilization; Rx, treat; WBC, white blood cell. *(From Turek PJ. Practical approach to the diagnosis and management of male infertility.* Nature Clin Pract Urol *2:1, 2005.)*

occurs in fertile men, but can be found in 5% of infertile men with normal semen analyses and 25% of infertile men with abnormal semen analyses.[17] Such testing can detect infertility that is missed on a conventional semen analysis. Often reversible, DNA fragmentation may be caused by tobacco use, medical disease (e.g., cancer), hyperthermia, air pollution, infections (e.g., leukocytospermia), chemotherapy, irradiation, sperm processing, and varicocele.[18]

Low Volume Ejaculate

The evaluation of low ejaculate volume is uniformly informative, as it is generally due to one of five problems: improper sample collection, congenital absence of the vas (and seminal vesicles), a hypoandrogenic state, retrograde ejaculation, or ejaculatory duct obstruction. These conditions can be systematically examined as outlined in Figure 24.3 on page 541. A low seminal pH (<7) can replace semen fructose in this diagnosis. Importantly, transrectal ultrasound criteria to diagnose ejaculatory duct obstruction, including the presence of cysts, calcification, or dilated seminal vesicles or ducts, may "over read" the diagnosis by 50% and should be confirmed with "functional" studies such as vasography or duct chromotubation prior to endoscopic treatment.[19] The response of retrograde ejaculation to alpha-agonist medications is best for nonsurgical nerve damage to the hypogastric plexus (e.g., diabetes mellitus).[20] Bladder harvest of sperm in nonresponders can often be used with intrauterine insemination (IUI) to overcome infertility.

Oligospermia-Asthenospermia

Low sperm motility is observed in 25% of abnormal semen analyses; an isolated low sperm concentration is much less common. Low sperm motility may be due to antisperm antibodies (>50% of sperm bound) or excessive white blood cells in the ejaculate (leukocytospermia), the latter resulting in overproduction of reactive oxygen species that can damage sperm.[21] Because "round cells" in the infertile ejaculate are more commonly immature germ cells (65%) rather than leukocytes,[22] special leukocyte stains are recommended before treatment. Semen cultures are uninformative in asymptomatic infertile men with leukocytospermia as 83% are normally positive with multiple organisms.[23] It is important to evaluate sexually transmitted diseases, penile discharge, prostatitis, or epididymitis. An expressed prostatic secretion is examined for leukocytes, and urethral cultures for *Chlamydia* and *Mycoplasma* can be considered. As a reference, the prevalence rate for PCR-based Chlamydia antigen detection is 0.3% among infertile men in general.[24] Although the findings vary by both methodology and laboratory, in general, more than 50% of sperm bound with antibodies is considered significant and merits treatment.

Low sperm concentration may be due to an endocrinopathy such as prolactinoma, varicocele, or genetic causes, as outlined in Figure 24.4. Although eternally debated, there is substantial evidence to support the role of varicocele in male infertility (Table 24.3). Increasingly, genetic abnormalities

Table 24.3 Controlled Trials Addressing the Treatment of Clinically Palpable Varicoceles

Study	Patients/Arm		Pregnancy Rate	
	Control (n)	*Treatment (n)*	*Control (%)*	*Treatment (%)*
WHO[38]*	109	129	16.7	34.8
Nieschlag et al[36]	63	62	25.4	29.7
Madgar et al[37]	25	20	10	60

WHO, World Health Organization.
*This study was conducted but never published. See Reference 38 for details.

Box 24.3 Current Indications for Genetic Testing of Infertile Men

1. A semen analysis with sperm concentration <5 million sperm/mL in a couple considering *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (Y microdeletion assay and karyotype analysis)
2. A semen analysis showing no sperm with evidence of testis atrophy in a couple considering testis sperm extraction with IVF and ICSI (Y microdeletion assay and karyotype analysis)
3. A semen analysis showing no or low sperm concentration with at least one absent vas deferens on physical examination (cystic fibrosis gene mutations)
4. A semen analysis showing no sperm with evidence of normal spermatogenesis (cystic fibrosis gene mutations)
5. A couple with other syndromes or conditions suggested by personal or family histories (e.g., Kallmann syndrome)

should be considered in men with sperm concentrations less than 5 million/mL.[25] Deletion of regions on the Y chromosome (microdeletions) occur in 6% of men with severely low sperm counts and in 15% of men with no sperm counts. In addition, 2% of men with low counts and 15% to 20% of men with no sperm counts will harbor chromosomal abnormalities detected by cytogenetic analysis (karyotype). These conditions include Klinefelter syndrome and translocations of non-sex chromosomes. Box 24.3 outlines current indications for genetic testing of infertile males. Importantly, varicocele repair for oligospermia in the setting of positive genetic findings is unlikely to improve semen quality or result in natural pregnancy.[26]

Azoospermia (Fig. 24.5)

If sperm are absent in a routine semen analysis, the differential diagnosis is excurrent ductal obstruction or testicular failure. The semen specimen should be centrifuged to assess for very low sperm numbers that effectively rule out complete obstruction.[27] If one or both vasa deferentia are not palpable, then a diagnosis of congenital absence of the vas deferens (CAVD) is made, and appropriate genetic testing for cystic fibrosis gene mutations is recommended prior to sperm retrieval and *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Affected patients exhibit a similar spectrum of wolffian duct defects as those with cystic fibrosis, but generally lack the severe pulmonary, pancreatic, and intestinal problems. Roughly 80% of azoospermic men with CAVD and one third of men with unexplained obstruction will harbor cystic fibrosis gene mutations.[28,29] Based on FSH values and a diagnostic testis biopsy, accurate differentiation of men into nonobstructive (testis failure) or obstructive azoospermia is possible. Genetic testing for nonobstructive azoospermia with Y chromosome microdeletion and karyotype analysis is recommended, as outlined in Box 24.3. The relative frequency of the different Y chromosome microdeletions among azoospermic men is outlined in Figure 24.6. Treatment of prolactinomas with dopamine agonists or transsphenoidal resection is indicated in men with hormonal disorders.

Unexplained Infertility

Infertile couples with an unremarkable female evaluation and normal semen parameters are termed, "unexplained infertility" (Fig. 24.1). In such cases, further evaluation of male factor infertility is warranted, yet a precise clinical care algorithm is lacking. Antisperm antibodies and poor chromatin structure, the latter reflective of increased levels of denatured sperm DNA, are each found in 10% of semen analyses in unexplained infertility.[18,30] Tests of sperm function such as the sperm penetration assay or the hemizona assay may also be considered. Finally, many couples proceed to intrauterine insemination. As our knowledge of the genes that control sperm capacitation, fertilization and egg activation improves, many cases of unexplained infertility will be more clearly defined. For example, the recent identification of the sperm factor phospholipase C zeta (PLCz) as the chemical responsible for calcium flux and egg activation in mammals will likely explain some cases of human pregnancy failure.[31]

Treatment of Male Infertility

Male infertility is associated with a defined cause in half of cases (Fig. 24.7). When associated with life-threatening disease, the disease should be treated (Box 24.4). Because of advances in assisted reproduction (including ICSI), there has been a concerning trend to avoid male factor treatments in favor of assisted reproduction. This is unfortunate, as many male factor treatments help infertile couples conceive without assisted reproduction.[32]

Certainly, maternal reproductive potential should be considered before correctable male factors are treated. This reasoning becomes obvious given that it takes 2 to 3 months to improve spermatogenesis after therapy. Thus, an important guiding principle in the decision to treat male infertility is to first determine whether or not the female partner has more than 1 year of stable reproductive potential. If so, then correcting male infertility is almost always warranted.

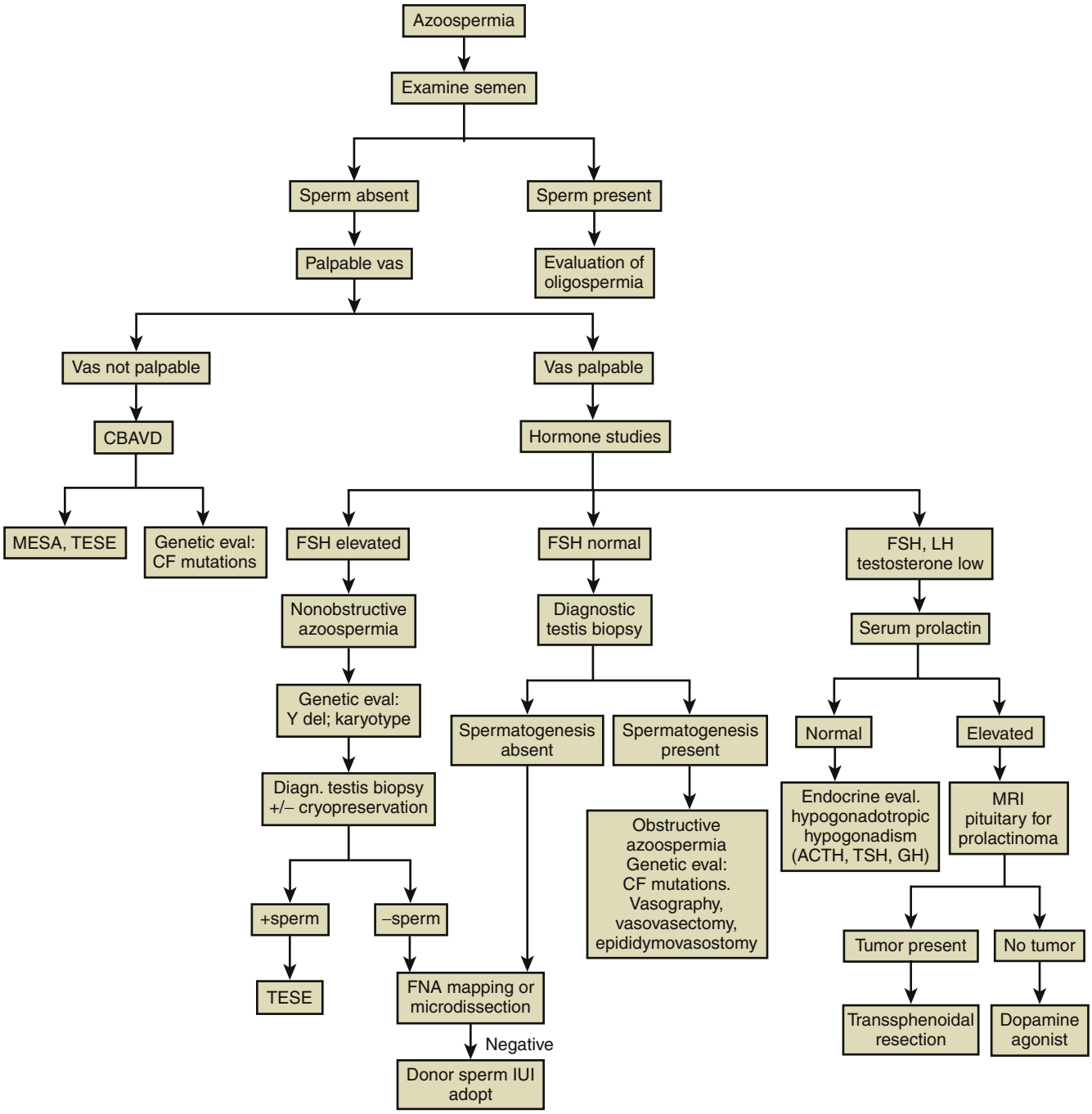


FIGURE 24.5 Algorithm for evaluation of azoospermia or no sperm in the ejaculate. ACTH, adrenocorticotropic hormone; CBAVD, congenital bilateral absence of the vas deferens; CF, cystic fibrosis; FNA, fine needle aspiration; FSH, follicle-stimulating hormone; GH, growth hormone; IUI, intrauterine insemination; LH, luteinizing hormone; MESA, microscopic epididymal sperm aspiration; MRI, magnetic resonance imaging; TESE, testis sperm extraction; TSH, thyroid-stimulating hormone. *(From Turek PJ. Practical approach to the diagnosis and management of male infertility.* Nature Clin Pract Urol *2:1, 2005.)*

Correctable Male Factor Problems

Coital Therapy

Simple counseling on issues of coital timing, frequency, and gonadotoxin avoidance can improve fertility. Coital lubricants should be avoided. Although scarcely published, hot baths and hot tubs are detrimental to sperm production.[13] Avoidance of tobacco, marijuana, excessive alcohol, and other recreational drugs is important. Maintaining a healthy weight and diet are germane to good fertility. Treating situational erectile dysfunction associated with timed intercourse with oral phosphodiesterase inhibitors is common and safe.

Ejaculatory Dysfunction

Retrograde ejaculation is diagnosed by finding sperm in the postejaculate bladder urine, and is treated with sympathomimetic medications or bladder harvest of sperm with

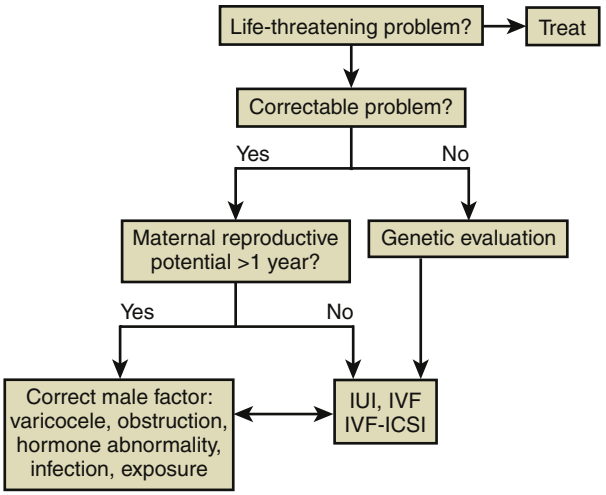


FIGURE 24.6 General algorithm for treatment of male infertility. ICSI, intracytoplasmic sperm injection; IUI, intrauterine insemination; IVF, *in vitro* fertilization. *(From Turek PJ. Practical approach to the diagnosis and management of male infertility.* Nature Clin Pract Urol *2:1, 2005.)*

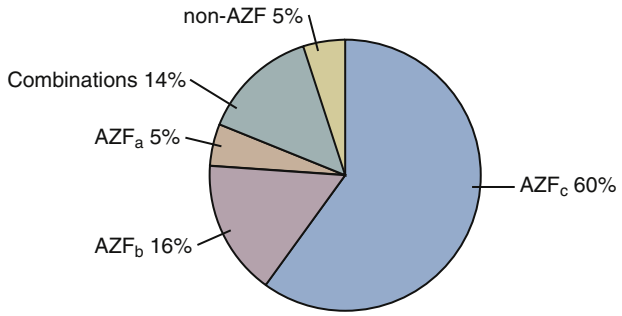


FIGURE 24.7 Relative frequency of specific AZF deletions in 5000 men with Yq microdeletions. *(From Shefi S, Turek PJ. Definition and current evaluation of subfertile men.* Int Brazil J Urol *32:385, 2006.)*

Box 24.4 Significant Medical Conditions Presenting as Male Infertility

Adult polycystic kidney	Pituitary adenoma
Cystic fibrosis	Prostate cancer
Diabetes	Retroperitoneal tumors
Hemochromatosis	Spinal cord tumors
Hypopituitarism	Testis cancer
Klinefelter syndrome	Thyroid disease
Multiple sclerosis	Urinary tract infection

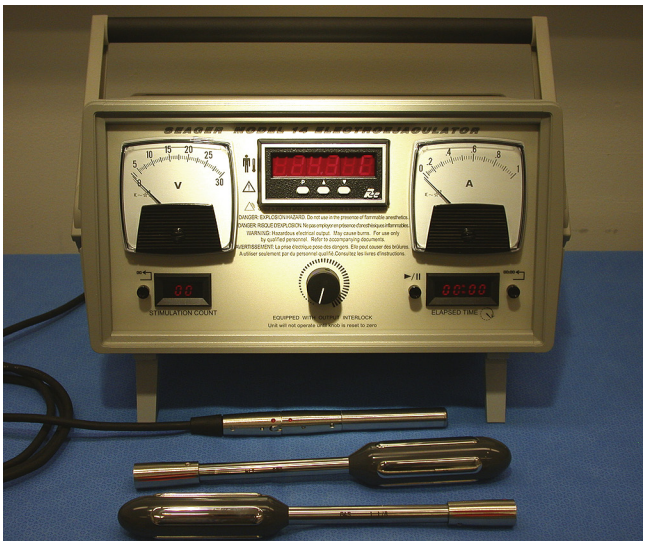


FIGURE 24.8 Rectal probe electroejaculator used for patients with primary or acquired ejaculatory dysfunction. Rectal probes (foreground) of different sizes are available to accommodate differences in patient anatomy. Ejaculates can be obtained in virtually all anejaculatory patients with this technology.

assisted reproduction. Premature ejaculation occurs when men ejaculate before their partner is ready. Although not an established cause of infertility, it can produce significant relationship stress. Sexual counseling combined with serotoninergic reuptake inhibitors or local anesthetics can be effective treatment. Ejaculatory failure or anejaculation has a variety of causes that include pelvic nerve damage from diabetes mellitus, multiple sclerosis, or abdominal-pelvic surgery; spinal cord injury; and psychosocial issues. Vibratory stimulation and rectal probe electroejaculation are common and effective techniques that help anejaculatory patients to conceive (Fig. 24.8).[20]

Leukocytospermia

Elevated leukocytes in semen (>1 million/mL) is termed pyospermia or leukocytospermia and is associated with (a) subclinical genital tract infection, (b) elevated reactive oxygen species, and (c) poor sperm function and infertility. Sperm are highly susceptible to the effects of oxidative stress induced by leukocytes because they harbor little cytoplasm and therefore little antioxidant activity. The treatment of leukocytospermia is controversial without overt bacteriological infection. When appropriate, the limited use of broad-spectrum antibiotics such as doxycycline and trimethoprim-sulfamethoxazole in combination with frequent ejaculation has been shown in a randomized controlled study to durably reduce seminal leukocyte concentrations.[33] Generally, the female partner is also treated.

Immunological Infertility

Antisperm antibodies are a complex problem for which available treatments include corticosteroid suppression, IUI, or IVF-ICSI. Corticosteroid suppression attempts to weaken an overactive immune system to reduce sperm antibodies and facilitate conception; however, two older, placebo-controlled, double-blind, crossover trials have shown conflicting findings regarding benefit of this treatment.[34,35] In addition, therapy must be undertaken for 6 to 9 months for best results. IUI places more sperm nearer to the ovulated egg to optimize the odds of fertilization. IVF and ICSI are very effective in overcoming infertility due to antisperm antibodies. Because the presence of antibodies is associated with obstruction in the genital tract, such lesions should be sought and corrected.

Varicocele

Although debated, there is substantial evidence to support the value of varicocele repair in male infertility. The outcomes of three controlled clinical trials that have examined treatment of palpable varicoceles generally favor varicocele repair, as summarized in Table 24.3.[36,37] Several meta-analyses have also examined outcomes of varicocele treatment and did not support varicocele repair as effective treatment.[38,39] However, one study has significant design flaws in that it examined trials that included subclinical varicoceles, the clinical relevance of which are unclear.[39] Furthermore, a controlled trial published by Madgar and colleagues[37] showed that a relative 6-fold benefit for varicocele repair was deemed an "outlier" in the meta-analysis, leaving only a single trial evaluable for clinical varicocele. Finally, the large WHO trial (238 patients) was excluded from the analysis.[36] Thus, more prospective trials are needed to prove that varicoceles improve fertility. However, clinically, semen quality will improve in 51% to 78% of men after varicocele repair and the associated pregnancy rate is 24% to 60%, with pregnancies occurring an average of 8 months after surgery.[32,40]

In addition to clinical care arguments that suggest varicocele repair is beneficial for infertility, economic analyses also support this treatment. Cost-benefit arguments demonstrate that varicocelectomy is more cost-effective than assisted reproduction[41] and "shift of care" analyses show that 30% to 50% of couples who begin as candidates for assisted reproduction due to low semen quality can be "rescued" from such procedures and conceive naturally after varicocelectomy.[32] Decision analysis research suggests that varicocele repair may have the greatest value in severe male factor cases that would otherwise require IVF-ICSI.[42] On the contrary, it is also apparent varicocele treatment is not worthwhile in the presence of coexisting genetic infertility.[26]

Several modalities are available for varicocele treatment, including vein ligation through retroperitoneal, inguinal, or subinguinal incisions, percutaneous transvenous embolization, and laparoscopy. Success rates do not differ significantly among approaches. Complication rates range from 1% for the incisional approach to 4% for laparoscopy. The most significant complication with embolization is the 10% to 15% technical failure rate (inability to access and occlude the culprit veins).

Ejaculatory Duct Obstruction

Ejaculatory duct obstruction is observed in 5% of azoospermic men, but is often overlooked in the male evaluation. Partial obstruction is likely more common than currently diagnosed, but is more difficult to diagnose than complete obstruction, as ultrasound findings are not specific for partial obstruction.[19] A 20% to 30% pregnancy rate can be expected from endoscopic treatment of the obstruction, and 70% to 80% of men will achieve significant, early, and durable improvements in semen quality.[19,43] Complications occur in 10% of cases and include hematuria, watery ejaculate, and epididymitis.

Vasovasostomy

Vasectomy reversal success depends on many factors, the most important of which are the surgeon's skill and the findings at surgery. Because of the need for superb tissue technique, experienced surgeons use a microscope. Evidence of inflammation or infection after the vasectomy, and a long interval from vasectomy-to-reversal are both associated with a decrease in surgical success.[44] If sperm are found at the cut edge of the vas during surgery, then 85% to 99% of patients can be expected to have a return of sperm after vasovasostomy.[45] With a healthy female partner, this is associated with a pregnancy rate of 40% to 65%.[45,46] If the vas fluid shows no sperm, the primary procedure involves connecting the vas to the epididymis in a procedure termed epididymovasostomy. In experienced hands, approximately 60% to 80% of men will have sperm in the ejaculate and a 30% to 35% pregnancy rate can be expected.[44,47]

A growing literature suggests that vasectomy reversal may be more cost-effective for pregnancy than IVF and ICSI.[47] Given the difficulty of randomizing patients to vasectomy reversal or sperm-retrieval and ICSI, decision analysis and Markov modeling research has been applied to this issue.[42,48] Decision analytical models are methods of estimating and calculating outcomes by identifying a clinical question, disaggregating the problem into discrete units to include all reasonable choices and consequences, and assigning probabilities and costs to the various events and outcomes. Decision science has revealed that reversal surgery is often more cost-effective than sperm retrieval and ICSI, but threshold values for surgical success are required to maintain its cost-effectiveness (Fig. 24.9).[42] Markov modeling is a form of decision analysis in which hypothetical patients proceed through health states over time based on predefined probabilities and costs. As patients are cycled, outcomes, including incurred costs and events, are tracked. Markov analyses have been used to better understand the relative impact of vasectomy obstructive interval and female partner age on fertility after vasectomy. These analyses have shown that female fertility potential impacts

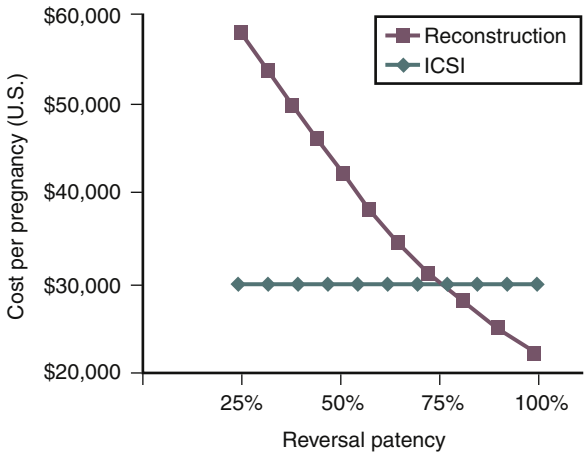


FIGURE 24.9 Sensitivity analysis from decision modeling of vasectomy reversal compared with intracytoplasmic sperm injection (ICSI) and sperm retrieval. This figure shows a cost comparison of both methods over a range of reversal patency rates when the pregnancy rate is held constant at 40%. This demonstrates that a minimum threshold vasectomy reversal patency rate (78%) is important to achieve if vasectomy reversal is to remain more cost-effective than ICSI. *(From Meng M, Greene K, Turek P. Surgery or assisted reproduction? A decision analysis of treatment costs in male infertility.* J Urol *174:1926, 2005.)*

cost-effectiveness more profoundly than does vasectomy obstructive interval (Fig. 24.10).[48] This suggests that, in the absence of randomized data comparing ICSI and sperm retrieval with vasectomy reversal, the primary driver of cost-effective care is the variable of female age and maternal reproductive potential. Even given this, respectable crude pregnancy rates have been reported in vasectomy reversal series in the setting of advanced maternal age.[49,50]

In addition to vasectomy, infertility can also result from idiopathic obstruction. In such cases, 65% of blockages are found within the epididymis, 30% in the vas deferens, and 5% in the ejaculatory duct.[51] In most cases of idiopathic obstruction, the blockage can be pinpointed and corrected with microsurgery. To distinguish obstructive azoospermia from that due to a sperm production problem, a testis biopsy is generally necessary.

Hormonal or Oxidative Dysfunction

Hormone therapy can and should be offered to patients with treatable diseases that predispose to infertility. Less effective treatments are those that seek to overcome ill-understood conditions or those without well-proved treatments. Examples of treatable conditions include hyperprolactinemia, hypothyroidism, congenital adrenal hyperplasia, and testosterone excess or deficiency (e.g., steroids or Kallmann syndrome). Examples of medical treatments that may not work in all men include clomiphene citrate, tamoxifen, human chorionic gonadotropin (hCG) therapy, L-acetylcarnitine, and antioxidant and herbal therapy. When given unselectively to infertile men, these measures should be considered empirical treatments for male infertility.[52] Like with cancer or aging, oxidants are thought to be a fundamental cause of male infertility as they clearly damage DNA and reduce sperm motility. Notably, a recent Cochrane review of 34 randomized controlled trials (2876 subjects) of antioxidant use in men whose partners were undergoing IVF demonstrated fourfold higher rates of conception and live births in the antioxidant arm.[53] The use of aromatase inhibitors in men with elevated estradiol: testosterone ratios have shown some benefit to semen quality in a single study.[54]

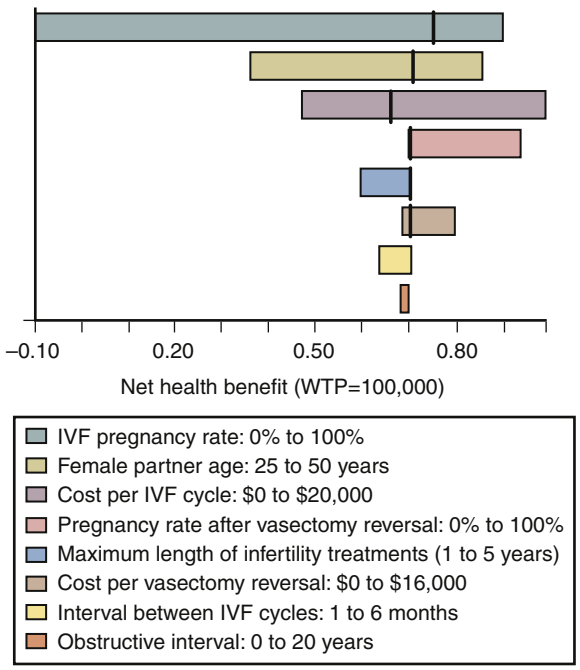


FIGURE 24.10 Tornado diagram from Markov modeling for intracytoplasmic sperm injection compared with vasectomy reversal. This figure shows the relative impact of female partner age, vasectomy obstructive interval, and other clinical parameters on net health benefit (NHB) and demonstrates that female age has the largest effect on cost effectiveness. Widths of horizontal bars indicate magnitude of impact of that parameter on NHB. The wider the bar, the greater the impact on NHB. Thick vertical bars are threshold values at which reversal and assisted reproductive technology switch being more cost-effective. IVF, *in vitro* fertilization; WTP, willingness to pay. *(From Hsieh M, Meng M, Turek PJ. Markov modeling of vasectomy reversal and ART for infertility: how do obstructive interval and female partner age influence cost-effectiveness?* Fertil Steril *88:840, 2007.)*

Assisted Reproduction

If neither surgery nor medical therapy is appropriate treatment for male infertility, assisted reproduction can be initiated. From a male infertility point of view, the choice of technique depends mainly on the degree of impairment in total motile sperm concentration (Fig. 24.11).

Intrauterine Insemination

Intrauterine insemination involves placement of washed ejaculated sperm within the female uterus, beyond the cervix. In this way, more motile sperm can progress to the fallopian tubes where fertilization normally occurs. The principal indication for IUI is a cervical factor, but IUI is also used for low sperm quality, immunological infertility, and in men with mechanical problems of sperm delivery (e.g., hypospadias). There should be at least 5 million to 40 million motile sperm in the ejaculate (volume × concentration × motility) to make this procedure worthwhile. Success rates vary widely and are directly related to female reproductive potential; given this, pregnancy rates of 30% for four cycles can be expected.[55]

In Vitro Fertilization and Intracytoplasmic Sperm Injection

First described in 1978, *in vitro* fertilization (IVF) is more complex than IUI and removes even more obstacles to sperm in the female reproductive tract. Following ovarian hyperstimulation and ultrasound-guided transvaginal egg retrieval, oocytes are fertilized *in vitro* with 500,000 to 5 million motile sperm. After fertilization, embryos are placed into the uterus transcervically. This technology can bypass moderate to severe forms of male infertility in which

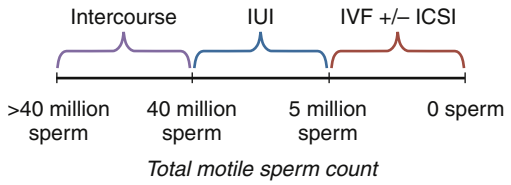


FIGURE 24.11 Level of assisted reproduction needed for conception based on semen quality determined by total motile count (volume × concentration × motile fraction). IUI, intrauterine insemination; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection. *(From Turek PJ. Practical approach to the diagnosis and management of male infertility.* Nature Clin Pract Urol *2:1, 2005.)*

there are low numbers of motile sperm. A revolutionary addition to IVF was described in 1992 and is referred to as sperm micromanipulation or ICSI, in which a single sperm is microinjected directly into the egg cytoplasm.[56] ICSI has lowered the sperm requirement for egg fertilization from hundreds of thousands to one viable sperm, thus allowing treatment of the most severe forms of male infertility. In a systematic review and meta-analysis, an association was found between the degree of sperm DNA fragmentation and pregnancy rates after IVF and ICSI, but this relationship was not deemed clinically significant.[57] The current indications for IVF and ICSI for male factor diagnoses are listed in Table 24.4.

Genetic Evaluation

High-technology solutions to pregnancy, including ICSI, are now known to be a two-edged sword. On one hand, ICSI is very enabling, allowing men who would otherwise have no chance for paternity the opportunity for fatherhood. However, because man and not nature selects sperm for ICSI, how it alters natural selection is not clear. Genetic evaluation of the infertile couple is important prior to IVF and ICSI for male infertility due to oligospermia or azoospermia that is not due to acquired blockage (Box 24.3). In addition, there is some debate about whether IVF-ICSI is as effective for men with defined genetic infertility, in particular Y chromosome microdeletions, as it is for other male factor indications (Table 24.5).[58-61] Although overall rates of major birth defects associated with ICSI (3.3%) are similar to those associated with intercourse,[62] chromosomal abnormalities are increased in offspring with ICSI[63] and increased rates of conditions such as hypospadias, and syndromes such as Angelman and Beckwith-Wiedemann have been reported.[64-66] In addition, subtle development defects and delays have been detected in children conceived with assisted reproduction.[67] For these reasons, genetic counseling is recommended for all couples considering IVF-ICSI.

Table 24.4 Indications for Assisted Reproduction Technologies with Male Factor Infertility

Male Factor Issue	IVF	IVF-ICSI
Retrieved sperm (testis and epididymis)	—	Yes
Low motile sperm count (<5 million)	Yes	Maybe
Poor sperm morphology (<4% strict or Kruger)	—	Maybe
Failed trial of IUI	Yes	Maybe
Immunological infertility (antisperm antibodies) and failed IUI	—	Yes
Abnormal sperm DNA integrity	?	Maybe
Correctable male factor with limited maternal reproductive potential	Yes	Maybe

ICSI, intracytoplasmic sperm injection; IUI, intrauterine insemination; IVF, *in vitro* fertilization.

Sperm Retrieval Techniques

ICSI has led to the development of aggressive surgical techniques for azoospermic men to provide sperm for egg fertilization. Since 1988, epididymal sperm aspiration has been performed either microsurgically (MESA) or percutaneously (PESA). Since 1995, testis sperm retrieved by needle (TESA) or biopsy (TESE) is routinely performed in men with obstructive azoospermia. Currently, there is insufficient clinical trial evidence to recommend a preferred sperm retrieval technique in azoospermic men.[68]

Although sperm retrieval in obstructive azoospermia is not difficult, there is a failure to obtain sperm for ICSI in 25% to 50% of men with nonobstructive azoospermia (Box 24.5).[69] In addition, clinical features of testicular size, history of ejaculated sperm, serum FSH level, or histological findings at biopsy do not accurately predict whether or not sperm will be recovered on exploration.[69] Because of this, strategies have been developed to more accurately determine which men with failing testes are candidates for ICSI, and surgical techniques have been

Box 24.5 Conditions Associated with Nonobstructive Azoospermia

- Idiopathic primary testicular failure
- Y chromosome microdeletions
- Karyotype disorder
- Sex chromosomes (Klinefelter syndrome)
- Non-sex chromosomes, translocations, aneuploidy
- Secondary testicular failure
- Noonan syndrome
- Kallmann syndrome
- Idiopathic gonadotropin deficiency
- Hypothalamic/pituitary tumor
- Hyperprolactinemia
- Cancer chemotherapy/radiotherapy treatment
- Varicocele effect
- Gonadotropin suppression
- Drug-induced (anabolical steroids, alcohol, glucocorticoids)
- Congenital adrenal hyperplasia
- Severe systemic illness (cancer, uremia)
- Cryptorchidism
- Sperm autoimmunity
- Pesticide/toxin exposure

Table 24.5 Summary of Studies of IVF-ICSI Outcomes in Couples with Yq Deletions

			Normal Fertilization Rate		Pregnancy/Cycle	
Author	Points	Yq deletions	Control	AZF	Control	AZF
Mulhall et al[58]	3	AZFc	45% (n = 25 cycles)	36% (n = 6 cycles)	—	—
Van Golde et al[60]	8	AZFc with oligospermia	71% (n = 107 pts)	55%	25%	16%
Oates et al[59]	26	AZFc	—	47%	—	27%
Choi et al[61]	17	AZFb and c with azoospermia and oligospermia	58%	49%	42%	33%

ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization.

refined to minimize the invasiveness of sperm harvest procedures. The first of these, the multi-biopsy method, involves taking as many biopsy samples as needed at the time of ICSI.[69] Another approach involves taking testis tissue by biopsy for both diagnosis (histology) and sperm retrieval simultaneously and *in advance* of ICSI to avoid cycle cancellation if sperm harvest fails.[70] Microdissection TESE uses microsurgical exploration of the widely opened testis to search for pockets of sperm.[71] Finally, diagnostic testis fine needle aspiration (FNA) "mapping" under local anesthesia literally maps out the geography of sperm production in the testis to determine patient candidacy for future testis sperm retrieval (Fig. 24.12).[72] Subsequently, at ICSI, needles or biopsies are "directed" to testis locations informed by the map. Most recently, preoperative factors such as FSH, inhibin B, and total testosterone have been used to construct formulas to predict sperm retrieval.[73] Regardless of approach, most men with nonobstructive azoospermia will have usable testis sperm by one of these methods.

Advances in Identifying Genes Involved in Male Infertility

It is widely believed that much of currently unexplained nonsyndromic male infertility will be found to have a genetic basis. And, as a complex disease, it is estimated that 1000 genes could be involved with normal male fertility. Past research aimed at discovering genetic loci responsible for male infertility involved targeting specific genes or gene regions or traditional genetic linkage approaches. These and other methods led to the identification of mutations associated with unique sperm phenotypes in selected populations. These include mutations in the aurora kinase C *(AURKC)*, a serine/threonine kinase, which cause macrozoospermia, characterized by sperm with large heads, increased chromosomal content, and a variable number of tails; and mutations associated with globozoospermia, sperm with round heads (*SPATA16;* spermatogenesis associated 16, a gene encoding a Golgi protein) and (*DPY19L2;* dpy-19-like 2, a gene encoding an inner nuclear membrane protein).

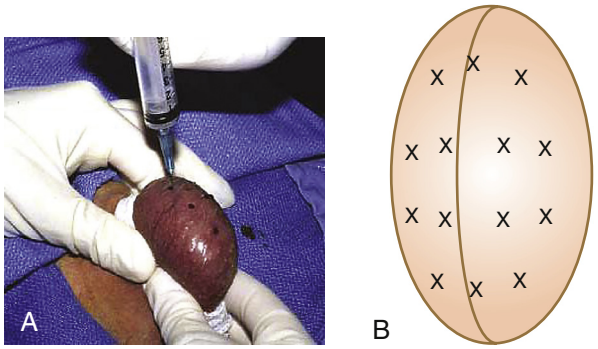


FIGURE 24.12 Technique of percutaneous fine-needle aspiration "mapping" for sperm in the testis. **A,** Samples are taken systematically from the testis for cytological examination for sperm. **B,** A recent sampling template. This is done in advance of assisted reproductive technology procedures. *(From Turek PJ, Cha I, Ljung B-M. Systematic fine needle aspiration of the testis: correlation to biopsy and the results of organ "mapping" for mature sperm in azoospermic men.* Urology *49:743, 1997.)*

The completion of the Human Genome Project in 2003 and the International HapMap Project in 2005 allowed the development of research tools and databases powerful enough to detect mutations using population-based approaches, genome-wide association studies (GWAS). GWAS techniques examine large numbers of genetic variations in a population, and associate variations with a specific trait or disease. To date, more than 1000 human GWAS have been carried out to examine 200 diseases and traits.[74] GWAS focus on small genetic variations termed single nucleotide polymorphisms (SNPs). SNPs are smaller variations in the human genome than are classic genetic deletions, insertions or copy number variants, but can be significant trait-altering events. In contrast to earlier genetic approaches that are able to test one or only a few genetic regions, GWAS investigate the entire genome. Such studies have been particularly useful in finding genetic variations in common, complex diseases such as asthma, cancer, diabetes, heart disease, and mental illnesses.

GWAS compare the DNA from those with a disease and those without a disease. From each person's DNA, millions of small genetic variations are read using SNP-based arrays. SNPs that occur commonly with a disease are "associated" with the disease, and therefore point to areas of the human genome that may influence the risk of that disease. Importantly, GWAS are unable to distinguish whether a genetic variant actually causes a disease. In addition, given the massive number of statistical tests needed to analyze GWA data, there can be an increased potential for false associations with this research.

Three recent GWAS have been reported that examined SNPs associated with male infertility (Table 24.6). In two studies of azoospermic Chinese men, a total of five genetic loci were observed to be associated with this condition, including several within the Human leukoctye antigen (HLA) region.[75,76] In a third study, fertile men were examined to determine associations between the fertility traits of family size and birth rate and potential genetic loci.[77] A total of nine loci were shown to be significantly associated with fertility traits and three were deemed reasonable candidate male fertility genes (Table 24.6). In summary, as a novel gene discovery tool, GWAS have great potential to delineate many of the thousand or so genes presumed to be associated with male fertility.

Table 24.6 Genome Wide Association Studies and Implicated Genetic Loci for Male Infertility

Study	Patient Population	Associated Genetic Loci
Zhao et al, 2012[75]	802 azoospermia cases 1863 controls	HLA-DRA, rs3129878, OR = 1.37 HLA-DRA, rs498422, OR = 1.42
Kosova et al, 2012[77]	269 married men	USP8, UBD, ESPSTI1, LRRC32
Hu et al, 2012[76]	2927 azoospermia cases 5734 controls	1p13.3 (PRMT6), OR = 1.25 1p36.32 (PEX10), OR = 1.39 12p.12.2 (SOX5), OR = 1.23

Note: OR = Odds ratio.

Conclusions

Evaluation of male factor infertility should be conducted to uncover life-threatening conditions and to detect correctable problems that present as infertility. The semen analysis may be viewed as a "biomarker" of men's health, as male infertility has also been shown to lead to an increased risk of later developing both testis and prostate cancers. Genetic causes of male infertility are assuming a greater role as our understanding of genomic medicine grows. Medical or surgical treatments should be undertaken after consideration of female partner reproductive potential. There is a need for adequately powered and well-controlled clinical trials to better understand both the value of classic male factor treatments, and the role of assisted reproduction in male infertility treatment.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 25

Endocrine Disturbances Affecting Reproduction

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The reproductive systems are vulnerable to disruption by internal and external forces including disease, malnutrition, and various forms of stress. The male and female axes are both susceptible to dysfunction from the same processes, although the female axis tends to be more sensitive. This chapter will review the influence of endocrine disorders of the pituitary, adrenal, and thyroid on reproduction. Each section will discuss the most important and relevant disorders of the specific gland and its effects on female reproduction first. Features specific for male reproduction will be discussed where relevant in each disease subsection. The reader is directed to chapters 3, 18, and 21 for review of neuroendocrine disorders; chapters 4, 17, and 18 for further discussion of congenital adrenal hyperplasia; chapter 21 for a review of Cushing disease; and chapter 28 for a review of endocrine disorders in pregnancy.

Pituitary Disorders

Overview

As the "master gland," the anterior pituitary gland controls the secretion of several essential hormones from other major endocrine glands, including the thyroid (releasing thyroxine and triiodothyronine), adrenal cortex (cortisol, dehydroepiandrosterone sulfate), and gonads (predominantly estradiol in females and testosterone in males). The anterior pituitary gland also produces growth hormone and prolactin, which act directly on target organs. The actions of growth hormone are largely exerted via the local or systemic production of insulin-like growth factor-1 (IGF1). The posterior pituitary regulates water metabolism via the production of vasopressin and induces milk letdown via the production of oxytocin. Disorders of the pituitary can be partial or complete, isolated to one hormone or multiple, and related to hormone deficiency or hormone excess. The axes controlled by the pituitary gland share the following basic principles:

1. Input from higher brain centers to the hypothalamus
2. Releasing and inhibitory factors influencing pituitary hormone secretion
3. Hypothalamic factor pulsatility, leading to pulsing of pituitary and target gland hormones
4. Feedback inhibition at both the hypothalamus and pituitary by active target gland hormones
5. Peripheral metabolism of target gland products
6. Influence of diurnal rhythm

The relative importance of these regulatory components varies for each axis and is described below in more detail. These axes are shown schematically in Figure 25.1.

Of the pituitary axes, the thyroid axis is the simplest for several reasons. The primary product of the thyroid is thyroxine (T_4), which is a precursor of the active hormone triiodothyroinine (T_3). Since T_4 is heavily protein-bound, T_4 has a long half-life (7 days), and T_4 is slowly metabolized to T_3. This scenario provides steady, well-dampened feedback, and the thyroid axis therefore shows high stability and low pulsatility. Hypothalamic thyrotropin-releasing hormone (TRH) pulses stimulate release of thyroid stimulating hormone (TSH); however, negative feedback by T_3 directly on the pituitary tightly regulates TSH biosynthesis and primarily regulates the thyroid axis. Thus the thyroid axis is a model endocrine feedback system due to its stability and simplicity.

In contrast, the adrenal axis is characterized by a strong diurnal rhythm, with the shorter half-life of cortisol (1 hour) leading to greater pulsatility. The adrenal axis is also more sensitive to factors beyond hypothalamic corticotropin-releasing hormone (CRH) and negative feedback by cortisol. Both vasopressin and various cytokines increase production of corticotropin (ACTH) in response to stress or illness. As a consequence, this intricate feedback system provides a more responsive axis to physiological changes and needs. However, this level of sophistication complicates clinical testing.

The growth hormone (somatotropin, GH) axis is primarily a two-component axis regulated by the hypothalamus. Growth-hormone releasing hormone (GHRH) serves as the major positive stimulus and somatostatin (SS) as the major negative stimulus. GH stimulates the production of IGF1 in the liver and locally in other tissues. Circulating IGF1 derives from the liver and exerts some negative feedback on the axis, but this influence is small relative to SS. Numerous hormonal and metabolical factors, however, also alter GH secretion by modulating the release of GHRH and SS. While the two hypothalamic hormones regulate GH pulsatility throughout the

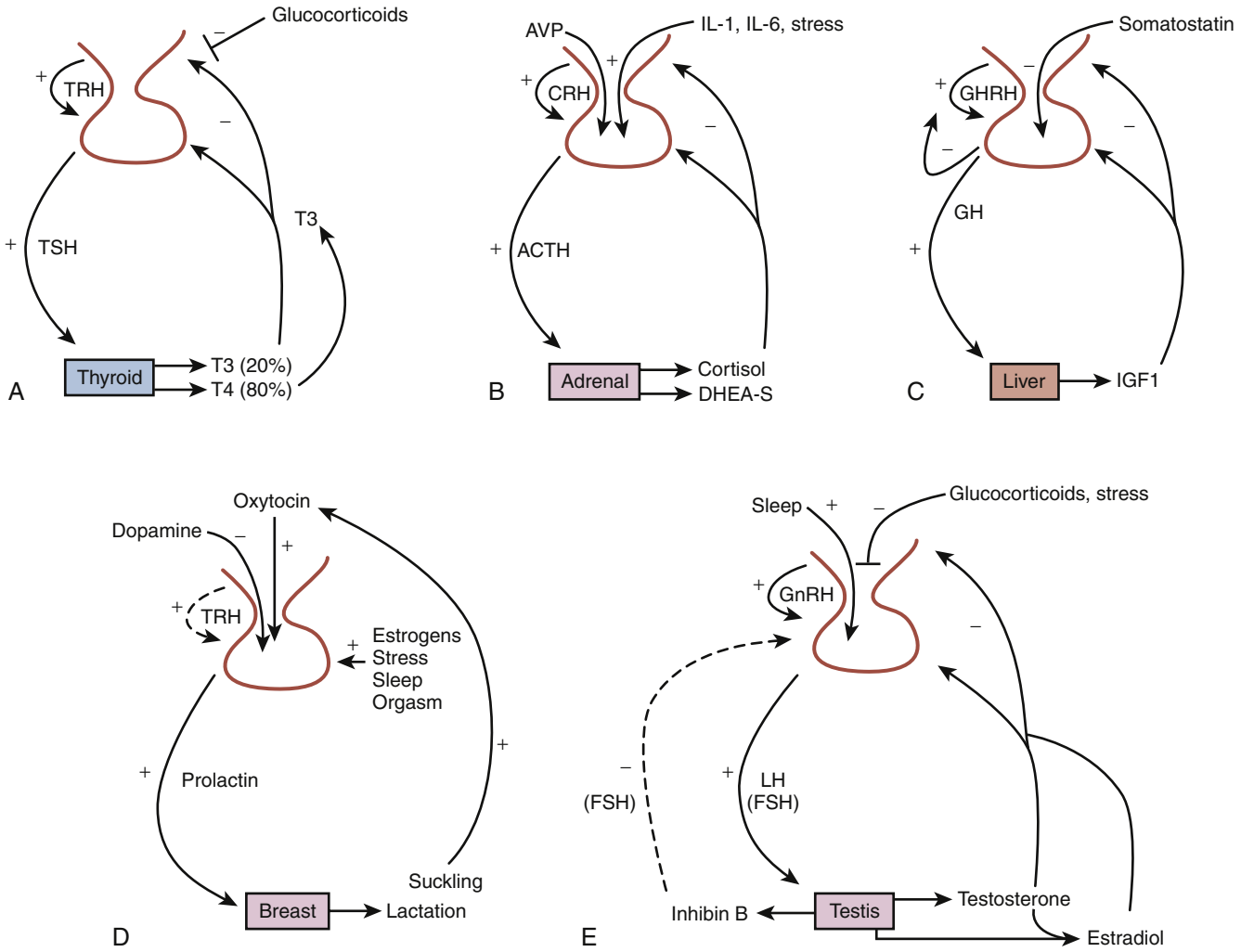


FIGURE 25.1 The major factors regulating the anterior pituitary axes. **A**, thyroid axis; **B**, adrenal axis; **C**, growth-hormone (GH)-insulin-like growth factor-1 (IGF1) axis; **D**, prolactin axis; **E**, gonadal axis. The male gonadal axis is shown here; the corresponding female axis is discussed at length in Chapter 8.

day, the greatest GH pulses are produced in deep sleep. Significant physical stressors, including hypoglycemia, also increase GH secretion.

Prolactin is the only anterior pituitary hormone that is primarily under negative control, mediated by dopamine (DA). For this reason, prolactin rises whenever blood flow to the pituitary from the hypothalamus is impaired. Therefore, hyperprolactinemia can result from a large pituitary tumor, which does not itself secrete prolactin, by blocking blood flow from the stalk. Prolactin is also increased by stress, nipple stimulation, and TRH, so prolactin rises transiently for several reasons and persistently for several others (Table 25.1). Prolactin acts on the breast to enable lactation, but there is no known hormonal product from the breast that exerts feedback on prolactin secretion. Estrogens also stimulate lactotrope growth.

The gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and their regulation by gonadotropin-releasing hormone (GnRH) are discussed in detail for the male and female in other chapters. Here, we simply emphasize that pulsatile GnRH secretion every 90 to 120 minutes is critical to LH and FSH production.

Table 25.1 Causes of Hyperprolactinemia

Cause	Characteristic features(s)
Prolactinoma	Mass effects if macroadenoma
Acromegaly	Headaches, heavy perspiration, acral changes
Macroadenoma (not prolactin secreting)	Peripheral vision loss, anterior pituitary defects
Other infiltrative or hypothalamic diseases	Anterior pituitary defects, maybe diabetes insipidus
Drugs	Other specific side-effects of drug
Pregnancy	Positive hCG, amenorrhea
Renal failure	Comorbidities of renal failure
Chest wall stimulation	Variable prolactin
Stress	Including phlebotomy
Primary hypothyroidism	See Table 25.4

hCG, human chorionic gonadotropin.

While this principle is true for both males and females, the influence on fertility and symptoms differs in other respects. The reproductive axis is particularly sensitive to disorders and disruptions of the pituitary-hypothalamic axes as discussed in further detail below.

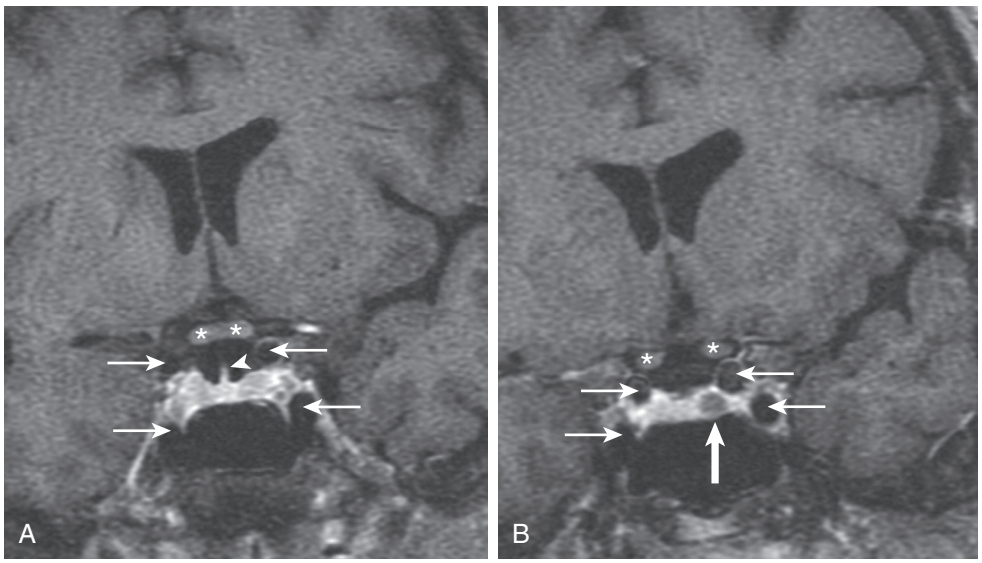


FIGURE 25.2 **A,** Normal pituitary. In this T1-weighted coronal image after gadolinium contrast, the pituitary gland is seen in the area of high-signal below the pituitary stalk *(indicated by chevron arrowhead)*. The carotid arteries are indicated by *thin arrows*, and the optic nerves are labeled with *asterisks (*)*. **B,** Pituitary microadenoma. The *thick arrow* shows the tumor as a hypointensity within the high-signal pituitary gland.

Loss of pituitary function is most commonly caused by drugs, exogenous hormones, or tumors. Tumors affect pituitary function by overproduction of hormones, including interference with the above axes (such as prolactin impairing gonadotropin secretion) or by mass effect. Any pituitary tumor >1 cm in maximal diameter is defined as a macroadenoma. The acquisition of pituitary hormone deficiencies due to tumors tends to follow the order GH first, then LH+FSH, then TSH, and finally ACTH.[1] Consequently, the reproductive axis is fairly vulnerable to disruption by macroadenomas of any cell type. Decompression by transsphenoidal surgery can restore pituitary function, particularly for the ACTH and TSH axes,[2,3] but iatrogenic hypopituitarism is a common risk of surgery. Radiotherapy tends to cause hypopituitarism over a period of 2 to 15 years and for this reason should be used judiciously in women of reproductive age.[4] In the following discussion, both the nature of the pituitary disorder and its treatment are addressed as they affect strategies for restoring reproductive function.

Pituitary Disorders that Affect Reproduction

Prolactinoma and Hyperprolactinemia

The combination of amenorrhea and galactorrhea in a young woman is a classic presentation of prolactinoma; however, these two symptoms may occur individually or not at all.[5] Hyperprolactinemia has many potential etiologies (Table 25.1). The mechanisms of reproductive dysfunction in hyperprolactinemia vary somewhat with etiology, but prolactin disrupts the pulses of GnRH and also directly reduces the production of LH and FSH.[6-8]

The diagnosis of hyperprolactinemia is established by measuring a serum prolactin at any time of day without dynamic testing, although the stress of phlebotomy can cause slight elevations.[9] Falsely elevated prolactin measurements can be caused by the presence of macroprolactin, also known as "big-big prolactin,"[10] which is a complex of prolactin and IgG detected variably in different immunoassays but lacking normal biological activity.[11,12] If the prolactin is clearly elevated and causes other than pituitary tumors are excluded, a dedicated MRI of the sella with gadolinium contrast agent should be performed.

Microprolactinomas (tumors <1 cm in greatest diameter) are found in about 1% of women age 20 to 40 years old.[13] The degree of prolactin elevation is roughly proportional to the size of the tumor.[14] A tumor not secreting prolactin might cause hyperprolactinemia via stalk compression and impaired dopamine delivery, but the prolactin rarely if ever rises above 250 ng/mL.[15] For example, a patient with a 3 cm pituitary mass and a prolactin of 150 ng/mL probably does not have a prolactinoma. In cases of large pituitary tumors with mild prolactin elevations, however, the prolactin measurement should be repeated with dilutions to identify the "high-dose hook effect," which artifactually lowers the assayed value.[16] GH is a full prolactogen in humans; consequently, galactorrhea with mildly elevated prolactin and a pituitary tumor could be secondary to a somatotropinoma rather than prolactinoma.[17] On T1-weighted MRI, microprolactinomas tend to be hypointense relative to the normally bright pituitary and usually do not distort the architecture of the gland (Fig. 25.2). Although the larger macroprolactinomas tend to enhance with gadolinium, the pattern is quite variable, and macroprolactinomas tend to distort the architecture of the gland (Fig. 25.3) with the inferior portion of the pituitary stalk often deviating away from the tumor.

Prolactinomas are generally very responsive to medical therapy with dopamine agonists (bromocriptine and cabergoline).[18] Even for large and invasive macroprolactinomas with visual changes, tumor shrinkage by dopamine agonists can be immediate and effective (Fig. 25.3). Treatment options are selected based on symptoms, tumor size, and patient goals. Indications for treatment include infertility, amenorrhea, galactorrhea (particularly if spontaneous and bothersome), hypopituitarism, and mass effect. A woman with a 5 mm microprolactinoma, serum prolactin of 60 ng/mL, regular menses, and only trace expressible galactorrhea does not require treatment, because these tumors rarely grow.[19] In contrast, the same woman with infertility would be treated if she desires pregnancy. Cyclic estrogen and progestin for endometrial and bone protection or symptoms of estrogen deficiency is an option for patients with

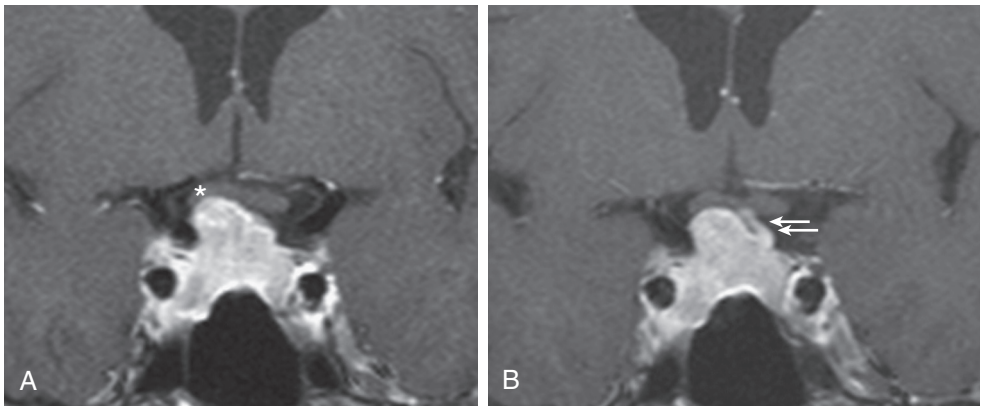


FIGURE 25.3 Macroprolactinoma and response to cabergoline. **A**, T1-weighted coronal image after gadolinium contrast at diagnosis with a serum prolactin of 350 ng/mL. The tumor is pressing against the optic nerve on the left (*). **B**, After 3 months of treatment with cabergoline at 0.5 mg twice weekly, the serum prolactin had fallen to 10 ng/mL. The stalk is now visible *(arrows),* and the tumor no longer abuts the optic nerve *(black rim separating the tumor from the optic nerve)*.

microprolactinomas and irregular menses but without galactorrhea and who do not desire pregnancy, with low risk of tumor growth.[20-22]

Bromocriptine is administered in 2 to 3 divided doses with a total of 2.5-40 mg/d. The main side effects of bromocriptine are nausea, lightheadedness, and nasal stuffiness. The dose is slowly advanced every 4 to 10 days as tolerated until the serum prolactin is normalized and the symptoms are relieved. Cabergoline, administered at 0.25-2 mg once or twice weekly, is much more potent and better tolerated than bromocriptine.[23] Bromocriptine is generally preferred if pregnancy is desired, stopping the medication upon confirming conception.[24] Cabergoline use early in pregnancy was not found to increase the risk for miscarriage or fetal malformations and is generally considered safe also.[25] Visual fields and serum prolactin should be monitored throughout pregnancy and during lactation, more closely for macroprolactinomas. MRI without contrast is performed if tumor growth is suspected, and cabergoline restarted to prevent vision compromise and/or hypopituitarism during pregnancy.

In some cases, particularly those without visible tumor on MRI scan, cabergoline can be discontinued after 2 to 5 years without recurrence of hyperprolactinemia.[26] Chronic treatment with high doses of cabergoline for Parkinson's disease has been rarely associated with cardiac valve disease[27,28] due to the serotonin receptor agonism by cabergoline but not bromocriptine. In one study, asymptomatic moderate tricuspid regurgitation was detected echocardiographically in 54% of cabergoline-treated prolactinoma patients versus 18% of controls.[29] Consequently, periodic cardiac exam is necessary in patients treated with cabergoline, and echocardiography is indicated if a murmur is appreciated or if high doses are required.

Surgery and radiotherapy are reserved primarily for rare tumors unresponsive to dopamine agonists and for patients intolerant to these drugs.[30] Surgery is most effective for microadenomas, with success rates approaching 90% for microadenomas but only about 60% for macroprolactinomas.[30,31] An immediate postoperative prolactin of less than 2 ng/mL is reliable evidence of cure. Radiotherapy takes at least a year to lower the prolactin significantly and to stop tumor growth.

Occasionally, patients present with normoprolactinemic galactorrhea and regular menses. If the galactorrhea is bothersome, treatment with bromocriptine or cabergoline to lower the prolactin to less than 2 ng/mL is effective in stopping the galactorrhea.[32] The duration of therapy required is roughly proportional to the duration of time that the galactorrhea has been present. Patients should be counseled to wear a tight bra or breast binder and to avoid both nipple stimulation and checking for expressible milk production during the course of therapy.

In men, the majority of patients with prolactinoma come to medical attention with macroprolactinomas and markedly elevated prolactin values.[33] Men present with symptoms attributable either to mass effect, such as vision loss and diplopia, or to hypogonadism, including fatigue, loss of libido, and erectile dysfunction.[33] Galactorrhea is rare but seen if gynecomastia is present and hyperprolactinemia is severe. Sperm count is usually reduced only after many years of hyperprolactinemia. Indications for treatment include mass effects and hypopituitarism. The hypogonadism associated with prolactinomas in men often responds well to dopamine agonist therapy, unless the duration of hypogonadism is prolonged. The erectile dysfunction of hyperprolactinemia does not always improve with testosterone replacement unless the prolactin is normalized.[34] Sperm count is not immediately restored by dopamine agonist therapy and may not return to normal after several months of therapy.

Acromegaly

Acromegaly results from overproduction of GH and IGF-1 accompanied by acral bone and soft tissue growth. Because symptoms are subtle and gradual in onset, the diagnosis may be delayed for several years. Additional symptoms and complications include fatigue, sleep apnea, hyperhydrosis, headache, and carpal tunnel syndrome. The majority of patients with acromegaly have a GH-secreting pituitary tumor (somatotropinomas), less than 10% have GHRH-producing tumors, usually pancreatic neuroendocrine tumors. Menstrual abnormalities are observed when tumor mass effect impairs delivery of hypothalamic releasing factors to the anterior pituitary or when hyperprolactinemia occurs. The direct action of GH can cause galactorrhea; however, tumors

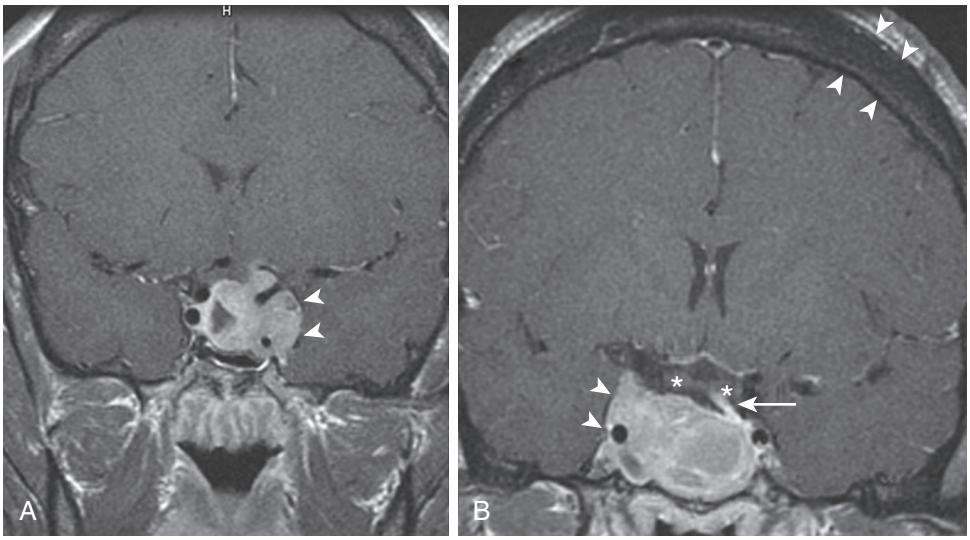


FIGURE 25.4 Magnetic resonance image (MRI) of invasive somatotropinomas. The tumor on the left **(A)** invades the carotid sinus on the right *(chevron arrowheads)* and obliterates the optic nerve on that side. The tumor on the right **(B)** has heterogeneous intensity from internal hemorrhage, and this tumor invades the carotid sinus on the left *(chevron arrowheads)*. The stalk *(arrow)* is visible on the right, but the normal pituitary is not discernable. The optic nerves (*) are not compromised, and diplöic space on the right *(between arrowheads)* is expanded.

that co-secrete GH and prolactin also exist, which complicates the evaluation.[35] Prolactin co-secretion does not predict an improved response to dopamine agonist therapy.[36]

A serum IGF1, corrected for gender and age or Tanner stage, is the best screening test for acromegaly. An elevated IGF1 plus acral changes in a patient with a pituitary tumor are usually sufficient to make the diagnosis. Formal GH suppression testing with 100 grams glucose (normal GH <0.1 ng/mL in males or <1 ng/mL in females[37]) is used mainly to gauge response to therapy. Gigantism occurs when the tumor forms prior to closure of the epiphyses. Genetic causes of acromegaly and gigantism include multiple endocrine neoplasia type 1 (MEN 1, *MEN1* gene), Carney complex (*PRKA1A* gene), and familial isolated pituitary adenoma (FIPA, *AIP* gene).[38]

Combined modality therapy is the norm for the typical invasive somatotropinoma (Fig. 25.4). Large tumors often require both transsphenoidal surgery and craniotomy to debulk the tumor sufficiently for drug or radiotherapy,[39] whereas for microadenomas, cure rates are high and hypopituitarism infrequent for experienced pituitary neurosurgeons.[40] Pure somatotropinomas occasionally respond to dopamine agonists with reduced GH secretion and/or tumor shrinkage, but at high doses.[41] Many somatotropinomas express somatostatin receptors, primarily type 2 (sst2) and remain responsive to somatostatin agonists such as octreotide or lanreotide, given as monthly long-acting intramuscular or deep subcutaneous injections of 10-40 mg or 60-120 mg, respectively. Although earlier reports suggested GH and IGF1 normalization in 70% of patients, more recent studies indicate that less than 40% of patients normalize on somatostatin agonist therapy.[42,43] Some tumor shrinkage occurs in over half of somatotropinomas treated with somatostatin analogs, but the degree of regression is not as dramatic as for prolactinomas treated with dopamine agonists. Pegvisomant, a growth-hormone receptor antagonist that is modified with polyethylene glycol, normalizes IGF1 in up to 95% of patients during the initial trials,[44] but long-term studies indicate that the success rate is 60% to 65%.[45] The drug is given by subcutaneous injection of 40 mg as loading dose, followed by 10-30 mg/day, and it is generally well-tolerated except for transaminase elevation in rare cases. Pasireotide, which binds to sst5 with higher affinity than octreotide, appears promising for the treatment of acromegaly[46] but causes more hyperglycemia than octreotide. GH normally rises in pregnancy due to the secretion of placental GH, which is the product of a separate gene from pituitary GH. Consequently, medical treatment is normally withheld during pregnancy.

Cushing Disease

Hypercortisolism from ACTH-producing pituitary tumors causes infertility both from the effects of glucocorticoids on the hypothalamic-pituitary-gonadal axis and from mass effect if it is caused by a macroadenoma. This topic will be covered in the adrenal section below, and the principles of hypopituitarism from macroadenomas are the same as discussed earlier.

Other Macroadenomas

Many pituitary adenomas are "nonfunctional," meaning that they do not produce significant amounts of biologically active hormones. Most of these tumors, however, derive from the glycoprotein hormone cell lineage and express mRNA for the common alpha subunit and/or the beta subunits of LH, FSH, or TSH,[47] but the glycoprotein products are biologically inactive due to improper glycosylation, dimerization and assembly.[48] In general, these tumors present with symptoms due to mass effect (vision loss, headache) or hypopituitarism. Unlike prolactinomas, these tumors tend to be resistant to medical therapy. Indications for surgery include vision compromise or other mass effect and severe hypopituitarism, such as ACTH deficiency or panhypopituitarism. For the woman with panhypopituitarism and infertility, ovulation induction with gonadotropins is required, as discussed in other chapters. For men, fertility is restored with human chorionic gonadotropins (hCG)

1000-2000 units 2 to 3 times weekly to normalize testosterone, with recombinant FSH 25 to 75 IU three times a week added if necessary.[49]

Lymphocytic Hypophysitis

Lymphocytic infiltration of the pituitary is a rare disorder, which most commonly occurs in postpartum women.[50] Common presentations are headache with inability to lactate postpartum due to impaired prolactin production or polyuria and polydipsia from diabetes insipidus.[51] Some women are relatively asymptomatic but develop amenorrhea or non-specific symptoms of hypopituitarism over time. The pattern of pituitary deficiencies is highly variable and often follows a pattern much different from that observed with macroadenomas, such as ACTH and TSH deficiency with diabetes insipidus but preserved GH and gonadotropins. During the active phase, MRI shows a symmetrically enlarged pituitary and stalk that enhances markedly with gadolinium. After the damage has occurred and the disease has subsided, the MRI can show a partial empty sella. Methylprednisolone in the acute phase is sometimes effective,[52] but pituitary function, when lost, rarely recovers.

Other Disorders Affecting the Pituitary

Granulomatous diseases such as sarcoidosis[53] and tuberculosis can involve the hypothalamus and pituitary, also causing central hypogonadism. Hypopituitarism can be the initial manifestation of sarcoidosis, while others have neurosarcoidosis with manifestations such as optic neuritis. MRI shows brisk enhancement of the meninges with or without hypothalamic and pituitary abnormalities. Other tumors also arise in the hypothalamus and pituitary, including germinomas and lymphomas. Carcinomas metastasize to the pituitary, primarily to the stalk. Diabetes insipidus is often a presenting manifestation, and hypogonadism is one of the most frequent manifestations of hypopituitarism in these neoplastic disorders.

Hemochromatosis (primary or secondary) and amyloidosis are infiltrative diseases that cause hypopituitarism.[54] The classic triad includes liver dysfunction, skin bronzing, and diabetes mellitus ("bronze diabetes"), but arthropathy, cardiomyopathy, and various endocrinopathies including adrenal insufficiency[55] and hypogonadotropic hypogonadism[56] are common as well. Iron deposition in the hypothalamus has predilection for impairing GnRH production among the releasing hormones. Hemochromatosis is one of the few disorders in which the male reproductive axis is more vulnerable to disruption than in the female, due to monthly menses in women.

Adrenal Disorders

Overview

The adrenal consists of a cortex, with three distinct zones, and a medulla. The medulla is an extension of the sympathetic nervous system and produces epinephrine. The steroid-producing cells of the cortex are arranged into the outermost zona glomerulosa, which produces aldosterone; the zona fasciculata, which produces cortisol, and the zona reticularis, which produces the androgen precursor dehydroepiandrosterone sulfate (DHEAS). DHEA and DHEAS are metabolized to testosterone in peripheral tissues. Cortisol and DHEAS production is regulated primarily by ACTH (Fig. 25.1), whereas aldosterone synthesis is mainly stimulated by the renin-angiotensin system and by potassium.

Diseases of the adrenal gland causing hormone deficiency rarely interfere with reproduction, but certain hormone excess states contribute to infertility, particularly for women. In the male, the contribution of adrenal DHEAS and other 19-carbon steroids to circulating testosterone concentrations is normally small, but in women, the majority of testosterone normally derives from adrenal-derived precursors. Consequently, disorders that increase adrenal DHEAS production cause hyperandrogenemia in women, which can impair fertility. Hypercortisolism may suppress gonadotropin production in women and, to a lesser extent, in men. We will not discuss primary aldosteronism, adrenal insufficiency, or pheochromocytomas in this chapter, because these disorders rarely impair fertility in isolation.

Adrenal Disorders that Affect Reproduction

Cushing Syndrome

The Cushing syndrome is categorized as iatrogenic or endogenous, and endogenous Cushing syndrome is dichotomized as being ACTH-dependent or ACTH-independent (Table 25.2). The majority of Cushing syndrome is ACTH-dependent, the most common cause being ACTH-producing pituitary tumors, which is called Cushing disease. The differential diagnosis of ACTH-dependent Cushing syndrome also includes ectopic production of ACTH or CRH by carcinomas, neuroendocrine tumors, and bronchial carcinoids.[57] ACTH-independent Cushing syndrome is caused by unilateral adenomas or carcinomas and by bilateral micronodular or macronodular hyperplasia. Cushing disease occurs most commonly in young women, and DHEAS production is more commonly elevated in ACTH-dependent forms of Cushing syndrome than in ACTH-independent forms. Consequently, Cushing disease in women is the most relevant form of hypercortisolism that impairs reproduction.

The clinical manifestations of cortisol excess can be subtle in the early stage when the diagnosis is most difficult but the benefits of treatment and potential for full recovery the greatest. Cortisol is a catabolical hormone that causes lipolysis and fat redistribution, as well as breakdown of body tissues such as muscle, skin, and bone. Central obesity is a prominent and common feature, with disproportionate fat accumulation around the face and neck. The dorsocervical fat pad ("buffalo hump") is a well-known feature of Cushing syndrome, but

Table 25.2 Etiologies of Endogenous Cushing Syndrome

ACTH-Independent	Adrenocortical adenoma
	Adrenocortical carcinoma
	Macronodular hyperplasia
	Micronodular hyperplasia
ACTH-Dependent	Corticotrope tumor (Cushing disease)
	Corticotrope hyperplasia
	Ectopic ACTH syndrome
	Ectopic CRH syndrome

supraclavicular fat pads are more specific for this disorder, especially in an otherwise nonobese individual (Fig. 25.5). Facial and upper chest plethora is observed (Fig. 25.5), and women often develop hirsutism.

Proximal muscle weakness and skin thinning are two very specific features of Cushing syndrome, and osteoporosis in an obese individual should raise suspicion as well.[57] Patients endorse difficulty arising from a chair and walking up stairs or getting out of a car, combing their hair, or changing an overhead light bulb. Skin thinning and capillary fragility cause easy bruising, and if weight gain is rapid, the striae formed are violaceous and non-blanching, reflecting hemorrhage into the new skin by fragile blood vessels. These striae are found on the abdomen and flanks, near the axillae, and on the thighs and breasts. If greater than 1 cm, purple striae are very specific for hypercortisolism; however, these findings develop late and only in severe disease.

The most important scenario in which Cushing syndrome should be considered is in the young woman with oligomenorrhea and hirsutism who could be mistaken for simple polycystic ovary syndrome (PCOS). A history of hirsutism onset after age 25 or specific findings for hypercortisolism, such as easy bruising, thin skin, proximal muscle weakness, and osteoporosis, should prompt screening for Cushing syndrome.

The diagnosis of hypercortisolism can be difficult early in the disease because of the large overlap with normals. Testing is based on the principles that the cortisol production rate is increased,[58] that the normal diurnal rhythm is disrupted, and that cortisol is not suppressible.[59] These principles are employed by the 24 hour urinary free cortisol,[60] late-night saliva[61] or serum cortisol measurements,[62] and overnight dexamethasone suppression testing,[63] respectively. The caveats of testing are beyond the scope of the chapter, but the reader should be aware that both false-positive and false-negative results are common. Consequently, tests often must be repeated several times before a diagnosis can be made or excluded.

Once hypercortisolism is confirmed, an ACTH measurement will determine if the disease is ACTH-dependent or ACTH-independent. If ACTH-independent (ACTH <5 pg/mL), an abdominal computed tomography scan of the adrenal glands is obtained. Patients with early ACTH-independent Cushing syndrome will have low but not suppressed ACTH (5-15 pg/mL), and these patients should have serial testing until the diagnosis is clarified.[64] If the ACTH is normal or elevated, the next step is magnetic resonance imaging of the pituitary with gadolinium contrast. The caveat of pituitary imaging is that small abnormalities of the pituitary are common, and about half of patients with Cushing's disease do not have a visible tumor by MRI. To conclusively exclude the ectopic ACTH syndrome, inferior petrosal sinus sampling is performed.[65] Blood draining both sides of the pituitary is sampled from the inferior petrosal sinuses under ovine CRH stimulation (100 μg bolus), and the ACTH values in these specimens are compared with those obtained in peripheral blood drawn simultaneously. An ACTH step-up greater than 3 from peripheral blood to the inferior petrosal sinus is reliable evidence for a pituitary source of ACTH.

Treatment of Cushing syndrome is primarily surgical. Cushing disease is treated with transsphenoidal pituitary adenomectomy, yet even in the hands of experienced surgeons, long-term cure rates are not above 80%. Repeat surgery, radiotherapy, and even bilateral adrenalectomy may be recommended if disease persists. Medical management with metyrapone, ketoconazole, and triolstane has been employed but are seldom very effective in severe disease. Mifepristone at high doses blocks the glucocorticoid receptor and improves the catabolical and metabolical features of all forms of Cushing syndrome,[66] but the renal and hemodynamic manifestations of hypertension and hypokalemia must be managed independently. Cabergoline is effective in reducing hypercortisolism for a subset of pituitary tumors that express dopamine type 2 receptors.[67] Furthermore, many corticotrope tumors express sst5 but not sst2, so while octreotide is rarely effective in reducing ACTH secretion, pasireotide reduces ACTH and cortisol production in the majority of cases.[68] In contrast, ACTH-independent Cushing syndrome is normally cured by unilateral or bilateral adrenalectomy, except in the case of adrenocortical carcinomas with inoperable or metastatic disease.

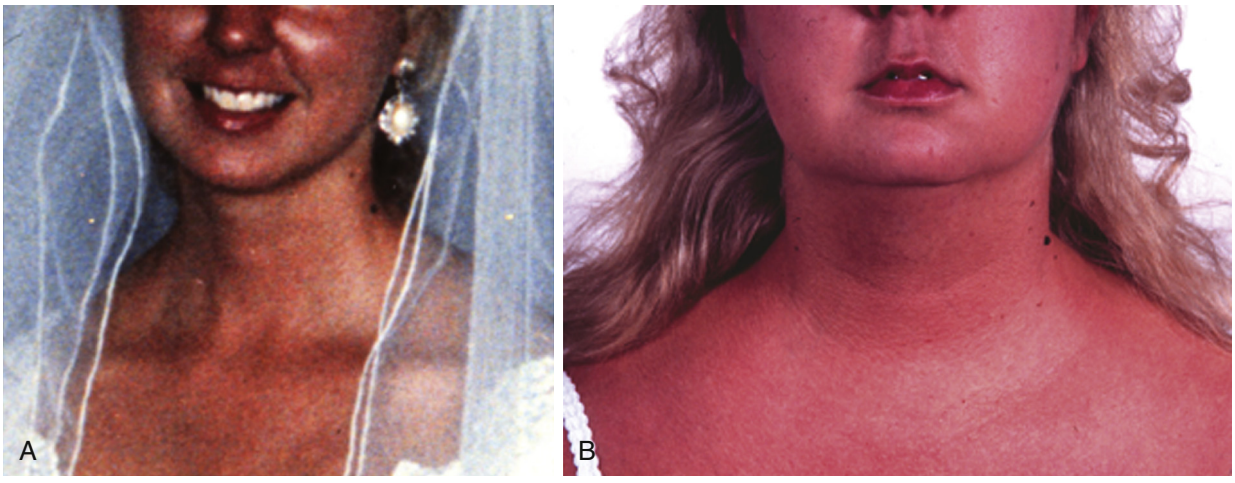


FIGURE 25.5 Subtle clinical signs of Cushing syndrome. This woman is shown 4 years before diagnosis **(A)** and at the time of diagnosis **(B)**. In part **B**, she demonstrates facial plethora and loss of clavicular silhouettes due to supraclavicular fat.

Congenital Adrenal Hyperplasia (CAH): 21-Hydroxylase Deficiency

The manifestations of 21-hydroxylase deficiency (21OHD) in the infant as an intersex disorder were discussed elsewhere, so this discussion will be restricted to adults with classical and non-classical forms of 21OHD. Among the forms of congenital adrenal hyperplasia (CAH), 21OHD is by far the most common form, accounting for >90% of cases.[69] Classical 21OHD is caused by mutations in the *CYP21A2* gene, which severely impair the activity of the encoded P450c21 enzyme to <2% of wild-type activity.[70] Nearly all of these patients have clinical manifestations and require therapy with glucocorticoid and mineralocorticoid replacement. Milder mutations, particularly the V281L allele, cause non-classical 21OHD (Table 25.3).[71] Males with non-classical 21OHD are rarely ascertained unless evaluation for premature pubertal features (body hair, acne, growth spurt) is sought. Females with non-classical 21OHD present with hyperandrogenism, which manifests with hirsutism, acne, and irregular menses,[72] but not all patients are symptomatic or require treatment. Similarly, patients with intermediate severity are often not diagnosed until adulthood, particularly males (Fig. 25.6). In many countries, 21OHD testing is part of newborn screening procedures, so most newborns are identified before adrenal crisis occurs, even if not ascertained by genital ambiguity.

The diagnosis of 21OHD is based on elevated circulating concentrations of 21-deoxysteroids, in particular 17-hydroxyprogesterone (17OHP). A random serum 17OHP > 10,000 ng/dL with serum cortisol <5 μg/dL establishes the diagnosis of classical 21OHD in males and females, and dynamic testing is rarely required.[73] In the case of non-classical disease, a morning serum 17OHP >800 ng/dL should prompt repeat measurement of cortisol and 17OHP 30 to 60 minutes after cosyntropin administration (250 mcg I.V. or I.M.). Postcosyntropin 17OHP values in the 1500-10,000 ng/dL range are typical of non-classical 21OHD.[74-76] If laboratory data are equivocal, genetic testing for mutations in the *CYP21A2* gene on DNA from peripheral blood cells is available.

The repertoire of mutations commonly found in 21OHD is limited due to the molecular mechanism responsible for most cases. The *CYP21A2* gene is located in a duplicated locus within the HLA region on chromosome 6p that includes the genes for the fourth component of complement. In the duplicated region, the DNA corresponding to the *CYP21A2* gene is replaced by the *CYP21A1P* pseudogene.[77,78] This pseudogene contains several mutations that render the cognate mRNA and protein non-functional. Most cases of 21OHD derive from gene conversion events, in which some or all of the *CYP21A2* gene is replaced by the corresponding region of the *CYP21A1P* pseudogene.[79] Consequently, the spectrum of mutations and the worldwide prevalence of 21OHD is fairly consistent, but certain mutations are particularly common in specific populations, such as V281L in Yupik Eskimos.[80] Occasionally, true point mutations are found instead.[81] Heterozygous carriers are identified with confidence by genetic testing, whereas 17OHP values, even after cosyntropin stimulation, show broad overlap with normal individuals of either gender.

The mechanisms of reduced fertility in women with classical 21OHD are complex, although affected women have given birth to normal female infants.[82] Chronic hyperandrogenemia, even though largely adrenal in origin, causes chronic oligo-anovulation. In addition, many women with 21OHD develop a secondary PCOS, with characteristic ovarian morphology, thecal hyperplasia, and ovarian androgen excess as well.[83] The block in 17OHP metabolism causes accumulation of adrenal-derived progesterone as well, and high circulating progesterone concentrations impair endometrial maturation and implantation.[84] Overtreatment with synthetic glucocorticoids, however, can suppress gonadotropins and cause glucose intolerance with its attendant reproductive disturbances.[85]

Women with virilization of the external genitalia face additional impediments to fertility related to anatomical considerations. Vaginal stenosis, either prior to reconstruction surgery or following suboptimal surgery and/or dilation, is known to cause dyspareunia,[86,87] which discourages coitus.[88,89] Vaginal anomalies also comprise sperm

Table 25.3 21-Hydroxylase Deficiency

Form	Common Mutations
Classical	
Salt-wasting	Large deletions, 656A/C-G*, G110del8nt, Q318X R356W*, R483P, I236N+V237E+M239K
Simple virilizing†	656A/C-G*, I172N, R356W*
Non-classical	P30L, V281L, R339H, P453S

*Can be associated with either salt-wasting or simple virilizing disease.
†All patients with classical 21-hydroxylase deficiency are prone to salt wasting with physiological stress, but the most severely affected experience spontaneous salt-wasting crises in infancy.

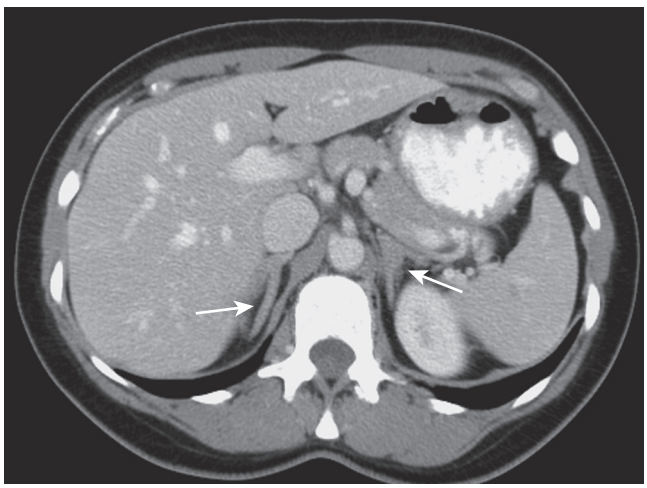


FIGURE 25.6 Computed tomography of adrenal glands in a woman with non-classical 21OHD diagnosed at age 50. She had both severe hirsutism and rapid somatic growth in childhood with normal external genitalia. She menstruated regularly during her reproductive years and gave birth to three children without glucocorticoid or ovulation therapies. Screening laboratory values included a testosterone of 179 ng/dL, SHBG of 52 nmol/L, DHEAS of 427 μg/dL, ACTH of 16 pg/mL, and 17OHP of 1100-2500 ng/dL. The 17OHP rose to >17,000 ng/dL with cosyntropin stimulation, establishing the diagnosis of 21OHD. The enlarged but normally-shaped adrenal glands are indicated by *arrows*.

deposition, and high circulating progesterone concentrations inhibit sperm penetration and fertilization. Psychosocial influences should not be underestimated. The fact that 21OHD is a genetic disorder associated with masculinization contributes to difficulties finding a partner and has been associated with preference for relationships with female partners in some but not all studies.[90]

Women with non-classical 21OHD are most commonly identified during an evaluation of hirsutism, oligomenorrhea, or infertility mimicking PCOS. It is difficult to distinguish non-classical 21OHD from idiopathic PCOS on clinical grounds.[72,91] More careful studies suggest that the frequency of PCOS in women with non-classical 21OHD is not much higher than in the general population. Nevertheless, if infertility and chronic anovulation are present in a woman with non-classical 21OHD, these women often benefit from glucocorticoid replacement. Curiously, about 70% of women with non-classical 21OHD in one large series are compound heterozygotes for one non-classical and one classical allele,[92] whereas the majority should be homozygous for non-classical allele given population estimates of carrier frequencies. This result suggests that primarily the less severely affected non-classical 21OHD patients do not come to medical attention. Furthermore, cryptic and largely asymptomatic non-classical 21OHD was found in 4% of parents with children affected by classical 21OHD,[93] all of whom were heterozygous carriers of classical alleles.

Little data exist to guide the optimal management of adults with 21OHD, either for long-term glucocorticoid replacement or for improving fertility.[85] A range of treatment regimens are used without obvious rationale, although the convenience of once-daily dosing favors use of the more potent glucocorticoids including prednisone, prednisolone, and dexamethasone.[94,95] In a large audit of 21OHD patients in England, many were short and obese, with poor health metrics and quality of life, and overtreatment with glucocorticoids appeared to be common.[96] Consequently, the minimum amount of glucocorticoid replacement to maintain testosterone in an acceptable range is recommended for chronic therapy in women, although this dose and target steroid values will vary among individuals. Serum 17OHP should not be normalized, as occurs with over-treatment, but dose adjustments should be based on serum testosterone and androstenedione values. Hydrocortisone is preferable for chronic therapy but must be given in at least two divided doses, although sustained-release preparations are in development.[97,98] Prednisolone and dexamethasone lower androgen production more effectively than hydrocortisone, particularly for the morning rise in ACTH and adrenal-derived steroids, where even bedtime hydrocortisone is not very effective. Fludrocortisone acetate, in doses sufficient to normalize plasma renin activity and serum potassium, eliminate orthostasis, and maintain fluid balance, should be maintained throughout life with rare exceptions.

Despite the many barriers to achieving pregnancy in women with 21OHD, fecundity rates in those women who attempt pregnancy are greater than 90%, equivalent to the general population.[99] The most critical parameter favoring conception appears to be follicular-phase serum progesterone, which should be lowered to less than 0.6 ng/mL (2 nmol/L) with multiple doses of hydrocortisone and/or prednisolone. A bedtime dose of 0.5-2 mg prednisolone, which is combined with either daytime hydrocortisone (two doses) or one dose of prednisolone, appears to be particularly important for achieving pregnancy. Ovulation induction with standard methods and agents might be necessary despite optimal adrenal replacement regimens. Despite clinical features of PCOS shared with 21OHD, metformin and other insulin sensitizers have not been studied adequately in women with 21OHD. Prenatal treatment with dexamethasone has been shown to reduce fetal virilization in couples who have one or more children with 21OHD,[100] but this treatment is considered experimental due to the paucity of long-term outcomes data in treated children with or without 21OHD.[73]

Almost all men with non-classical 21OHD are asymptomatic and can be identified only with genetic testing. Men with classical 21OHD, in contrast, frequently develop infertility, particularly if poorly controlled. Severe adrenal androgen excess rarely causes gonadotropin suppression with subsequent Leydig cell atrophy, reduced testicular testosterone production, and impaired spermatogenesis.[101,102] Glucocorticoid replacement often normalizes testicular function, although high doses are often required initially. More commonly, adrenal rests are the cause of male infertility in 21OHD.[103] The steroid-producing cells of the adrenal cortex and the gonads derive from the same pool of precursors during embryogenesis. The migration of adrenal cells to the suprarenal space can be imperfect, and adrenal cortex cells or progenitors appear to reside in the testis or within the groin and abdomen. ACTH is trophic for these adrenal rest cells as well, and when ACTH is high as in poorly controlled 21OHD, these cells will form masses, most often in the testis itself. These tumors are present in most men with 21OHD, and their size correlates better with adrenal volume than with single steroid measurements.[104] Testicular ultrasonography is the most sensitive method of diagnosis, although many are palpable.[105] Because the testis is confined to a rigid capsule, the growth of intra-testicular adrenal rests impairs testicular blood flow and efflux of sperm into the ejaculate. Treatment of adrenal rests requires potent glucocorticoids, such as dexamethasone, 1-2 mg at night, although even this therapy is not always successful in reducing tumor size and restoring fertility. Combination therapy with daytime hydrocortisone and low-dose (0.1 mg) bedtime dexamethasone also appears effective and less toxic than dexamethasone monotherapy at higher doses.[106] Surgical resection of the adrenal rests by a competent urologist is associated with a low risk of recurrence but with little improvement in spermatogenesis.[107] This result, however, could reflect a selection bias in reserving surgery for the most severely affected men.

Other CAH

The other forms of CAH associated with androgen excess in women are 11-hydroxylase deficiency (11OHD) and 3β-hydroxysteroid dehydrogenase/isomerase deficiency (3βHSDD). In 11OHD, caused by mutations in the *CYP11B1* gene, the 11-deoxysteroids 11-deoxycorticosterone (DOC) and 11-deoxycortisol are markedly elevated, and excess DOC causes hypertension and hypokalemia unlike 21OHD.[108] Serum 17OHP may be elevated in 11OHD, but much less than in 21OHD with similar hyperandrogenemia. In 3βHSDD, caused by mutations in the *HSD3B2*

gene, the diagnostic steroid ratios are those of delta-5 to delta-4 steroids, such as 17-hydroxypregnenolone/cortisol ratios greater than 6 standard deviations above normal.[109] Note that circulating testosterone concentrations are paradoxically elevated in women with 3βHSDD. The delta-5 steroids DHEA and DHEAS are metabolized to active androgens in the periphery and converted to delta-4 steroids by 3βHSD type 1, which is abundant in liver and skin, explaining this phenomenon.

The mechanisms responsible for infertility in men and women with 11OHD are similar to those in 21OHD. Treatments are similar, except that mineralocorticoid receptor antagonists (spironolactone, eplerenone) are useful to control hypertension during chronic therapy. Note that spironolactone is also an androgen receptor antagonist and is contraindicated in women who have unprotected intercourse. Women with 3βHSDD, even in the most severe cases, have mild clitoromegaly and little labioscrotal fusion, so anatomical impediments to pregnancy are less important, and high progesterone is less of a negative factor for reproduction than in 21OHD and 11OHD.[109]

Forms of CAH that impair both androgen and estrogen production include combined 17-hydroxylase/17,20-lyase deficiency,[110] caused by mutations in the *CYP17A1* gene encoding P450c17,[111] and isolated 17,20-lyase deficiency,[112] due to mutations in *CYP17A1*[113,114] or its redox partner proteins cytochrome P450-oxidoreductase (*POR* mutation G539R)[115] and cytochrome b_5.[116,117] Lipoid CAH, due to mutations in the *STAR* gene encoding the steroidogenic acute regulatory protein (StAR)[118] or rarely the *CYP11A1* gene encoding P450scc,[119] abrogates all steroid production, although a non-classic form with partial StAR deficiency mainly impairs cortisol production.[120] POR deficiency manifests as a spectrum of phenotypes with disordered steroidogenesis,[121] from primary amenorrhea in phenotypically normal women to the Antley-Bixler syndrome, with skeletal dysmorphologies.[122,123] The discussion of these diseases is beyond the scope of this chapter, and fertility is very rare in these patients despite the optimal therapy.

Thyroid Disorders

Overview

Before discussing the specific effects of thyroid disorders on reproductive function, it is important to understand some basic principles and potential areas of interaction between elements of thyroid hormone physiology and reproductive function.

Within the thyroid gland, millions of follicles are the factories and storage silos for all of the circulating T_4 and 20% of the active T_3 thyroid hormone. The thyroid follicle is composed of simple secretory epithelial cells surrounding a lumen containing colloid—a gelatinous substance composed primarily of thyroglobulin and T_4 (Fig. 25.7). The unique aspect of the thyroid gland is its ability to store enormous quantities of thyroid hormone in the follicular colloid. Iodide imported from the circulation and concentrated in colloid is used for iodination of the thyroglobulin backbone by thyroid peroxidase (TPO) (Fig. 25.8) to produce inactive precursor mono- and diiodotyrosines. Synthesis of T_4 and T_3 using these precursors is also catalyzed by TPO. Reuptake

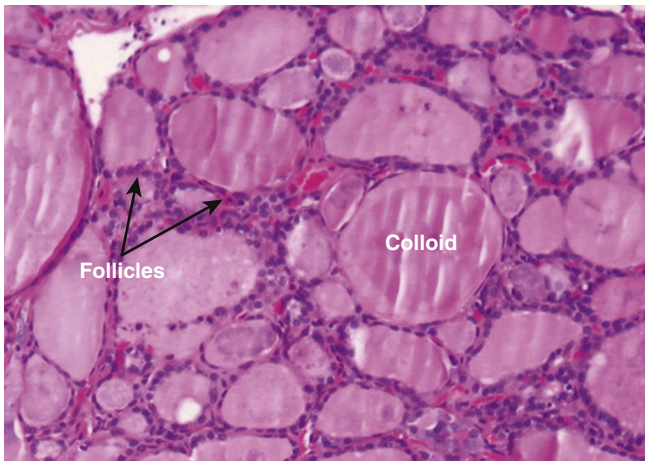


FIGURE 25.7 Histology of a normal thyroid gland. Shown is a 100× magnification of a hematoxylin and eosin-stained section of normal thyroid. Single-layer, epithelial-lined follicles of variable sizes are filled with pink colloid where thyroglobulin, T_3, and T_4 are stored.

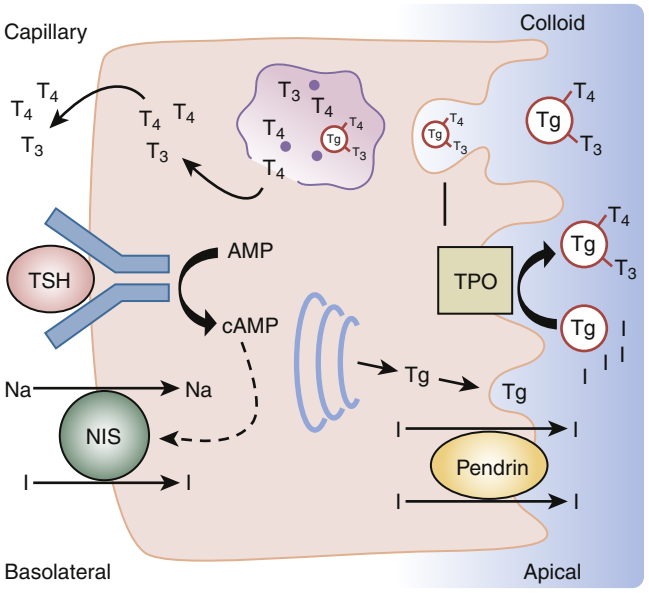


FIGURE 25.8 Production of thyroid hormone in the follicular epithelial cell. This schematic illustrates the variety of functions carried out in the follicular epithelial cell from iodine (I) transport via the sodium iodide symporter (NIS), thyroglobulin (Tg) synthesis, iodination, and T_4 production by thyroid peroxidase (TPO) at the apical membrane where Tg and Tg complexed with T_3 and T_4 are stored. Reuptake and Tg digestion from the complexes allows for secretion of T_3 and T_4 through the basolateral membrane into the abundant capillary network. Stimulation by thyrotropin (TSH) of the TSH receptor results in cyclic AMP (cAMP) production, which augments hormone production by stimulating many cellular functions.

and Tg digestion off of the stored T_3 and T_4 from the colloid allows for secretion through the basolateral membrane into the abundant capillary network.

As described in the pituitary section overview, the hypothalamic-pituitary-thyroid axis is a straightforward model of an endocrine feedback system. The anterior pituitary secretes TSH, which regulates multiple steps in thyroid hormone production: (1) growth of the follicles, (2) iodine uptake, and (3) iodinaton of thyroid hormone by

the secretory epithelial cells. Control of TSH secretion, and therefore, ultimate control of all elements required for thyroid hormone production, is tightly regulated by negative feedback of circulating T_4 and T_3, suppressing production of both TRH from the hypothalamus and TSH by the anterior pituitary (Fig. 25.1A).

Outside of the hypothalamic-pituitary-thyroid axis, the major regulator of thyroid hormone production is the concentration of thyroid hormone globulin (TBG). Circulating T_4 is almost entirely (99%) bound to plasma proteins—approximately 70% bound to TBG, 20% to transthyretin (prealbumin), and 10% to albumin. In women, significant changes in TBG glycosylation in the liver are induced by rises in estradiol during pregnancy or exogenous estrogens, prolonging TBG half-life, and increasing circulating TBG concentrations.[124] The rise in TBG can transiently decrease free T_4 concentrations, leading to an increase in the TSH and total T_4 to maintain normal free T_4. During pregnancy, this may also lead to a visible enlargement of the thyroid gland or goiter. Peripheral conversion of T_4 to T_3 is mediated by deiodinases, types 1 and 2 located in different locations both at the tissue and cellular level. Interestingly, type 3 deiodinase is almost exclusively expressed in the placenta where it is tightly regulated by thyroid hormone concentrations.

Thyroid Disorders that Affect Reproduction

Diseases of the thyroid are more common in women than men. The most common conditions are Hashimoto's thyroiditis and Graves disease, both autoimmune diseases of the thyroid. Despite the importance of thyroid hormone in maintaining normal organ and endocrine function throughout the body, the exact mechanisms for thyroid hormone's effects are not well understood. Notably, it is the absence of thyroid hormone (hypothyroidism) and the multitude of accompanying symptoms that demonstrates its importance throughout the body. Similarly, for reproduction, hypothyroidism has more significant effects on menstrual cycles and fertility than hyperthyroidism, or more generally, thyrotoxicosis. Hypothyroidism may exert more of an effect on reproduction through a decrease feedback to the hypothalamic-pituitary axis, subsequent hyperprolactinemia, and interference with GnRH pulsatility and ovulation. The effect of low and high thyroid hormone concentrations on reproduction may also be mediated indirectly by effects on sex hormone binding globulin (SHBG) function and production as well as directly on steroidogenesis and sperm morphology, production, or function.[125] Subclinical hypothyroidism and autoimmune thyroid disease have been associated with decreased fertility and miscarriage, although the mechanism and benefit from treatment with thyroid hormone replacement are still not clear.[126-128]

Hypothyroidism

The overall prevalence of hypothyroidism is 3.1% in reproductive age women.[129] Women with hypothyroidism present with any of numerous symptoms and signs (Table 25.4). The reproductive age woman may experience irregular menses, anovulatory cycles, menorrhagia, or present only with infertility.[125] A significant mechanism for alterations in the reproductive system is through the hypothalamic-pituitary axes. While basal gonadotropins are normal, elevated TRH can stimulate lactotrophs to produce prolactin, which interferes with GnRH pulsatility as described earlier in the pituitary section. In women and men, hypothyroidism reduces SHBG binding affinity through altered sialic acid content, which reduces total and often free fractions of estrogens and androgens[130]; conversely, hyperthyroidism increases SHBG production.[131] Hypothyroidism may also interfere with ovarian function. Experimentally, T_4 has been shown to stimulate ovarian steroidogenesis in granulosa cells cultured *in vitro*.[132] In men, hypothyroidism has been associated with decreased libido, erectile dysfunction, and abnormalities of sperm morphology or motility.[125] As opposed to women, the hypogonadotropic hypogonadism associated with hypothyroidism is not associated with hyperprolactinemia, but the hypogonadism can correct after thyroxine replacement.[133] Abnormalities of sperm count, motility, and morphology as well as erectile dysfunction and poor testosterone response to hCG injections also improve after T_4 replacement.[134,135]

Table 25.4 Common Clinical Presentations of Thyroid Disorders

Hypothyroidism	Hyperthyroidism
Weight gain	Weight loss
Fatigue	Fatigue
Constipation	Increased frequency of bowel movements
Cold intolerance	Heat intolerance
Hair loss	Anxiety
Menorrhagia	Dyspnea on exertion
Infertility	Palpitations
Dry skin	Diaphoresis
Periorbital edema	Lid lag or "stare"
Hoarseness	Tremor
Delayed reflexes	Hyperreflexia
Anemia	Supraventricular arrhythmias

Subclinical Hypothyroidism and Autoimmune Thyroid Disease

While more severe hypothyroidism clearly interferes with successful ovulation and fertility, more subtle abnormalities of the pituitary-thyroid axis or the underlying autoimmune disease may also affect fertility. In one study of women presenting to an infertility clinic with normal thyroid function tests, women with lower or higher than normal responses to TRH stimulation had lower pregnancy rates than women with normal responses.[136] Few studies have investigating the role of subclinical hypothyroidism in male reproduction. In one study, men with subclinical hypothyroidism had the same hypogonadotropic hypogonadal profile as hypothyroidism, but there was no association with changes in sperm quality.[137] Treatment of subclinical hypothyroidism with T_4 replacement has only been shown to improve pregnancy outcomes in women positive for anti-TPO antibodies.[138]

Multiple studies have reported an association of antithyroid antibodies and miscarriage and preterm birth in euthyroid women.[127,139] However, it is not clear whether this association represents a more generalized autoimmunity or a need for thyroid hormone replacement. There are only two small studies with T_4 replacement in euthyroid women with antithyroid antibodies with two meta-analyses in disagreement

whether there is a significant risk reduction of miscarriage and preterm delivery with treatment.[127,128,140,141] Until a large, randomized placebo-controlled trial provides stronger evidence of a treatment effect, T_4 replacement is not recommended to prevent miscarriage in women with anti-TPO antibodies if they are euthyroid. T_4 treatment should only be considered in women with anti-TPO antibodies who have subclinical or clinical hypothyroidism.[126]

The most common cause of hypothyroidism is chronic autoimmune thyroiditis (Hashimoto thyroiditis). In addition to Hashimoto thyroiditis, hypothyroidism may also be seen in patients with acute (silent) and subacute thyroiditis (Table 25.5). Postpartum thyroiditis is an acute thyroiditis that occurs within 6 months of delivery and is usually self-limited. Thyroiditis typically has an acute thyrotoxic phase followed by a hypothyroid recovery phase and eventually a euthyroid phase. Postpartum thyroiditis typically presents with symptoms in the hypothyroid phase, whereas subacute thyroiditis presents with pain during the hyperthyroid phase. Although most return to a euthyroid state in the short-term, 20% to 64% of these women will develop chronic hypothyroidism over the next few years.[142] An important secondary cause of hypothyroidism is radioactive iodine ablation or thyroidectomy for definitive treatment of Graves disease, thyroid nodules, or thyroid cancer.

The diagnosis of hypothyroidism itself is easily made with modern immunoassays. Clinical hypothyroidism is defined by both higher than normal TSH and lower than normal free T_4 concentrations. An elevated TSH greater than 10 mIU/L is a clear indicator of hypothyroidism with very rare exceptions (Fig. 25.9). Subclinical hypothyroidism describes the mild or early changes when the TSH rises before the free T_4 falls below normal. The mild elevation in TSH compensates for the early decline in T_4 production by the thyroid gland. During pregnancy, trimester-specific and lab-specific reference ranges should be used to determine the optimal TSH range and the diagnosis of clinical hypothyroidism.[126] Although not necessary for the diagnosis of hypothyroidism, additional tests of anti-TPO and anti-thyroglobulin antibodies may influence treatment decisions by establishing underlying Hashimoto's thyroiditis. The presence of anti-TPO antibodies increases the risk for developing clinical hypothyroidism from 2.1% to 4.3% per year.[143] Age and female gender also increase the risk of progression to overt hypothyroidism.

The treatment of hypothyroidism involves replacement of thyroid hormone with synthetic T_4 (levothyroxine). A general guide for replacement is a weight-based dose of 1.6 μg/kg once daily, but actual requirements may vary. After initiation or dose adjustment, follow-up tests should be ordered after at least 6 to 8 weeks. To minimize problems with absorption, levothyroxine should be taken without other drugs, especially calcium or iron-containing tablets.

For the woman known to have hypothyroidism prior to pregnancy, very close monitoring of the TSH is required to maintain a euthyroid state given the increase in TBG and therefore total T_4 requirements. This dose increase is particularly important for the fetus, with strong evidence that maternal hypothyroidism may affect fetal intellectual development during the first trimester when the fetus is dependent on maternal thyroid hormone.[144] Maternal hypothyroidism and subclinical hypothyroidism are associated with a higher risk for miscarriage, hypertension in pregnancy, low birth weight from premature delivery,[145,146] and gestational diabetes.[147] Guidelines suggest achieving a target of TSH less than 2.5 μU/mL before pregnancy to lower the risk for maternal hypothyroidism in the first trimester.[126,148] The T_4 dose usually needs to be increased by anywhere from 10% to 20% to as high as 30% to 50% within the first month of gestation, and therapy should be monitored at least every 4 weeks in the first trimester after any adjustment.[126,149]

Table 25.5 Common Causes of Thyroid Disorders

Primary Hypothyroidism	Primary Hyperthyroidism
Chronic autoimmune thyroiditis (Hashimoto)	Graves disease
Radioiodine ablation	Toxic multinodular goiter
Thyroidectomy	Solitary toxic nodule
Silent/postpartum thyroiditis (hypothyroid phase)	Silent/postpartum thyroiditis (lymphocytic)
Subacute thyroiditis (hypothyroid phase)	Subacute thyroiditis (granulomatous)
Iodine deficiency	Iodine-induced (e.g., amiodarone)
Drugs (thionamides, lithium)	Exogenous thyroid hormone ingestion

Hyperthyroidism

Women and men with hyperthyroidism present with typical findings of weight loss, palpitations, anxiety, and increased frequency of bowel movements. For women, although oligomenorrhea and amenorrhea may be more common in women with hyperthyroidism than euthyroid women, there is less evidence of ovulatory dysfunction and infertility in hyperthyroidism than hypothyroidism. Fewer studies have

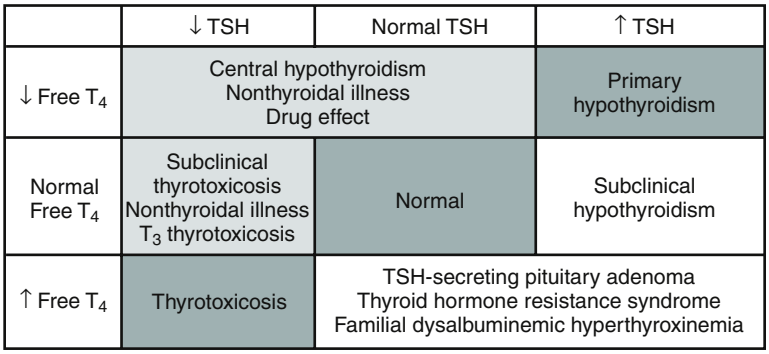


FIGURE 25.9 Differential diagnosis of thyroid disorders based on TSH and free T_4 results.

evaluated reproductive function in hyperthyroid women. Hyperthyroidism is associated with increased SHBG and estrogen concentrations. Abnormalities in basal and stimulated gonadotropins have been observed in women with hyperthyroidism, which reverses after treatment for hyperthyroidism.[125] During pregnancy, there can be subtleties to the diagnosis of thyrotoxicosis and distinguishing hyperthyroidism from normal physiological changes, hyperemesis gravidarum, and gestational trophoblastic disease. As opposed to association of antithyroid antibodies with fertility and miscarriage rates, the thyroid receptor stimulating antibodies of Graves disease have more of an impact on the fetus and the risk for neonatal thyrotoxicosis.[126]

In men with Graves disease, symptomatic hypogonadism and bioavailable testosterone lower than normal controls have been observed despite the overall increase in total testosterone and SHBG. These findings have been associated with impaired gonadotropin response to hCG and significant abnormalities in semen quality. Changes in semen parameters could be reversed with anti-thyroid treatment.[150] Older men are more likely to be clinically affected because of a lower gonadal reserve to compensate for the increases in SHBG. Elevated estradiol may result in gynecomastia and also likely explains the normal gonadotropin concentrations with low free testosterone.[125]

Graves disease is the most common cause of hyperthyroidism. In addition to the symptoms of thyrotoxicosis and goiter, patients may present with the typical stare and extraocular muscle paralysis resulting from infiltrative orbitopathy and opthalmopathy. Graves disease is an autoimmune disease with the thyroid stimulating immunoglobulin or anti-TSH receptor antibody driving unregulated stimulation of thyroid hormone production.

Thyroiditis refers to any inflammatory state of the thyroid. The thyrotoxicosis of thyroiditis results from release of stored thyroid hormone, which may persist for up to 2 months. As discussed above, the usual course is then transient hypothyroidism followed by recovery of thyroid function, although chronic hypothyroidism may also develop. Subacute (de Quervain's) thyroiditis is distinguishable by a history of neck pain and recent viral infection and physical findings of fever and exquisitely tender thyroid. The serum thyroglobulin is often elevated in thyroiditis, and the erythrocyte sedimentation rate is particularly elevated in subacute but not silent thyroiditis.

Other potential causes of thyrotoxicosis include an autonomous thyroid nodule and multi-nodular goiter. Toxic adenoma, which is less common than Graves' disease or toxic multinodular goiter, is usually a benign, solitary tumor with autonomous T_4 and T_3 production. In a large proportion of toxic adenomas, a somatic or germline mutation in the genes encoding the TSH receptor or G protein subunits results in constitutive activation of TSH receptor signaling.[151] Multi-nodular goiter is less likely to cause hyperthyroidism in reproductive age women and is more common in older patients. Growth and the development of autonomous function take years to manifest as hyperthyroidism, and most toxic adenomas are greater than 3 cm in diameter.

The diagnosis of thyrotoxicosis is clear with an elevated free T_4 and low TSH. However, a low TSH with a low or normal free T4 has several additional diagnoses to consider other than subclinical hyperthyroidism (Fig. 25.9). Diagnosis of thyrotoxicosis during pregnancy should be made more cautiously than hypothyroidism, because the TSH level can be low in the first trimester due to hCG-mediated thyroid stimulation. Because total T_4 is elevated due to the increased TBG during a normal pregnancy, pregnancy-specific ranges for total T4 should be used. Alternatively, free T4 concentration can be measured and will only be elevated above non-pregnant ranges in overt hyperthyroidism.

Radioactive iodine scans are not necessary to make the diagnosis of hyperthyroidism but are most useful for distinguishing Graves disease from toxic adenoma (Fig. 25.10), thyroiditis, and factitious disorder. Usually, clinical factors obviate the need for diagnostic scans. Especially since radioactive iodine scans are contraindicated during pregnancy, another useful test is the thyroid stimulating immunoglobulin (TSI) or thyroid receptor antibody (TRAB). Elevated TSI or TRAB supports the diagnosis of Graves disease and is recommended for measurement in the week 20 to

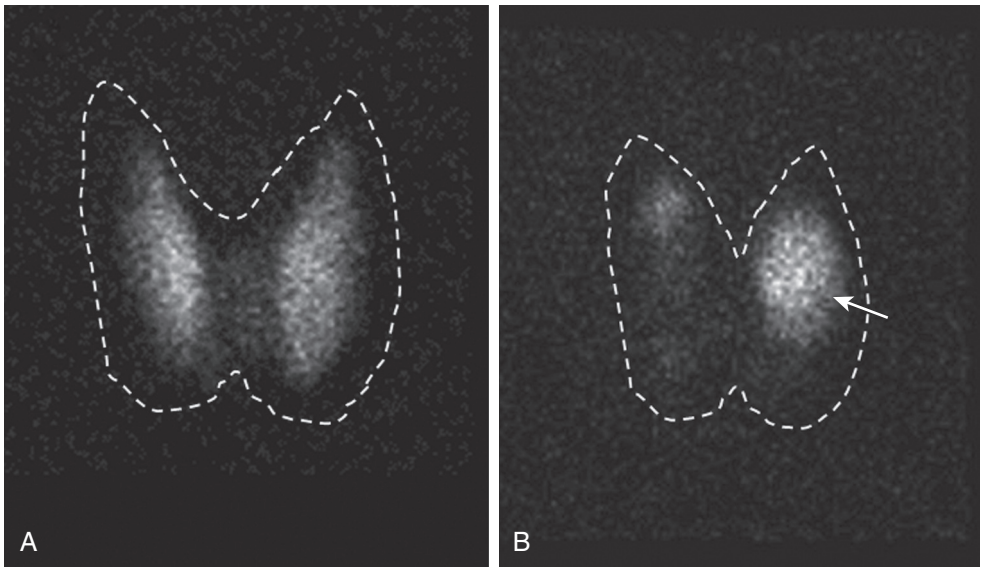


FIGURE 25.10 Radioactive iodine-123 scans to distinguish Graves disease from toxic adenoma. **A**, Increased radioactive iodine uptake seen symmetrically throughout a thyroid gland with Graves disease. **B**, Increased uptake by a toxic adenoma, indicated by *arrow*, with little uptake elsewhere. The dotted lines approximate the outline of the thyroid gland with minimal uptake in comparison to the adenoma.

24 of pregnancy to determine the need for increased fetal monitoring. In pregnancy, breastfeeding, or women trying to conceive, ultrasonography with Doppler blood flow may be useful for distinguishing Graves disease from thyroiditis. The need for experienced technicians and radiologists, however, limits the ability to recommend the general use of ultrasonography for this purpose.[126]

The pharmacological treatment of Graves disease is antithyroid treatment with a thionamide—typically propylthiouracil from 50 mg tid to 100 mg qid or methimazole 5 to 40 mg per day in one or two divided doses, although thionamide doses twice as high are used for the first few days or weeks in severe or life-threatening thyrotoxicosis. The goal of therapy initially is a T_4 in the normal range, as the TSH rise may lag behind. Patients may experience a sustained remission after 12 to 18 months of therapy. Elevated T_4 from thyroiditis is both transient and will not respond to anti-thyroid treatment. Symptoms of palpitations, anxiety, and tremor can be managed with a beta-blocker such as propranolol or metoprolol. In the case of subacute painful thyroiditis, pain responds to non-steroidal anti-inflammatory drugs or a course of corticosteroid therapy. Thionamides are useful for restoration of euthyroidism in patients with toxic adenomas and multi-nodular goiters, but usually definitive therapy (below) is required.

Radioactive iodine-131 ablation is an alternative to antithyroid agents for hyperthyroidism (aside from thyroiditis). When counseling reproductive age women and men about ablation, there is no evidence that previous radioactive iodine ablation at low doses used for hyperthyroidism will affect subsequent fertility in women or men or increase the risk for congenital abnormalities.[125,126,152] In higher doses used for thyroid cancer remnant ablation, radioactive iodine may be associated with azospermia, and sperm cryopreservation should be discussed with young men to preserve fertility for the first few months to years after therapy.[125] Otherwise, guidelines suggest women wait at least 6 months after ablation to ensure that target TSH values are obtained on T_4 replacement therapy.[126]

Methimazole is the preferred anti-thyroid treatment for hyperthyroidism over propylthiouracil due to concerns of hepatotoxicity. One important exception is the first trimester of pregnancy because of the rare association of methimazole with congenital abnormalities. After the first trimester of pregnancy, it is recommended that pregnant women are switched to methimazole for the remainder of the pregnancy.[126,153] In practice, methimazole is often used throughout pregnancy if unable to successfully control T_4 concentrations with PTU due to tolerability and efficacy issues. The goal for therapy in hyperthyroidism prior to conception or while pregnant is a free T_4 at or slightly above the upper limit of the normal range to avoid hypothyroidism.[126]

Surgery—thyroidectomy or resection of a toxic adenoma—is generally reserved for treating hyperthyroidism in disease refractory to medical therapy, for glands that are producing obstructive symptoms, for large nodules, and when radioactive iodine is contraindicated or refused. In pregnancy, surgery is reserved for cases when disease is severe and refractory to medical therapy, or when antithyroid agents cannot be used due to the side effect of agranulocytosis or liver damage.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 26

Endometriosis

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Endometriosis is a chronic condition characterized by the growth of hormone-responsive endometrial tissue outside the uterine cavity. Typically, ectopic endometriotic implants are found on the peritoneal surface, within the ovary or invading the rectovaginal septum; however, many examples of more widely distributed lesions have been described. Several studies suggest that the overall prevalence of endometriosis among reproductive-age women is around 11%.[1] This disease is accompanied by pelvic pain or infertility in up to 90 million women worldwide. Its symptoms are associated with work absenteeism, social isolation, and high costs of therapy. In addition, endometriosis is the third most common indication for hysterectomy in the United States.[2] This outcome can be particularly devastating in women who undergo hysterectomy for endometriosis before the age of 30, because they are likely to have residual somatic symptoms and a psychological sense of loss.[3] Careful estimates of healthcare costs for 2002 in the United States were $22 million.[4] Furthermore, in a cohort study of more than 1.4 million births in Sweden, women with endometriosis were associated with an increased risk of cesarean section, preterm delivery, preeclampsia and antepartum hemorrhage.[5]

History and Histogenesis

Classical Theories of the Origins of Endometriosis

Scholars of this disease have traced original sources that describe the pathological features of endometriosis in Dutch and Belgian women of the late 1600s.[6] Curiously, the prevalence of endometriosis remains high in that part of the world.[7] The great German pathologists von Rokitansky and Meyer wrote extensively about this disorder in the latter part of the 19th century. In the United States, the first clear pathological description of an ovarian endometrioma was reported to the Johns Hopkins Medical Society in 1899.[8] Dr. John A. Sampson graduated from Johns Hopkins that same year. After more than 25 years in the practice of gynecological surgery, he put forward the hypothesis that endometriosis lesions arose from endometrial tissue that escaped through the fallopian tubes at the time of menstruation. This tissue, he postulated, implanted on peritoneal surfaces and regenerated an endometrial epithelial lining.[9] Contemporaneously, Halban proposed that viable endometrial cells might travel via hematogenous or lymphatogenous vascular transport.[10] Although this metastatic theory explains rare manifestations of distant, extraperitoneal lesions (e.g., endometriosis in the brain[11] or nose[12]) it does not explain the gravity-dependent, intraperitoneal location of most foci of endometriosis.

Retrograde Menstruation and Implantation

Even today, the prevailing theory concerning the etiology of endometriosis is the implantation hypothesis set forth by Sampson.[9] This theory has been supported by the documentation of reflux menstruation[13] and intraperitoneal spillage of viable endometrial cells[14] in ovulating women (Fig. 26.1).[15] Furthermore, the incidence is increased in cases of menstrual outflow obstruction; in women with known endometriosis, ablation of the eutopic endometrium dramatically reduced the risk of recurrence.[16] Interestingly, the recurrence rate of endometriosis was lower in women who had vaginal deliveries than in nulliparous women or those undergoing cesarean section suggesting cervical dilatation following vaginal parturition reduces resistance to menstruum.[17] That more than 60% of unilateral lesions occur in the left hemipelvis also is consistent with accumulation of refluxed cells in this location, due to the position of the mesentery of the sigmoid colon.[18]

The adhesion of shed endometrial cells to peritoneal and subperitoneal surfaces involves the expression of extracellular membrane adhesion molecules and their coreceptors. Although some researchers have argued that implants of endometrium have never been clearly visualized microscopically in the process of attaching to the peritoneum,[19] the *in vitro* findings of Witz and colleagues[20] indicate that the process of implant invasion into the subperitoneal space is so rapid that one would not expect to observe adherent fragments of endometrium (Fig. 26.2).[21] This origin has been proposed by Nisolle and Donnez[22] to be the most likely source of intraperitoneal lesions *in vivo*.

Coelomic Metaplasia

An alternative hypothesis, historically attributed to Meyer,[23] suggests that metaplasia of the coelomic epithelium is the origin of endometriosis. This theory is logical, because cells from both the peritoneum and endometrium derive from a common embryologic precursor: the coelomic cell. However, this theory has been difficult to support scientifically.

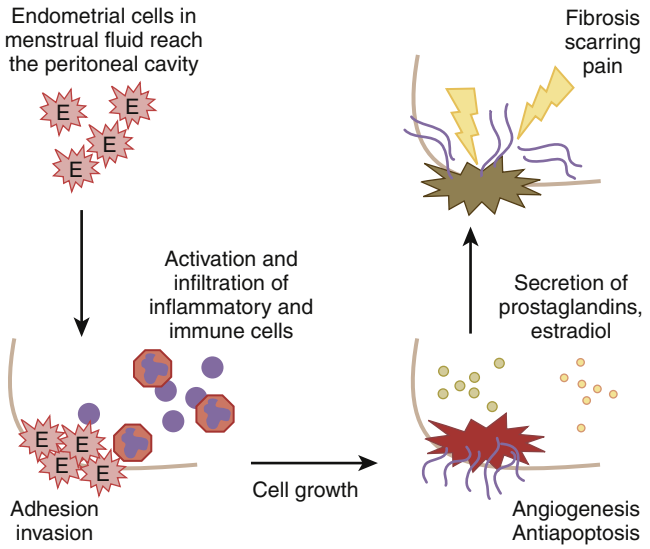


FIGURE 26.1 Proposed establishment of peritoneal endometriotic implants via retrograde menstruation, attachment, proliferation, migration, neovascularization, inflammation, and fibrosis. E, endometrial cell. *(From Flores I, Rivera E, Ruiz LA, et al. Molecular profiling of experimental endometriosis identified gene expression patterns in common with human disease.* Fertil Steril *87:1180–1199, 2007.)*

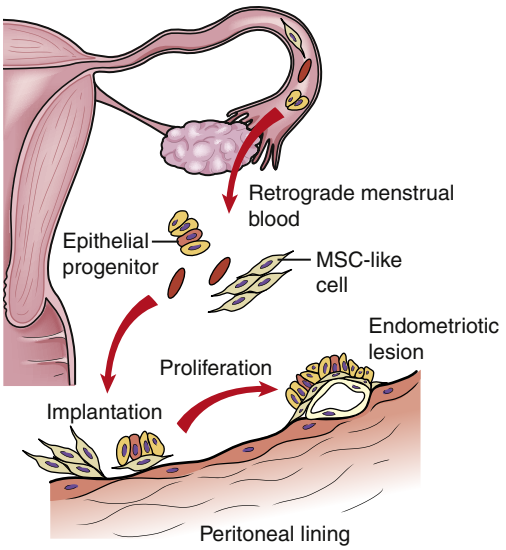


FIGURE 26.3 Role of endometrial stem cells in the pathogenesis of endometriosis. Endometrial epithelial progenitor cells and mesenchymal stem-like cells (MSC) enter the peritoneal cavity via retrograde menstruation wherein they establish endometriotic lesions. *(Adapted from Gargett CE, Masuda H. Adult stem cells in the endometrium.* Mol Hum Reprod *16:818–834, 2010.)*

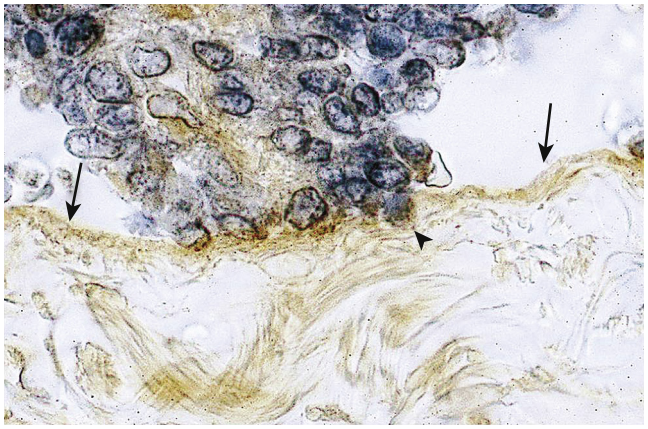


FIGURE 26.2 Early invasion of an endometrial implant through the mesothelium. The mesothelium is labeled with monoclonal antibody to cytokeratin and stained with diaminobenzidine *(arrows)*. An endometrial stromal cell *(arrowhead)* passing through the mesothelium is thought to represent the initial step of invasion into the stroma of the peritoneum. Original magnification, ×31,000. Counterstained with hematoxylin. *(From Witz CA, Monotoya-Rodriguez IA, Schenken RS. Whole explants of peritoneum and endometrium: a novel model of the early endometriosis lesion.* Fertil Steril *71:56–60, 1999.)*

If the postulate were correct, one would expect much higher rates of pleural endometriosis because that mesothelium is the same as that which lines the abdominal cavity. Furthermore, investigators have been unable to show that peritoneal cells can be experimentally differentiated into endometrial cell types. Lastly, metaplasia is an age-related process. Endometriosis occurs early in reproductive-aged women, and its incidence peaks in the third decade. This pattern would not be predicted by a linear, progressive likelihood of metaplasia. Nevertheless, this has been postulated as the most likely source of deep, adenomyotic nodules of endometriosis in the rectovaginal septum[22] and in some cases of endometrioma.[24] A related theory, that is more straightforward in pathophysiology, is the embryogenetic theory: atypical locations of müllerian duct cells deposited during migration at the time of embryogenesis. With the onset of menarche and stimulation with estradiol, these ectopic cells may then grow into classic endometriotic lesions. Signorile and colleagues buttressed this theory when they found 9% of 101 human female fetuses possessed primitive endometrium outside the uterine cavity.[25]

Neoclassical Theories of Endometriosis Histogenesis

In the past decade, scholars of endometriosis have considered integrated models for the etiology of this disorder. We currently recognize that genetic, anatomical, endocrine, immunological, and possibly environmental factors influence the risk of developing endometriosis. Hence, the neoclassical view of this syndrome requires a broader conceptualization of its etiology and the mechanisms whereby endometriosis produces its clinical symptoms. Based on histological and biochemical similarities of endometriosis lesions with the basalis layer of the endometrium, Leyendecker and colleagues suggested that endometriosis is derived from the subendometrial zone that they refer to as *archimetra*.[26] More recently, authors have posited that endometriosis may arise from rare adult stem cells residing within the uterus.[27-29] Endometrial mesenchymal stem cells have been isolated in both the basalis and functionalis; these appear to have a predominant perivascular localization (Fig. 26.3).[30]

Epidemiology

Endometriosis has been found in women between the ages of 12 years old and 80 years old, with the average age being

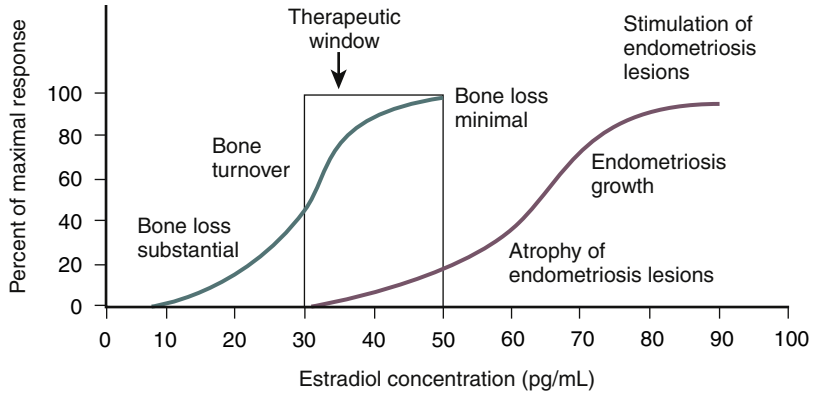


FIGURE 26.4 The estrogen threshold hypothesis postulates that different estrogen target tissues have differential hormone sensitivity. In this model, a therapeutic window of serum estradiol concentration, between 30 and 50 pg/mL, should protect against bone mineral loss and prevent stimulation of endometriosis lesion growth. *(From Barbieri R. Hormone treatment of endometriosis: the estrogen threshold hypothesis.* Am J Obstet Gynecol *166:740–745, 1992.)*

approximately 28 years old. Exposure to ovarian hormones appears to be essential to fostering lesion growth, with the vast majority of the cases found in women aged 20 years old to 50 years old. The estrogen dependency of this condition led Barbieri to propose the hypothesis that concentrations of estradiol over 50 pg/mL were needed to support the growth of endometriosis lesions (Fig. 26.4).[31] This hypothesis will be discussed in more detail when the medical treatment of endometriosis is considered later in this chapter.

In severe disease there seems to be a familial correlation; however, no clear mendelian genetic trait has been identified and most researchers believe it to be complex and multifactorial.[32] These points will be discussed more fully later in this chapter. Retrograde menstruation and increased exposure to menstrual flow (reduced parity, longer duration of menstrual periods, and shorter cycle length) have been identified as risk factors for endometriosis.[33] Recently, environmental factors, such as dioxin[34,35] and other presumed endocrine disrupters have been implicated.[36]

When adolescent girls present with chronic pelvic pain, endometriosis may be found in up to 38% and nearly 70% for those with recalcitrant symptoms.[37,38] In contrast to adults who often present with cyclic pain, these adolescents have a greater prevalence of acyclic pain. Initial surgery usually reveals stage I (77% to 92%) or II disease (8% to 23%) with a preponderance of red lesions (75%) as opposed to that normally seen in adults (25%).[39] Since the disease is progressive,[40] treatment ought to be initiated with oral contraceptives or a progestin regimen in order to impede disease progression as well as preserve fertility.

Demography

The prevalence of endometriosis ranges from 2% to 50% of reproductive-aged women. Unfortunately, this number is widely disparate depending upon the study. In women with infertility, the prevalence ranges from 21% to 47%. This high frequency may be due to the fact that endometriosis plays a causative role in infertility, but it also may be due to a diagnostic selection bias. Women with infertility are more likely to undergo laparoscopy as part of their clinical evaluation, thereby confirming the diagnosis. Childhood and early adulthood body size inversely influence the risk of endometriosis such that a lower body mass index (BMI) is associated with a lower risk.[41] This may be related to the finding that in adolescents, a lower BMI is associated with a later age of menarche and a lower incidence of the disease (OR, 0.3; 95% CI, 0.1-0.6).[42] Asian women have a ninefold greater risk of developing endometriosis compared to white women.[43]

Observations of a high prevalence of endometriosis in primate colonies exposed to dioxin,[44] a particularly potent polychlorinated biphenyl (PCB) industrial contaminant, have suggested that this compound might influence the risk of endometriosis in women. Recent case-control studies revealed increased adjusted odds ratios for endometriosis in women exposed to dioxins and other PCB chemicals of this type.[45,46] *In utero* exposure to PCBs was shown to induce an endometriosis-like uterine phenotype (e.g., reduced progesterone receptor levels) in females of the F1 generation.[47]

Genomics, Genetics, and Epigenetics

Genes seem to influence susceptibility for endometriosis[48]; however, as previously noted, the mode of hereditary transmission appears to be polygenic and likely to involve multiple loci.[32] Several lines of evidence implicate genetics as a risk factor. In one study, first-degree female relatives (mothers and sisters) of women with severe endometriosis had a 7% incidence, whereas primary female relatives of their partners (who typically have similar ethnic and socioeconomic status) had less than 1% incidence of endometriosis.[49] Consistent with this observation is the finding of Kennedy and colleagues that familial cases of endometriosis tend to be more severe and have an earlier onset of symptoms than sporadic cases.[50] Large population studies based in Iceland and Australia and the United Kingdom identified loci on chromosome 9q and 10q, respectively, significantly associated with endometriosis, but retrospective sib-pair analyses of this type can be complicated by diagnostic misclassification.[51,52]

Over the past several years, complementary DNA (cDNA) microrray methods have been used to identify endometriosis-related genes. Giudice and colleagues[53] were among the first to use Affymetrix chips with arrayed oligonucleotides and observed that many progesterone-regulated genes are relatively downregulated in eutopic (intrauterine) endometrium of women with endometriosis. This effect appears to be most dramatic for gene expression in the early secretory phase of the cycle.[54] Genes involved in oxidative stress, and the wingless-related integration site (WNT) and mitogen-activated protein (MAP) kinase signalling pathways have been consistently reported.[55] Our group[56] focused on the reduced expression of glycodelin mRNA, which encodes

a progesterone-regulated, secreted glycoprotein associated with immunological tolerance of the semiallogeneic blastocyst. These findings are consistent with a comprehensive meta-analysis showing lower embryonic implantation rates in women with advanced endometriosis undergoing *in vitro* fertilization. This study is discussed in more detail later in the chapter.

Another powerful genomic screening technique was introduced by Liang and Pardee in 1992.[57] Differential display–polymerase chain reaction (dd-PCR), in a fashion akin to subtraction hybridization, employs random sets of oligonucleotide primers and PCR to amplify fragments of differentially expressed cDNAs from two different tissues. Endometriotic implants and biopsies of eutopic endometrium from normal and endometriosis cases were obtained in the midproliferative phase of the ovulatory cycle and analyzed by Lundeen and colleagues[58] using this technique. One interesting PCR product was observed to be consistently up-regulated in ectopic and eutopic endometriotic tissue relative to normal eutopic endometrium. The cDNA was shown to correspond to the zinc-finger transcription factor, early growth response-1 (EGR-1). EGR-1 is an interesting candidate because it can be activated by a variety of upstream signaling molecules that are known to be associated with endometriosis, including estradiol, interleukin (IL)-lβ, IL-6, and TNF-α.[56]

Lebovic and colleagues[59] compared cDNAs expressed in normal endometrial stromal cells with those derived from endometriotic implants, with and without pretreatment with the inflammatory cytokine IL-lβ. Endometriotic stromal cells substantially down-regulated a tumor suppressor gene *(TOB1)* in response to IL-1β treatment, whereas normal endometrial stromal cells showed no effect of IL-1β. *TOB1* is an endogenous inhibitor of the *ERBB2* receptor, a member of the epidermal growth factor receptor family that is overexpressed in many human cancers. Monoclonal antibodies directed against this receptor, such as trastuzumab, and other inhibitors of *ERBB2* have been shown to reduce the progression of metastatic breast cancer.[60] In a similar study of normal endometrial stromal cells, Rossi and colleagues found angiogenic and extracellular matrix remodeling genes were upregulated by IL-1β.[61] The significance of these findings in endometriosis is that endometriotic stromal cells appear to have a growth advantage in an inflammatory environment, mediated by the attenuation of a natural inhibitor of mitotic activity induced through the *ERBB2* receptor pathway.

A new development in the genetics of endometriosis is the identification of micro-ribonucleic acid (miRNA) expression. These small RNAs hybridize with mRNA transcripts and induce their degradation, functionally repressing gene actions. MiRNAs have been identified that would be expected to reduce production of steroid receptors known to be down-regulated in endometriosis. These may become interesting new targets for endometriosis therapeutics.[62-65]

Cytogenetics of Endometriosis

Cytogenetic methods that assess gene content in intact or microdissected endometriotic tissues include chromosome satellite painting and comparative genomic hybridization. Kosugi and colleagues[66] used fluorescence *in situ* hybridization (FISH) to find substantially increased aneuploidy and loss of heterozygosity of chromosome 17 in endometriotic lesions. Interestingly, this chromosomal region is the location of several tumor suppressor genes important in the normal regulation of the mitotic cell cycle. Among these is the *p53* locus, which is lost in cases of severe endometriosis,[67] and the *TOB1* tumor suppressor, noted to be preferentially down-regulated in endometriotic cells treated with IL-1β.[59] A 50% loss of heterozygosity (LoH) for chromosomes 1p and 22q was noted by Gogusev and colleagues[68] in endometriotic implants. Lower levels of LoH also have been reported for chromosomes 9p and 11q.[69] These findings suggest that genomic instability is a feature of endometriosis.

Comparative genomic hybridization is a technique based on the competitive *in situ* hybridization of differentially labeled DNA from endometriosis and normal endometrial tissue to human metaphase spreads. Regions of gain of DNA sequences are seen as an increased color ratio of the two fluorochromes labeling the respective DNAs. Using this method, Gogusev and colleagues in Paris identified chromosomal regions in which gene copy numbers are altered in endometriosis.[68] In an established endometriosis cell line (FbEM-1), these investigators observed that the *c-ERBB2* protooncogene was overexpressed.[70] This result complements the finding that *TOB1* is reduced in endometriosis and suggests that *ERBB2* signalling is important in endometriosis.

Genetic Polymorphisms and Endometriosis Risk

Several candidate gene polymorphisms have been evaluated in women with endometriosis. In general, these have fallen into three major classes, each representing presumed pathogenetic abnormalities:

1. Genes involved in xenobiotic metabolism
2. Genes that mediate inflammatory responses
3. Genes regulating steroid action

An N-acetyltransferase 2 allele *(NAT2*4/*6)* was significantly more common among affected women (35.2%) than population control subjects (8.1%) or unaffected women (4.2%; $P = 0.02$). More affected women (57.4%) were found to be "fast acetylators" than were population control subjects (32.3%) or unaffected women (33.3%; $P < 0.05$). Altered N-acetyltransferase 2 *(NAT2)* enzyme activity, which would enhance the rate of xenobiotic acetylation, may be a predisposing factor in endometriosis.[71]

Based on the observation that women with vaginal agenesis carried N314D mutations in the galactose-1-phosphate uridyl transferase *(GALT)* gene regulating galactose metabolism,[72] Cramer et al.[72] speculated that this defect also might be associated with increased retrograde menstruation and may predispose to endometriosis.

A proteomic analysis of plasma proteins from 56 endometriosis cases and 20 unaffected controls identified elevated concentrations of vitamin D binding protein among the cases. Based on trypsin digestion patterns it was discerned that the guanine-cytosine (GC)*2 allele was over represented in women with endometriosis.[73] This allele has been associated with impaired macrophage phagocytosis, as postulated in the impaired immunosurveillance of endometriosis, reviewed below.

Viganò and colleagues reported a polymorphism of the intercellular adhesion molecule-1 *(ICAM-1)* in women with severe endometriosis.[74] The implication of such a protein, where abnormal cellular adhesion is suspected, deserves further investigation. Despite the hypothesis that dioxins and other environmental toxicants might play an etiological role in endometriosis,[44] Japanese women with this condition were not observed to have an increased incidence of polymorphisms in the aryl hydrocarbon receptor gene or associated factors studied to date.[44] Variants in the glutathione S-transferase genes were reported by Baranova and colleagues[75] in a group of French and Slavic women with endometriosis, but the same polymorphisms were not identified in subjects from the United Kingdom.[76] A polymorphism in the 5′ untranslated region of the human *VEGF* (vascular endothelial growth factor) gene (+405G>C) also has been correlated with an increased risk of endometriosis development in Italian Caucasian, South Indian, and Korean populations.[77]

Several genome wide association studies (GWAS) have been published. The first included 1907 cases and 5292 controls from Japan and identified polymorphisms on chromosome 9p21 (located within the intron of a gene encoding a cyclin-dependent kinase inhibitor antisense RNA) and chromosome 1p36 (near the *WNT* locus).[78] Another study[79] of 3194 cases and 7060 controls, drawn from a population of women from Australia, the UK and the US, reported a locus on chromosome 7p15.2. The latter site is located 1.35 megabases upstream from the plausible candidate genes homeobox A *(HOXA)10* and *HOXA11*. Yet another GWAS study and meta-analysis confirmed an association with an SNP on chromosome 7p15.2, and an SNP near the *WNT4* gene. Several other new loci were also discovered. There was a significant overlap in Japanese and European ancestry populations suggesting that risk alleles contribute to endometriosis across populations. It should be noted that the variants associated with endometriosis in these GWAS studies had relatively low odds ratios (≤1.44).

Inconsistencies among these reports reflect some of the inherent methodological problems with GWAS. In particular, the recruitment of pre-existing cases and the willingness of these subjects to participate in research can bias the results. There also are pitfalls in control recruitment, including the use of patients who have other diseases as control subjects. Failure to replicate findings in a different racial or ethnic group, as noted above, may not mean that the original study was flawed, but could reflect differences in allele frequency between populations. Finally, if gene–environment interactions affect the magnitude of a genetic risk factor, another population may not be exposed to the same environmental factor as in the original study.

Nuclear Receptor Genes as Biomarkers of Endometriosis Risk

Homozygosity for a *Pvu II* polymorphism in the estrogen receptor *(ER)-α* gene was found less frequently in women with endometriosis when compared with a disease-free group.[80] A variant progesterone receptor *(PR)* allele, called *PROGINS*, is characterized by an *Alu* insertion in intron G and two additional mutations in exons 4 and 5. The *PROGINS* allele codes for a progesterone receptor with increased stability and augmented hormone-induced transcriptional activity. Homozygosity for this allele was present in 3.2% of women with endometriosis and 0.9% of control subjects.[81] This result is at odds with the evidence that progesterone action is decreased in endometriotic tissue, due to a relative decrease in the ratio of PR-B (which generally activates gene transcription) to PR-A (which generally suppresses transcription) isoforms.[82]

Bulun and colleagues observed that ERβ mRNA is overexpressed approximately fortyfold and PR-B is underexpressed by 88% in endometriotic tissue. The apparent mechanism in the former case involves hypomethylation of cytosine and guanine-rich (CpG) islands in the ERβ gene promoter, indicating that epigenetic regulation may, in part, explain this observation.[83]

Familial aggregation of endometriosis also could be attributed to epigenetic factors, and although sparse, recent evidence supports this interesting hypothesis. Another pertinent gene that was found to be hypomethylated in endometriosis is the homeobox transcription factor HOXA10. This protein also is expressed in midsecretory phase endometrium and its expression is reduced in clinical endometriosis and in an induced baboon model of the disorder.[84-86]

Clinical Presentation

Symptoms and Signs

The most common symptom in women with endometriosis is progressive, secondary dysmenorrhea. The pain usually begins before menses and continues throughout the duration of menstrual flow. It may be accompanied by dyspareunia, dysuria, or dyschezia. The pain also may be referred to musculoskeletal regions, such as the flank or low back. Cyclic *in situ* menstruation at the site of an endometriotic lesion is believed to result in a chronic inflammatory nidus. Cytokine release and activation of prostaglandin-mediated pain perception, or direct infiltration of endometriotic cells into afferent nerves have been proposed.[87] Furthermore, the enhanced inflammatory milieu may result in sensitization of nociceptors and central neurons.[88] New hypotheses suggest that coordinated neural and vascular growth, "neuroangiogenesis," and secondary central neuropathic sequelae contribute to pelvic pain.[89]

The second most common symptom is infertility with monthly fecundity rates similar to unexplained infertility patients at 0.02 to 0.10. It has long been accepted that women with moderate and severe endometriosis have fertility problems due to the mechanical interference with sperm-egg union or zygote transport, as a result of pelvic adhesions and disruption of normal anatomy. Interestingly, women with minimal or mild endometriosis also have decreased fertility outcomes when compared with those patients without clinical evidence of disease. The exact causes remain elusive to date, but several postulated mechanisms are addressed later in this chapter.

Unfortunately, due to the diffuse and pusillanimous nature of endometriotic lesions, the physical examination is commonly unrevealing. The bimanual examination is of limited precision in either localization or diagnosis of endometriosis. However, the astute clinician can appreciate pain or induration in the vicinity of otherwise nonpalpable lesions, most commonly in the cul-de-sac or rectovaginal septum.

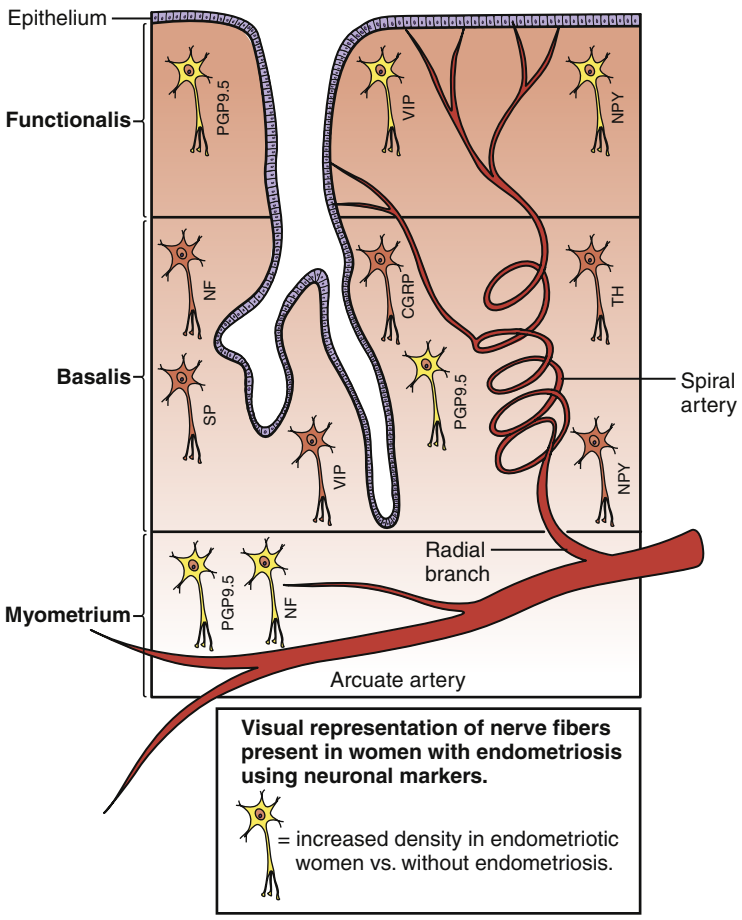


FIGURE 26.5 Neuronal markers denoting which nerve fibers are present in the various layers of the endometrium. *(Adapted from Medina MG, Lebovic DI. Endometriosis-associated nerve fibers and pain.* Acta Obstet Gynecol Scand *88:968–975, 2009.)*

Tender nodules may be palpable along the uterosacral ligaments or within the cul-de-sac, especially if the examination is done just before the menses. Rarely, impaired renal function and azotemia can develop in women with retroperitoneal ureteric fibrosis.

Ectopic endometrium within the uterine corpus is termed adenomyosis, and although this entity is not covered in this chapter, there seems to be an association between adenomyosis and endometriosis. Utilizing junctional zone measurements from magnetic resonance imaging (MRI) to diagnose adenomyosis in patients with endometriosis, the prevalence of adenomyosis was 90% in one recent study.[90] Interstitial cystitis is another comorbidity in at least 60% of women with endometriosis.[91]

Diagnosis

Direct visualization of endometriotic implants is the current gold standard for diagnosis. Although endometriosis is associated with certain biochemical characteristics (described later in this section), none of these features have adequate sensitivity or specificity for clinical application. Hence, laparoscopy, and rarely laparotomy, is the most common means to establish a firm diagnosis. The histopathological confirmation of endometriosis is established on the microscopic identification of ectopic endometrial epithelium and stroma, often with fibrosis and the infiltration of hemosiderin-laden macrophages. These pathognomonic features are documented in only approximately 70% of clinically suspicious cases.

Radiological imaging techniques have been evaluated as noninvasive approaches to endometriosis diagnosis, but these methods have not replaced surgical verification of lesions. The diagnostic sensitivity and specificity of imaging modalities are reviewed later in this chapter. With the discovery that a greater density of nerve fibers is present in the functionalis layer of eutopic endometrium in endometriosis than those without endometriosis (Fig. 26.5), a minimally invasive endometrial biopsy may help reduce the delay in diagnosis and subsequent treatment of endometriosis.[84,92,93]

Because disease is found in the dependent areas of the pelvis, performance of a systematic rectovaginal examination is critical. Some authors have advocated the use of needle biopsy to confirm the histological features of the nodular rectovaginal lesion.[94]

Variable Lesion Appearance

Visually, peritoneal endometriosis can take on a variety of appearances. Classically, physicians were taught that endometriosis implants were blue-black "powder burns" or "mulberry lesions" of the peritoneum. In recent years, several stages of implant development have been appreciated, each with a corresponding appearance. Early, active lesions can appear as papular excrescences or vesicles, and can range in color from clear to bright red.[95] About one third of lesions are in phase with the eutopic endometrium and have a tendency to spontaneously grow and regress. This characteristic suggests a fluctuating proliferation in association with hormone production during the menstrual cycle.[96]

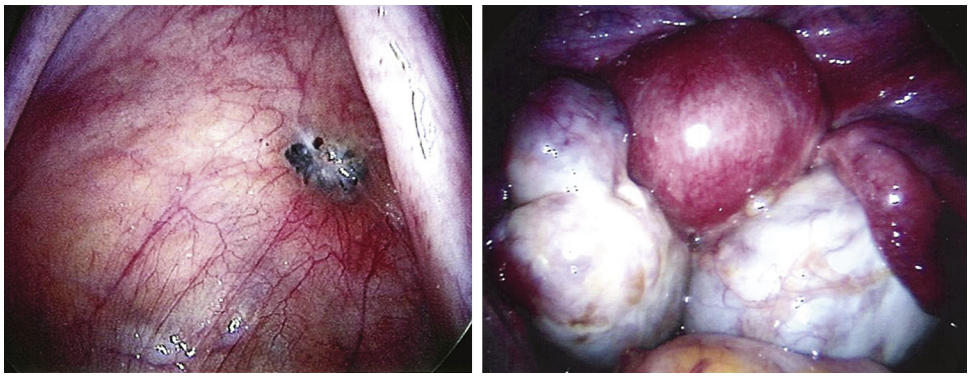


FIGURE 26.6 Laparoscopic photographs of pigmented peritoneal implant *(left)* and bilateral ovarian endometriomas, or so-called "kissing ovaries" *(right)*. *(Images courtesy of Dr. Michael D. Mueller, University of Bern, Switzerland.)*

Table 26.1 Natural Course of Endometriosis

Study	Increase in Disease	No Change	Decrease in Disease *[Elimination]*
Thomas EJ et al, 1987[100]* Stage I-III, *n* = 17 6 mo follow-up L/S	47% (8)	24% (4)	29% (5) *[18% (3)]*
Telimaa S et al, 1987[99]* Stage I-II, *n* = 17 6 mo follow-up L/S	25% (4)	63% (10)	19% (3) *[13% (2)]*
Cooke ID et al, 1989[101]* Stage I-II, *n* = 17 6 mo follow-up L/S	47% (8)	24% (4)	29% (5) *[18% (3)]*
Mahmood TA et al, 1990[102]* Stage I-III, *n* = 11 9-18 mo follow-up L/S	64% (7)	9% (1)	27% (3) *[9% (1)]*
Overton CE et al, 1994[103]* Stage I-II, *n* = 15 6-9 mo follow-up L/S	27% (4)	20% (3)	53% (8) *[NR]*
Sutton CJ et al, 1997[104] Stage I-III, *n* = 24 6-39 mo follow-up L/S	29% (7)	42% (10)	29% (7) *[4% (1)]*
Harrison RF et al, 2000[105]* All stages, *n* = 43 4-6 mo follow-up L/S	9% (4)	28% (12)	63% (27) *[44% (19)]*
Abbott J et al, 2004[106] Stage II-IV, *n* = 19 6 mo follow-up L/S	42% (8)	42% (10)	29% (7) *[4% (1)]*
Totals (*n* = 162)	**31% (50)**	**32% (50)**	**38% (62) *[23% (29)]***

*Infertile patients.
L/S, laparoscopy; NR, not reported.

Advanced, active lesions are associated with inflammation, fibrosis, and hemorrhage, and they take on the more classic pigmented appearance (Fig. 26.6). These lesions can express myriad colors—black, brown, purple, red, or green—due to the presence of heme degradation products as the foci undergo periodic hemorrhage and fibrosis. Dormant and healing lesions appear white or calcified, reflecting remnants of glands embedded in fibrous tissue.[95] In a histological study, visually white and mixed-color lesions were more likely to contain histological evidence of endometriosis than were black or red lesions.[97]

Although difficult to prove in cross-sectional clinical studies, endometriosis appears to be a progressive disease, as evidenced by longitudinal laparoscopies in female baboons.[98] At least eight studies were reported of repeat laparoscopy in women assigned placebo treatment.[99-106] Combining these placebo participants yields a total of 162 women that confirm that endometriosis is a disease in flux. There was a nearly equal distribution among women whose disease stage deteriorated (31%), was unchanged (32%), or improved (38%). In fact, all but one study noted that 23% of placebo patients had complete regression of disease over intervals of 4 to 39 months (Table 26.1). Regrettably, this means for over two thirds of women the disease will either persist or progress, and this situation must be addressed with postoperative medical management.

An evolution of the visual appearance of the lesions from clear to red to black to white has been suggested to correlate with the subject's age.[107] This observation implies that the appearance of endometriotic lesions may correlate with their biology. Deeply invasive lesions of the rectovaginal septum have the histological characteristic of smooth muscle hyperplasia, so-called *adenomyotic nodular endometriosis*. Some argue that this reflects a unique histogenesis from müllerian remnants,[22] but other authors accept that these lesions also may be manifestations of retrograde menstruation and implantation.[108,109]

A specific manifestation is the *endometrioma*, or *chocolate cyst* (Fig. 26.6). These ovarian cysts gained their moniker by the characteristic chocolate syrup appearance of

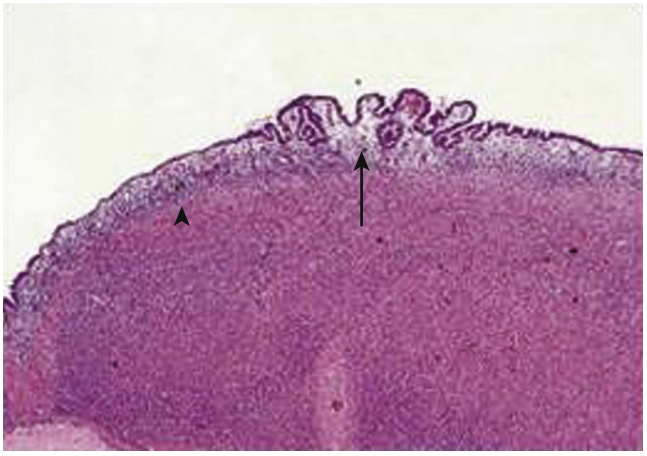


FIGURE 26.7 Presumptive development of ovarian endometrioma via surface epithelial metaplasia. Low-power view of an endometriotic lesion arising in ovarian surface epithelium. The typical or well-developed area of endometriosis is seen in the center of the lesion *(arrow)*, with transition from minimal epithelial and stromal changes from normal ovarian surface epithelium on the sides of the focal lesion *(arrowhead)*. *(From Zheng W, Li N, Wang J, et al. Initial endometriosis showing direct morphologic evidence of metaplasia in the pathogenesis of ovarian endometriosis.* Int J Gynecol Pathol *24:164–172, 2005.)*

their contents, often seen at rupture. These cysts arise after implantation of ectopic endometrial tissue and subsequent invasion into the ovarian cortex or alternatively as a result of metaplasia (Fig. 26.7).[24] The cell types present in these cysts include endometrial epithelium (both as glands and flattened cells), endometrial stroma, and hemosiderin-laden macrophages. In some cases, ciliated cells, similar to those of oviduct epithelium, have been observed.[110] It is reported that pelvic pain associated with endometrioma is positively correlated with ovarian blood flow and microvascular density, suggesting that enhanced angiogenesis may relate to symptoms.[111] Techniques to culture the endometriotic lining of these cysts[112] have been widely adapted and allow a reliable and reproducible source of primary endometriosis cells for *in vitro* research.

Under scanning electron microscopy, microscopic lesions have been found in the normal-appearing peritoneum of women with and without endometriosis.[113] The clinical significance of these findings is a matter of controversy at present, but researchers argue that the prevalence of histological endometriosis may be underestimated.

Classification

The system most widely used to classify the extent of endometriosis was promulgated by the American Fertility Society in 1985.[114] This scheme designates disease extent based upon total three-dimensional volume of endometriosis. Importance is placed upon depth of invasion, bilaterality, and ovarian involvement, as well as density of associated adhesions and extent of cul-de-sac involvement. Using this classification, scores are tallied to objectify and compare natural history and treatment outcomes among different centers and surgeons. Scores of 1 to 15 reflect minimal or mild disease; 16 to 40, moderate; and over 40, severe. It is important to note that this staging system has not been shown to correlate with predicting pregnancy or the more common symptom of pelvic pain.[115] New classifications are being promoted to try to correct inadequacies of the current schema, but their complexity and lack of sensitivity have hindered adoption.[116-118] Some authors have suggested the incorporation of objective biochemical markers of inflammation as a means to assess disease parameters beyond laparoscopically visible lesions.[119,120] Future studies will evaluate the utility of the latter recommendations.

Radiological Imaging

Economic pressures and perioperative risks associated with surgical staging have stimulated attempts to develop noninvasive tests for endometriosis. Radiological evaluation by MRI and ultrasonography show some promise.

Compared with other imaging modalities, MRI is the best technique to identify endometriosis. It lacks, however, sufficient sensitivity and specificity, especially in the detection of small implants and adhesions. MRI also may have a role in evaluating treatment response with endometriosis patients receiving medical therapies such as danazol, progestins, or gonadotropin-releasing hormone analogs.[121,122]

Overall sensitivity of MRI in the detection of endometrial implants is 77%, with a specificity of 78%.[99] MRI identification of endometriomas is more satisfactory and has high sensitivity (90% to 92%) and specificity (91% to 98%) rates.[121] MRI scans of endometriomas are characteristically identified by "shading," with homogeneous hyperintensity on T1-weighted images and relative hypointensity on T2-weighted images. The signal intensity pattern is consistent with the presence of intracellular methemoglobin (Fig. 26.8). Endometriomas may have a complex appearance, usually related to the presence of blood clots or debris within the cyst, and in this setting use of intravenous contrast material may be helpful in distinguishing these products from more worrisome ovarian neoplasms.

Ultrasonography

Endometriomas may be difficult to differentiate from ovarian neoplasms by ultrasonographic features alone. Recurrent episodes of bleeding and surrounding inflammation sometimes result in a heterogeneous internal appearance (suggesting solid components), a thickened wall, or apparent septations. Apparent septations may be particularly misleading because they can reflect compressed ovarian parenchyma intervening between the endometrioma and a satellite cyst. Endometriomas tend to be well vascularized and Doppler blood flow may erroneously suggest a neoplastic mass.

Although endometriomas may mimic neoplasms, they typically demonstrate a characteristic sonographic appearance. Because of ease of use, cost-effectiveness, and accessibility, transvaginal ultrasonography has become the imaging modality of choice when evaluating the ovary for an endometrioma.[123] The classic endometrioma has diffuse low-amplitude internal echoes, enhanced sound transmission, and a visible uniform wall, sometimes containing bright reflectors (Fig. 26.8).[124,125] The reflectors have been postulated to represent red blood cell breakdown products. About 50% of ovarian masses that are histologically

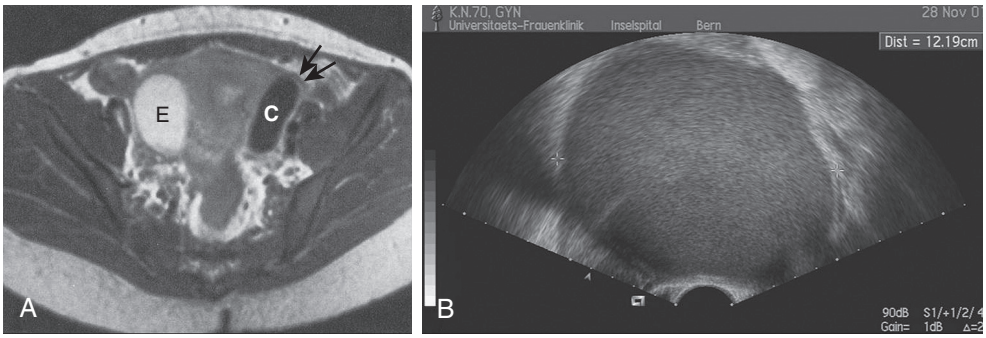


FIGURE 26.8 **A,** Magnetic resonance imaging of the pelvis reveals characteristic high signal intensity in the right ovarian lesion suggesting an endometrioma *(E)*. The low signal intensity lesion of the left ovary is consistent with a simple ovarian cyst *(C)*. Note enhancement of the periphery of the cyst *(arrows)*. **B,** Transvaginal ultrasonography reveals a cystic adnexal mass with diffuse, homogenous moderate-level echoes consistent with an endometrioma. *(**A** from Jaffe R, Pierson RA, Abramowicz JS (eds). Imaging in Infertility and Reproductive Endocrinology. Philadelphia, Lippincott, 1994; **B** courtesy of Dr. Michael D. Mueller, University of Bern, Switzerland.)*

confirmed endometriomas demonstrate this characteristic sonographic appearance.[126] Although the same appearance may occur in an acutely hemorrhagic cyst, the latter will evolve or resolve over time but an endometrioma usually remains stable or enlarges. Serial sonograms are often necessary before the physician may reliably assume that a mass is an endometrioma.

Serum Markers of Endometriosis

Although they might provide great clinical value, serum markers of endometriosis have proved to be elusive.[127] The best studied of these is the tumor marker cancer antigen (CA)-125. This transmembrane glycoprotein, initially identified in serous ovarian carcinomas, was noted by Barbieri and colleagues to circulate at elevated concentrations in women with endometriosis.[128] The CA-125 gene has yet to be fully characterized, but it encodes a 4-megadalton product with repetitive peptide motifs and O-linked polysaccharides, which is synthesized and secreted by cells of coelomic origin.[129] Some authors have found this marker to be a better correlate of active endometriotic lesions than peritoneal adhesions, and it may have utility in monitoring the progression of disease or recurrence during therapy.[130] Although the sensitivity of preoperative CA-125 for the detection of endometriosis is too low for a screening test, high concentrations may be predictive of advanced disease.[131] The addition of serum soluble ICAM-1 only modestly improved the sensitivity of concomitant CA-125 screening for endometriosis.[132]

Two recent papers have addressed the use of serum biomarkers in the identification of women with endometriosis. Othman and colleagues reported significantly elevated levels of IL-6, monocyte chemoattractant protein-1 (MCP-1), and interferon-γ (IFN-γ) in the serum of women with endometriosis relative to infertile controls without laparoscopic evidence of endometriosis.[133] Among these, IL-6 was the most predictive biomarker. An algorithm including several of the same cytokines was found to perform better than any individual analyte in separating endometriosis and control subjects in a similar study.[134] Fassbender and colleagues used plasma proteomic analysis to identify five unique proteins in cases of endometriosis.[135] This unbiased strategy may be a fruitful approach to discover new biomarkers.

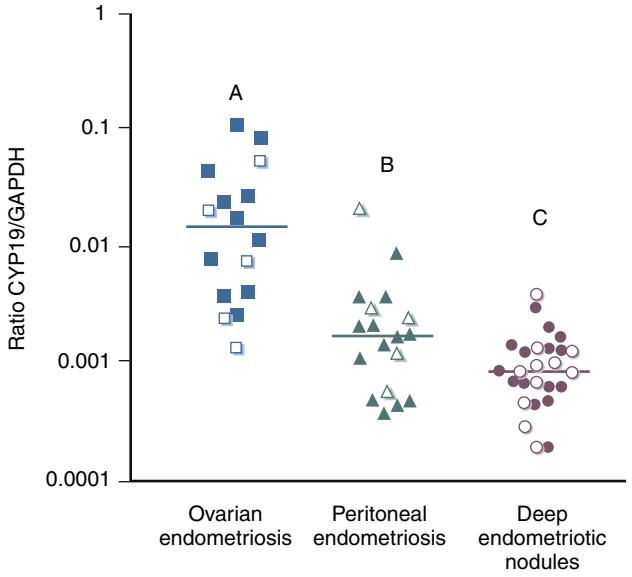


FIGURE 26.9 CYP19 (P450 aromatase) expression in ovarian endometriosis *(A)*, peritoneal endometriosis *(B)*, and deeply invasive endometriosis *(C)* of the rectovaginal septum. Data are expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Filled symbols represent patients under medical treatment; scatters with different letters are significantly different at a level of P = 0.05 (bar indicates median, note log scale along abscissa). *(From Heilier JF, Donnez O, Van Kerckhove V, et al. Expression of aromatase [P450 aromatase/CYP19] in peritoneal and ovarian endometriotic tissues and deep endometriotic [adenomyotic] nodules of the rectovaginal septum.* Fertil Steril *85:1516–1518, 2006.)*

Pathogenesis

Hormone Responsiveness (Steroids and Nuclear Receptors)

One of the defining characteristics of endometriosis is its endocrine responsiveness. Typically endometriosis only becomes symptomatic after the onset of menses[136] and, in the majority of cases, resolves spontaneously with menopause. An unusual case of persistent postmenopausal endometriosis led investigators to the discovery that aromatase expression and estradiol biosynthesis occurred within the endometriotic implant itself.[137] Aromatase activity is particularly high in ovarian endometrioma (Fig. 26.9).[138] Relative

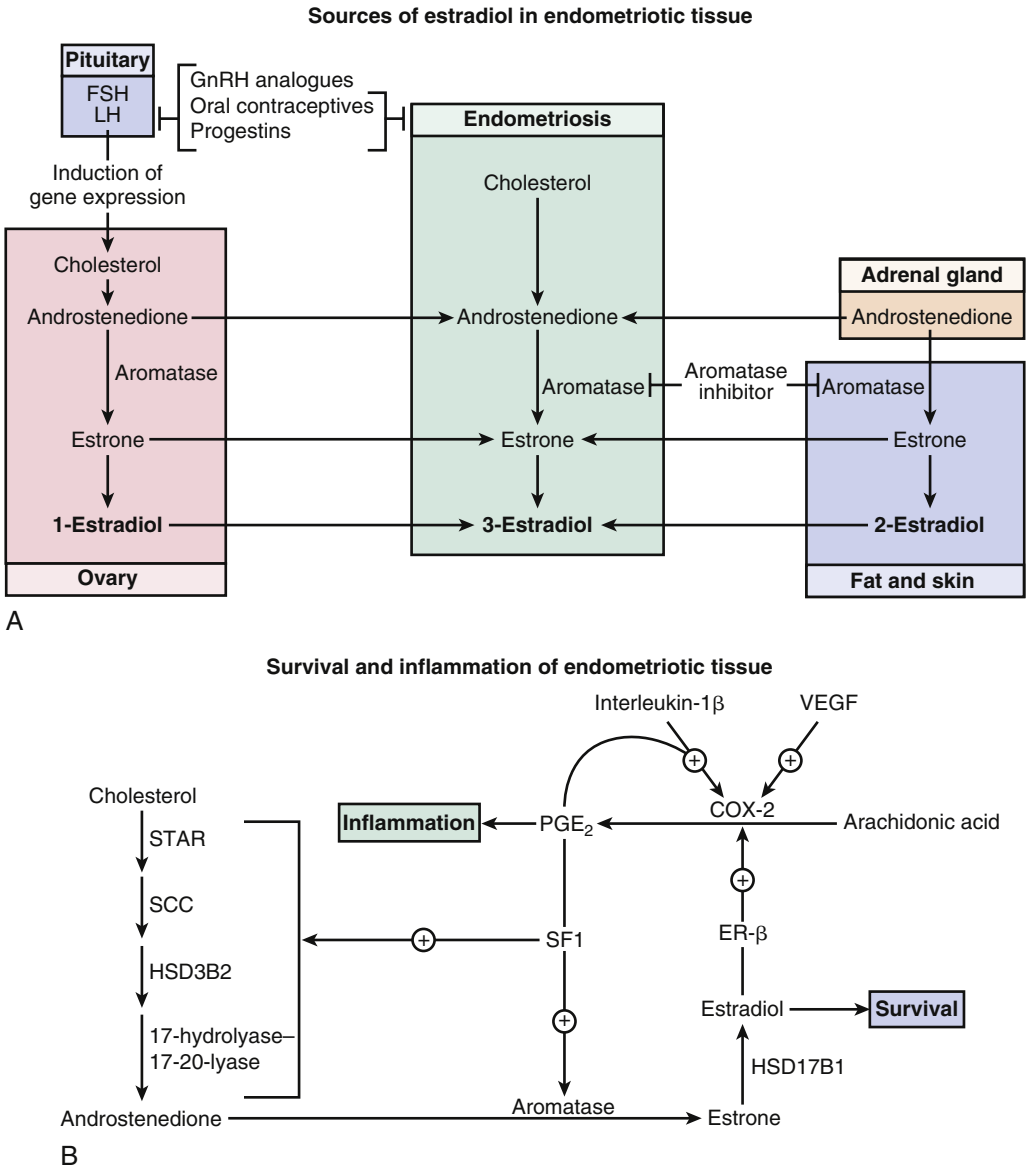


FIGURE 26.10 **A**, There are multiple sources of estradiol in endometriotic tissue. One source is via the pituitary follicle-stimulating hormone (FSH) and luteinizing horomone (LH), which induce ovarian biosynthesis of estradiol. The additional sources are from aromatase activity in fat, skin, and local production within endometriotic tissue. **B**, High levels of estradiol and prostaglandin E_2 (PGE_2) are produced by a feedforward loop. All the enzymes necessary for *de novo* steroid biosynthesis from cholesterol are expressed in stromal cells isolated from endometriotic implants. Among these, the steroid acute regulatory (StAR) protein, aromatase P450, and 17β-HSD type 1 appear to be the most highly regulated in this cell type. StAR and aromatase activities are modulated via the cAMP pathway and the transcription of these genes is activated by the aberrant expression of steroidogenic factor-1 (SF-1), resulting in estradiol synthesis and lesion proliferation. Prostaglandin synthesis is regulated by cyclooxygenase-2 (COX-2), which in turn is controlled by estradiol, cytokines, and vascular endothelial growth hormone (VEGF), contributing to inflammation and a feedforward effect on local estradiol production. *(Adapted from Bulun SE. Endometriosis.* New Engl J Med *360:268–279, 2009.)*

overexpression of the orphan nuclear receptor SF-1 in endometriotic cells appears to be a major reason for increased aromatase activity in these lesions.[139] Steroid biosynthesis and metabolism are clearly intrinsic activities within these lesions. Also identified in ectopic endometrial tissues were messenger RNA transcripts encoding steroid acute regulatory (StAR) protein,[140] which controls the delivery of cholesterol to the inner membrane of mitochondria, cholesterol P450 side chain cleavage (SCC) enzyme, 3β-hydroxysteroid dehydrogenase type 2, and 17α-hydroxylase. Further, oxidative metabolism of estradiol to the less biologically active estrone by 17β-hydroxysteroid dehydrogenase type 2 is decreased in endometriosis, aggravating local estrogen action.[141] Thus, endometriotic lesions appear to have co-opted the ability to generate their own estrogenic milieu (Fig. 26.10).

Like their derivative eutopic endometrium, endometriotic lesions express estrogen and progesterone receptors. Over the years, however, several authors have indicated that concentrations of these steroid receptor proteins may be altered in endometriotic implants.[142-144] In cultured stromal cells from these lesions, both estrogen[145] and progesterone receptor[82] isoform expression appear to differ from those

in normal endometrium. These differences may impart a growth advantage to endometriotic implant cells. Estrogen receptor β is highly expressed in endometriosis and is an emerging target for new therapeutics.[146] The roles of androgen action in endometriosis are as yet poorly defined; however, androgen receptors are expressed in normal endometrium and endometriosis lesions,[147] and clinical evidence supports the efficacy of danazol therapy for relief of pain associated with endometriosis.[148]

Lesion Establishment (Adhesion, Proteolysis, Invasion)

As discussed previously, the Sampson hypothesis[149] posits that viable endometrial cells spill into the peritoneal cavity apposing the peritoneal surface and adhere to the mesothelial monolayer. Although some controversy exists as to whether or not such invasion requires an *a priori* breach in the mesothelial surface[150] or if the cells are capable of invading intact mesothelium,[151] this is a necessary step in lesion establishment. The expression of hyaluronic acid (on peritoneal cells) and its receptor, cluster determination (CD)44 (on endometrial cells), have been proposed to play a role in this early attachment step.[152] Enhanced cytokine (e.g., IL-lβ, IL-6, and TNF-α) expression and action in the eutopic endometrium of women with endometriosis,[153] may predispose the adhesion of shed endometrial fragments in these cases. The ability of endometriotic cells to invade the peritoneal surface has stimulated extensive research into their cancer-like proteolytic properties.[154] The identification of specific matrix metalloproteinases (MMPs), which themselves are regulated by cytokines and ovarian steroid hormones, has been a particularly active area of investigation.[155,156] In the eutopic endometrium, these enzymes and their endogenous inhibitors (tissue inhibitor of metalloproteinases-[TIMPs]), are activated and repressed, respectively, following hormone withdrawal in the late luteal phase of the cycle. Implications of this work suggest that hormonal medications, particularly progestins, may inhibit invasive behavior. However, relative steroid hormone insensitivity and increased cytokine activity observed in endometriotic tissues may enhance MMP action in endometriosis, facilitating peritoneal invasion.

Proliferation and Apoptosis

A variety of human growth factors stimulate endometrial cell mitogenic activity. Many of these factors also have been identified in endometriotic tissues and in the peritoneal fluid of women with endometriosis. Although their precise roles in endometriosis are not well known, these factors are potential regulators of endometriosis implant proliferation. Table 26.2 summarizes the principal mitogens detected in the pelvic fluid of women with endometriosis and compares their concentrations with women without evidence of this disorder. The table provides reference sources for more detailed information.

Programmed cell death (apoptosis) is also an important mechanism for the cyclical remodeling of the endometrium.[157] Eutopic endometrial apoptosis was found to be decreased in women with endometriosis[158] and in endometriotic epithelium.[159,160] These findings indicate another potential selective advantage for the persistence of endometriotic lesions within the peritoneal cavity.[161]

Table 26.2 Cytokines and Growth Factors in Peritoneal Fluid

Concentrations Increased in Endometriosis	
Complement	Badawy et al, 1984[337]
Eotaxin	Hornung et al, 2000[338]
Glycodelin	Koninckx et al, 1992[339]
Interleukins (IL)	
IL-1	Anderson and Hill, 1987[340]
IL-6	Rier et al, 1994[341]
IL-8	Ryan et al, 1995[173]
Monocyte chemoattractant protein 1 (MCP-1)	Akoum et al, 1996[342]
Platelet-derived growth factor (PDGF)	Halme et al, 1988[343]
RANTES (regulated upon activation, normal T cell expressed and secreted)	Khorram et al, 1993[172]
Soluble intercellular adhesion molecule-1 (ICAM-1)	Daniel et al, 2000[207]
Transforming growth factor β (TGF-β)	Oosterlynck et al, 1994[184]
Vascular endothelial growth factor (VEGF)	Shifren et al, 1996[175]
Concentrations Unchanged in Endometriosis	
Epidermal growth factor (EGF)	De Leon et al, 1986[344]
Basic fibroblast growth factor (FGF)	Huang et al, 1996[345]
Interferon-γ (IFN-γ),	Khorram et al, 1996[172]
IL-2	Keenan et al, 1995[346]
IL-4	Gazvani et al, 2001[347]
IL-12	Mazzeo et al, 1998[348]
Concentrations Decreased in Endometriosis	
IL-13	McLaren et al, 1997[213]
IL-1RA	Zhang et al, 2007[214]

Angiogenic Factors

Given the ubiquity of retrograde menstruation, it is not clear why endometriosis only develops in a subset of women. Using the analogy of tumor metastasis[162,163] some authors have postulated that the angiogenic potential of the intraperitoneal environment may predict the likelihood that lesions become established.[164,165] Indeed, on visualization, endometriotic implants are often surrounded by exaggerated vascularity (Fig. 26.11); extrapelvic endometriosis, while rare, typically occurs in well-vascularized sites.[166] This concept is consistent with other analogous aspects of endometriosis pathogenesis and cancer biology. In addition to increased angiogenesis, endometriotic lesions manifest enhanced proliferative, adhesive, and invasive capacity; more LoH and genomic instability; and decreased apoptosis.

The sprouting of new blood vessels from preexisting vessels is complex and includes the proteolytic degradation of extracellular matrix, proliferation and migration of endothelial cells, and ultimately the formation of patent capillary tubules supplying the angiogenic stimulus. Many different growth factors and cytokines have been shown to exert chemotactic, mitogenic, or inhibitory activities on endothelial cells, smooth muscle cells, and pericytes. Increased IL-1β sensitivity increases the angiogenic potential of endometriosis lesions.[167] An attractive interpretation of this finding (assuming the Sampson hypothesis is accepted) is that

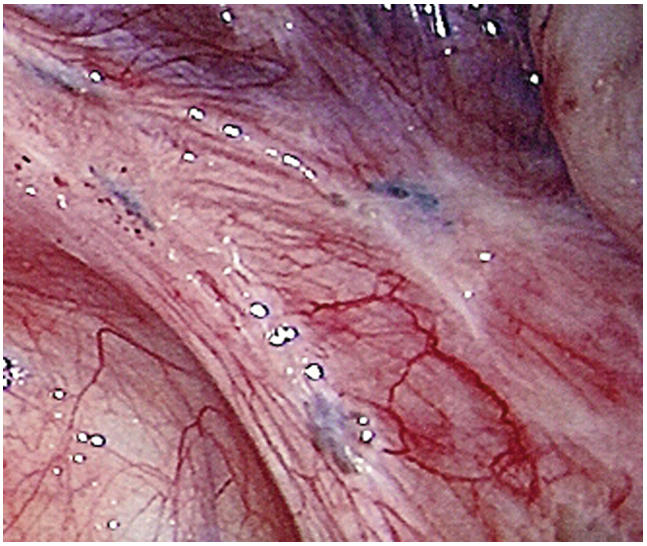


FIGURE 26.11 Laparoscopic photograph of endometriotic implants on the peritoneum overlying the uterosacral ligament. *(From Taylor RN, Yu J, Torres PB, et al. Mechanistic and therapeutic implications of angiogenesis in endometriosis.* Reprod Sci *16:140–146, 2009.)*

the endometrial lining of women destined to develop endometriosis already manifests an "activated" angiogenic phenotype. As an analogy to Knudson's "two-hit" hypothesis of oncogenesis,[168] women susceptible to the development of endometriosis may carry a "first hit" genetic trait that enhances endometrial cell angiogenic factor production. This proposal is in keeping with the familial trends observed in cases of endometriosis.[49,169,170] Angiogenic activity within advanced stage endometriomas appears to be particularly robust.[171]

Although pelvic fluid is a complex medium containing and influenced by multiple cell types, we and other investigators have found this to be a useful biological medium to define intercellular mediators of the local reactions associated with endometriosis.[172-174] Peritoneal fluid seems to play an important role in the process of neovascularization. Oosterlynck and colleagues[174] used the chick chorioallantoic membrane bioassay to show that pelvic fluid from women with endometriosis had more angiogenic activity than pelvic fluid from healthy control subjects. We confirmed this observation using an *in vitro* model of human endothelial cell proliferation. Thymidine incorporation into nascent cellular DNA demonstrated that peritoneal fluid from women with endometriosis contains substantially more angiogenic activity than pelvic fluid from normal control subjects.[164]

A vast array of angiogenic principles are synthesized by human endometrium and endometriosis. Among these are basic fibroblast growth factor, IL-6, IL-8, macrophage migration inhibitory factor, platelet-derived growth factor, prokineticin 1, and vascular endothelial growth factor (VEGF), which is the most potent of these (Table 26.2). We initially reported that endometrial VEGF levels were expressed highest in the mid-secretory phase of the cycle, a time when estradiol and progesterone levels were elevated, and confirmed VEGF expression in endometriotic implants.[175] Other studies using immunohistochemistry and endometrial cultures corroborate these findings.[176] These two ovarian steroid hormones have direct transcriptional effects on the human VEGF gene promoter.[177,178] VEGF, IL-6, and IL-8 mRNA were noted to be substantially up-regulated in endometriomas in relation to normal ovarian tissue.[179,180] In totality, these findings suggest that the local expression of angiogenic cytokines in endometriosis implants contributes to implant neovascularization and may correlate with symptoms.[111]

Fibrosis

Biochemical principles secreted by endometriotic lesions (perhaps in response to apoptosis) or their neighboring cells may contribute to the fibrosis and adhesions that classically surround the implants and adjoining pelvic organs.[181,182] Indeed, TGF-β, a growth factor associated with other types of intraperitoneal adhesion formation,[183] was noted to be elevated in peritoneal fluid of women with endometriosis.[184]

Oxidative Stress and Endometriosis

The role of oxygen free radicals as mediators of the cellular pathology associated with endometriosis has been postulated in recent years. *Free radicals* are highly reactive molecules that transiently contain one or two unpaired electrons. The transfer of electrons to other structural or regulatory molecules can induce cell damage. Most free radicals of biological significance are referred to as *reactive oxygen species* (ROS), such as hydroxyl (HO•), peroxyl (ROO•), or superoxide ($O2^{\bullet -}$) radicals. Oxidative stress implies a homeostatic deviation, wherein the pro-oxidant environment exceeds the defensive ability of antioxidant vitamins and enzymes in a physiological system. Endometrial stromal cells *in vitro* have a mitogenic response to oxidative stress[185] and free radicals may contribute to lesion proliferation and invasion.

Murphy and colleagues found that peritoneal fluid levels of the antioxidant vitamin E were decreased and the ratio of lysophosphatidylcholine to phosphatidylcholine increased in women with endometriosis.[186] These findings were interpreted to reflect increased lipid peroxidation in endometriosis. However, attempts by others to directly assay lipid peroxidation by measurements of malondialdehyde[187] or 2,2′-azino-di-3-ethylbenzthiazoline sulfonate reduction[188] failed to detect differences between endometriosis subjects and control subjects. Nevertheless, this novel hypothesis is an excellent focus of further investigation.[189]

Inflammation (Immune Cells, Chemokines, Cytokines, Prostaglandins)

The accumulation of immune cells within the peritoneal cavity and endometriotic lesions has been recognized in women with this disorder for over 15 years.[190-192] This phenomenon is thought to be initiated via the innate immune system, which is not antigen-specific and has no memory. Granulocytes, natural killer (NK) cells, and macrophages are the major cells providing this type of defense and they are recruited into tissues via the expression of soluble chemoattractant proteins referred to as *chemokines* (Fig. 26.12), demonstrates the elaborate interplay between the nervous, endocrine, and immune systems. Given the predominant monocytic inflammatory response in endometriosis, chemokines specific for this cell lineage have been

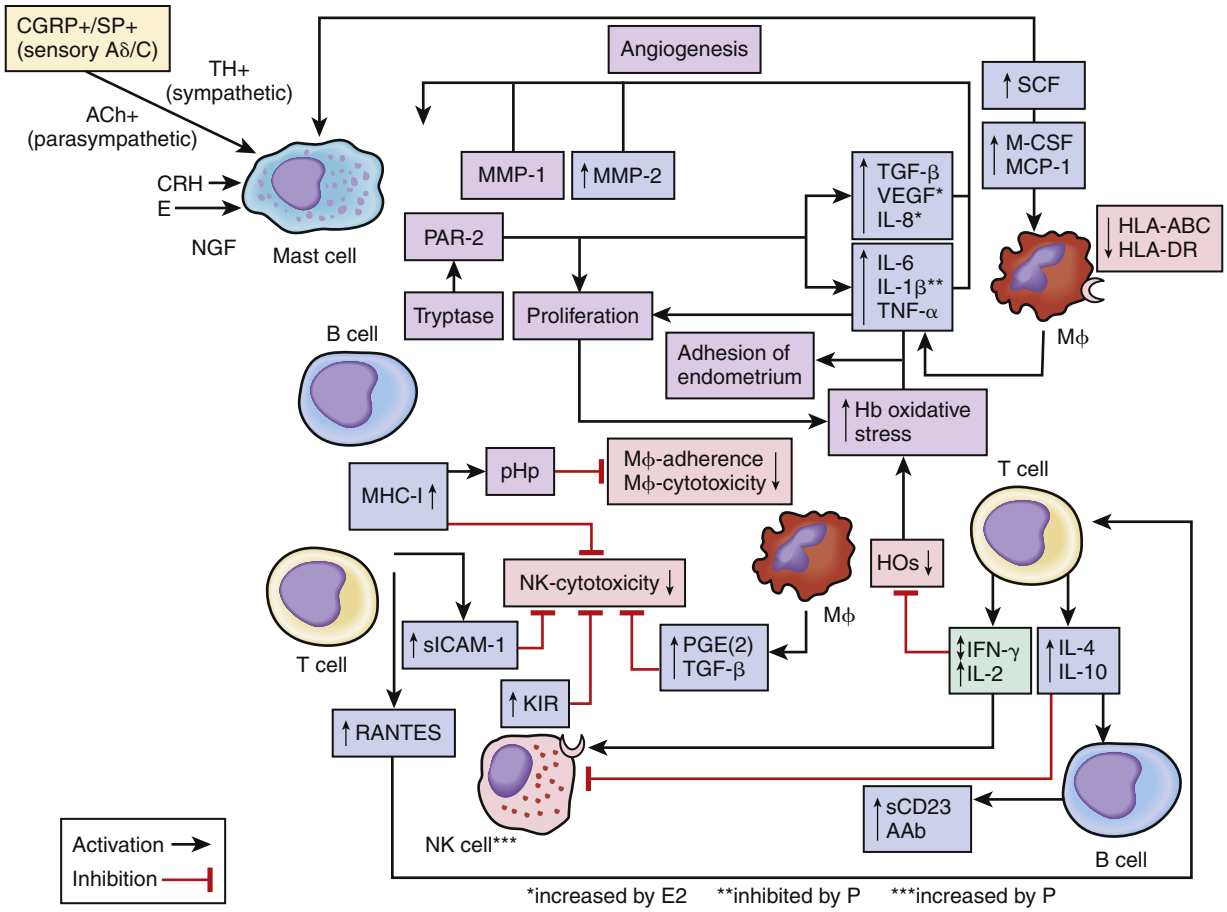


FIGURE 26.12 Neuroendocrine-immune disequilibrium in endometriosis. In endometriosis, elevated levels of M-CSF, MCP-1, RANTES, and SCF in peritoneal fluid may lead to increased recruitment of macrophages, T cells, and mast cells. Although markers of antigen presentation on macrophages such as HLA-ABC and HLA-DR are decreased in endometriosis, macrophage-derived IL-6, IL-1β, TNF-α, TGF-β, VEGF, and IL-8 are increased in peritoneal fluid, together with MMP-1 and MMP-2, stimulating angiogenesis. IL-6, IL-1β, and TNF-α support adhesion of endometrial cells to the peritoneum, and TNF-α stimulates the proliferation of ectopic tissue, resulting in high levels of Hb. T cell-derived IL-2 and IFN-γ decrease HO, leading to oxidative stress. IFN-γ has been inconsistently described as increased or decreased. Increased T-cell-derived IL-4 and IL-10 inhibit cellular immunity and stimulate B cells to AAb production. sCD23 is increased in peritoneal fluid in endometriosis and might derive from activated B cells. Lymphocytes are increased in peritoneal fluid and abundantly present in ectopic tissue. Decreased NK cell cytotoxicity might be due to high anti-inflammatory T cytokines, increased KIR, high macrophage-derived PGE_2 and TGF-β, high MHC-I expression on ectopic cells, and high sICAM-1 levels in peritoneal fluid. Nerve fibers found within lesions are positive for CGRP, SP, TH, and Ach, and NGF and CRH were demonstrated. CRH and accumulated E can activate mast cells to release tryptase, activating PAR-2, which leads to increased secretion of VEGF, IL-8, and IL-6 and proliferation of ectopic tissue. pHp, expressed by ectopic tissue, decreases adherence and, in stage III and IV, cytotoxicity of peritoneal macrophages. E2 further increases RANTES, IL-8, and VEGF, whereas P inhibits IL-1β secretion from peritoneal macrophages and increases NK cell numbers. AAb, autoantibodies; Ach, acetylcholine; CGRP, calcitonin-gene-related peptide; CRH, corticotropin-releasing hormone; E, estrogen; E2, estradiol; Hb, hemoglobin; HO, heme oxygenases; IL, interleukin; HLA, human leukocyte antigen; IFN-γ, interferon-γ; KIR, killer cell inhibitory receptor; M-CSF, macrophage colony stimulating factor; MCP-1, monocyte chemotactic protein-1; MHC-I, major histocompatibility complex class-I; MMP, matrix metalloproteinase; NGF, nerve growth factor; NK, natural killer; P, progesterone; PAR-2, protease-activated receptor-2; PGE_2, prostaglandin E_2; pHp, peritoneal haptoglobin; RANTES, regulated upon activation normal T cell expressed and secreted; sCD23, soluble CD23;, SCF, stem cell factor; sICAM-1, soluble intercellular adhesion molecule-1; SP, substance P; TGF-β, transforming growth factor-β; TH, tyrosine hydroxylase; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor. *(Adapted from Tariverdian N, Theoharides TC, Siedentopf F, et al. Neuroendocrine-immune disequilibrium and endometriosis: an interdisciplinary approach.* Semin Immunopathol *29:193–210. 2007.)*

evaluated and identified in peritoneal fluid and endometriotic lesions. In particular, IL-8, MCP-1, and regulated upon activation normal T cell expressed and secreted (RANTES) have been studied intensively. Specific references to these important chemokines are provided in Table 26.2, and the general topic of peritoneal chemokines has been reviewed in recent literature in detail.[193]

We have postulated that the accumulation of peritoneal macrophages initiates a rather complex network of chemokines, cytokines, and growth factors that contribute to the pathophysiology of endometriosis.[194] This hypothesis is summarized in Figure 26.13. The activation of autoregulatory loops may contribute to a feedforward amplification of local inflammation within endometriotic implants.

Adaptive immunity, by contrast, refers to that "learned" by experience and induces an enhanced anamnestic response with repeated antigen exposure. Thymus and bursa-dervied (T and B) lymphocytes and antibody-producing plasma cells

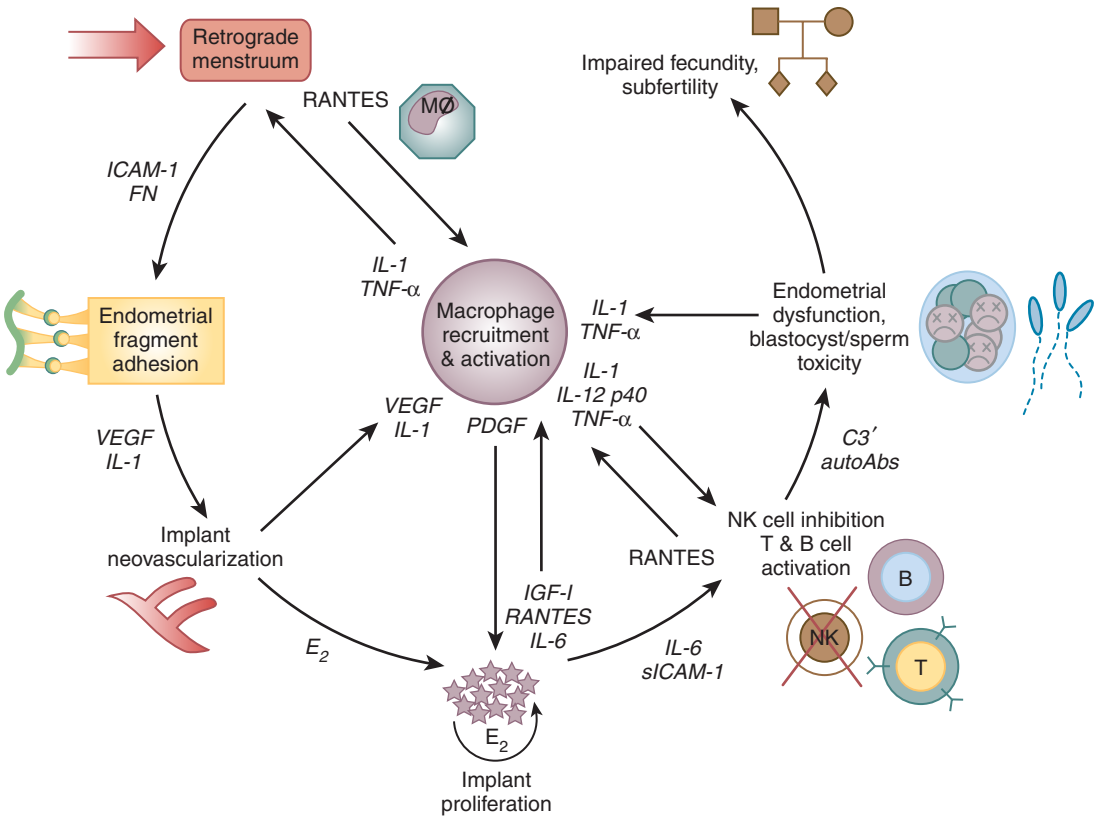


FIGURE 26.13 Schematogram depicting the network of chemokines, cytokines, and growth factors in the pathophysiology of endometriosis. autoAbs, autoantibodies; C3′, complement 3; E_2, estradiol; FN, fibronectin; sICAM, soluble intercellular adhesion molecule; IGF-1, insulin-like growth factor-1; IL, interleukin; MØ, macrophage; NK cell, natural killer cell; PDGF, platelet-derived growth factor; RANTES, regulated on activation, normal T cell expressed and secreted; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

are the primary cell types providing adaptive immunity. Weed and Arquembourg[195] first observed immunoglobulin G (IgG) and complement deposits in the eutopic endometrium of women with endometriosis and proposed that development of endometrial autoantibodies might explain poor reproductive outcomes in some women with this syndrome. Elevated levels of complement component C3 also were noted in peritoneal fluid of women with endometriosis.[337] Increased concentrations of T cells were observed in baboons with spontaneous endometriosis but not in those with surgically induced disease. This observation led the authors to suggest that an underlying immunocyte abnormality might precede, rather than reflect a response to, intraperitoneal endometrial implants.[196]

In the 1980s, serum of women with endometriosis was used to identify endometrial autoantigens by Western blotting methods. Similar findings were confirmed with enzyme-linked immunosorption assays against solubilized endometrial antigens and endometrial cell monolayers.[197] It has been suggested that a generalized adaptive immune response, manifested by polyclonal B-cell activation, occurs in some cases of endometriosis.[198]

Leukocytes recruited and activated by endometriosis-derived chemokines secrete a variety of soluble inflammatory mediators. Particularly potent among these are proinflammatory cytokines (reviewed in Table 26.2) and prostaglandins. These mediators also have intrinsic vasoactive and pain-activating properties and are thought to lie along a critical common pathway involved in the symptoms of endometriosis.[194]

Although no clear associations were found in early genotyping studies assessing the major histocompatibility type I and II loci human leukocyte antigen (HLA)-A, -B, and -DR),[199,200] more recent immunohistochemical analyses of tissues from women with endometriosis suggest an increased expression of HLA-DR antigen in endometrial and endometriotic epithelial cells than what is seen in other infertile control subjects.[201] Moreover, the coincidence of surgically confirmed endometriosis with other autoimmune disorders, including systemic lupus erythematosus, Sjögren syndrome, rheumatoid arthritis, and multiple sclerosis, was documented in a self-reported, cross-sectional survey of more than 3500 members of the North American Endometriosis Association.[202] In a recent histochemical and microarray study of ovarian endometriosis, macrophages, plasma cells, complement proteins, and a B-lymphocyte stimulating cytokine (BLyS), all were found to be markedly upregulated relative to matched eutopic endometrium. These same markers are elevated in other autoimmune diseases, including systemic lupus erythematosus, Sjögren syndrome, and rheumatoid arthritis.[203]

Immunosurveillance

An intriguing concept in the etiology of endometriosis is defective immunosurveillance in susceptible women. If

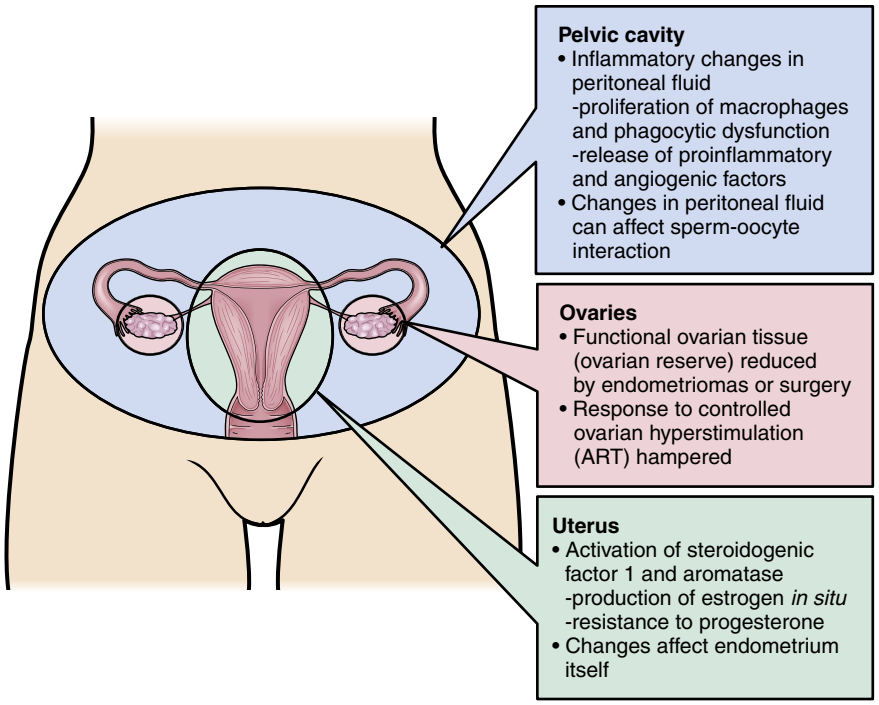


FIGURE 26.14 Mechanisms of endometriosis-associated infertility. ART, assisted reproductive technologies. *(Adapted from de Ziegler D, Borghese B, Chapron C. Endometriosis and infertility: pathophysiology and management.* Lancet *376:730–738, 2010.)*

normal eutopic endometrium elicited an immune response, antiendometrial autoantibodies would be prevalent in all women rather than a subset of cases with endometriosis.[204] Hence, adaptive immunity toward these cells seems to occur as a result of their ectopic location. As an overwhelming majority of cycling women regurgitate menstrual endometrium into the peritoneal cavity, why does endometriosis occur in only 10% to 15% of these individuals?

In normal women, intraperitoneal menstrual debris appears to be eliminated without loss of immune tolerance, presumably mediated by $CD8^+$ T cells and immunosuppressive cytokines (e.g., IL-13, IL-1RA). However, as noted in other autoimmune disorders, immune tolerance might break down in endometriosis, resulting in the recognition of ectopic implants by a chronic inflammatory response. Induction of so-called "blocking antibodies" or tolerance to endometrial antigens[205] have been postulated but never confirmed. Nevertheless, defects in NK cell activity and reduced production of immunosuppressive cytokines have been reported in cases of endometriosis. For example, ICAM-1 may interfere with peritoneal immunosurveillance and allow refluxed endometrium to escape clearance from the peritoneal cavity.[206] Increased concentrations of this protein have been noted in pelvic fluid of women with endometriosis.[207] Endometrial cells also are inherently resistant to apoptosis and phagocytosis,[208] yet endometriosis only occurs in a minority of menstruating women, as previously noted. Some researchers have suggested that mediators of the innate immune system, particularly macrophages and NK cells, clear regurgitated endometrial cells from the peritoneal cavity. Oosterlynck and colleagues first described decreased NK cell cytotoxicity against endometrial and hematopoietic cells in women with endometriosis.[209] The same group showed that peritoneal fluid from women with endometriosis contained more soluble NK cell suppressive activity than peritoneal fluid from fertile control subjects. In addition, the phagocytic ability of endometriotic peritoneal macrophages is reduced by suppressed expression of the scavenger receptor CD36.[210]

Hornung and colleagues postulated that expression or secretion of the non-classical major histocompatibility antigen HLA-G by endometriotic cells might inhibit NK cell function in this setting, but were unable to detect this protein in ectopic or eutopic endometrial stromal cells or tissues by Western blotting, and no soluble HLA-G protein was detectable in peritoneal fluid.[211] However, using *in situ* methods, Barrier and colleagues observed HLA-G in endometriotic glands.[212] Interestingly, reduced expression of other anti-inflammatory cytokines (i.e., IL-13 and IL-1RA) was reported in the peritoneal fluid of women with endometriosis.[213,214]

Possible Mechanisms of Endometriosis-Associated Infertility

How mild to moderate endometriosis reduces fertility is among the most controversial aspects of research in this field (Fig. 26.14). A case-control study found a significantly lower serum level of the ovarian reserve marker anti-müllerian hormone (AMH) in 87 women with moderate-to-severe endometriosis compared to 306 without the disease.[215] In addition to mechanical effects of lesions and adhesions on the adnexa, evidence supports the hypothesis that endometriosis causes an immunologically hostile environment within the pelvis. Evidence from oocyte donor programs indicates that egg or early embryonic development are disturbed in women with endometriosis[216,217] and specific chemokine concentrations are elevated in the follicular fluid of affected women undergoing *in vitro* fertilization (IVF).[218] The resolution of this critical issue will advance our understanding of endometriosis-associated infertility and should pave the way to improved therapies.

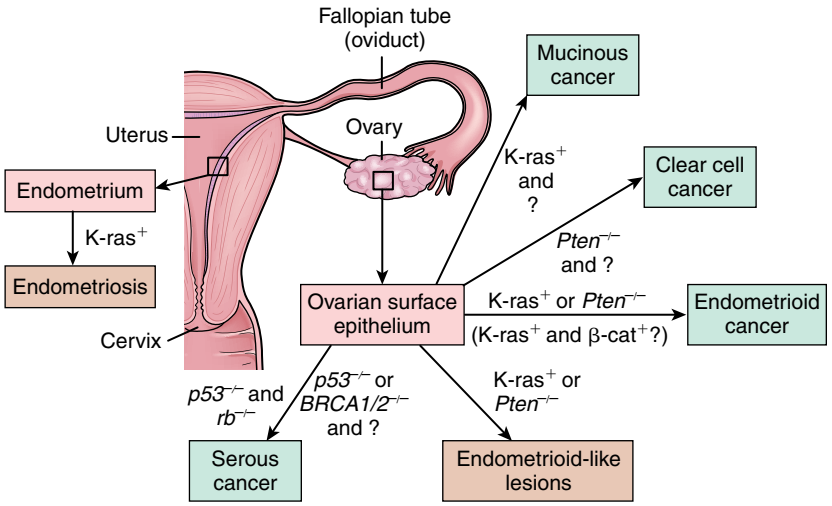


FIGURE 26.15 Proposed genetic alterations in the development of endometriosis and epithelial ovarian cancers. *Pink rectangle*, normal tissue; *brown rectangle*, benign disease; *green rectangle*, cancerous lesions; +, activating mutations in oncogenes; –/–, inactivating mutations in tumor suppressor genes; ?, indicates that the second genetic hit is unknown. *(Adapted from Matzuk MM. Gynecologic diseases get their genes.* Nat Med *11:24–26, 2005.)*

In general, inflammatory responses in the vicinity of the ectopic endometrial implants are thought to impair sperm transport,[219] tubal motility,[220] and oocyte development.[221] *In situ* menstruation within endometriotic lesions has been proposed to cause adhesion formation, fibrosis, and proximal tubal obstruction.[222,223] Moreover, the eutopic endometrium of women with endometriosis manifests a variety of histological and biochemical perturbations, including increased immune cell infiltration[224] and increased cytokine and chemokine expression.[153,225,226] Increased concentrations of complement component C3,[162] E-cadherin, total β-catenin and decreased αvβ3 integrin,[227] glycodelin,[228] HOXA10, and HOXA11[84] have been reported. Based on our current knowledge of their functions, dysregulation of these gene products would be predicted to cause impaired implantation. Altered αvβ3 integrin expression may be corrected by aromatase inhibitor therapy.[229]

High peritoneal fluid concentrations of prostaglandins have been noted by some authors but not by others. As these are rapidly acting autocrine and paracrine substances, circulating levels may not be revealing. Prostanoids offer an attractive mechanism for endometriosis-associated pain and also may affect fertility via interference with tubal motility and ovum pick-up.[230] When sperm were incubated with peritoneal fluid from healthy controls compared to those with endometriosis the latter samples significantly induced sperm DNA fragmentation.[231] There is some evidence that women with endometriosis have abnormal[17] or suppressed uterine peristalsis which may have a negative impact on sperm or oocyte transport.[232]

Some data indicate that the spontaneous abortion rate is increased in women with endometriosis,[233] although this finding has been disputed in other studies.[234] In a meta-analysis of IVF outcomes, women with advanced endometriosis had substantially lower fertilization and implantation rates compared with other indications.[235] These observations are discussed in further detail later in this chapter.

Endometriosis and Genital Tract Carcinoma

Endometriosis is identified concurrently in approximately 17% of ovarian carcinoma cases. In a recent pooled analysis of case-control studies involving more than 23,000 participants (7911 with ovarian cancer) a history of endometriosis was associated with a 1.5% life-time risk of developing ovarian cancer (endometrioid, clear-cell, and low-grade serous).[236] A murine model that combined an oncogenic Kirsten rat sarcoma *(K-ras)* allele activation with a tissue-specific conditional knock-out of the tumor suppressor phosphatase and tensin *(Pten)* resulted in aggressive and widely metastatic endometrioid ovarian adenocarcinoma, recapitulating the histomorphological appearance seen in the human disease (Fig. 26.15).[237] Using an intact immunocompetent murine model of endometriosis, Cheng and colleagues were able to activate *K-ras* in donor endometrial tissue to promote endometriotic lesion development.[238] Recently, *K-ras* variant alleles were identified in 31% of endometriosis cases, compared to 5% of controls.[239]

A rare but serious complication of cervical endometriosis is the misclassification of cervicovaginal cytologic smears as harboring a high-grade intraepithelial lesion, atypical glandular cells of undetermined significance, or even an adenocarcinoma *in situ*.[240]

Treatment

Surgical Therapy for Pain Associated with Endometriosis

Owing to their peritoneal location, most endometriotic lesions are amenable to laparoscopic extirpation. Techniques involving aggressive implant excision, electrofulguration, or laser vaporization have been adapted through endoscopic ports and these procedures have been taught widely to gynecological surgeons. It is relatively rare that laparotomy or hysterectomy is necessary in the treatment of women who wish to preserve their uterus.

One of the first evidence-based evaluations of laparoscopic treatment of endometriosis-associated pelvic pain was carried out in the United Kingdom in the early 1990s. Sutton and colleagues conducted a randomized, prospective, double-blind trial in 63 patients with minimal to moderate endometriosis. Half of the subjects underwent laser ablation of endometriotic deposits and laparoscopic uterine nerve ablation, and the others received a diagnostic laparoscopy

and expectant management. Pain symptoms quantified by a visual analog scale were significantly improved in 62.5% of those receiving laser ablation, compared with 22.6% in the expectant group at 6 months after surgery.[241] In a long-term follow-up study of the laser-treated cohort (approximately 72 months after surgery), continued symptom relief was reported in 55.3% of the respondents.[242] Laparoscopic uterosacral nerve ablation does not add any further relief of pain beyond ablative surgery alone.[243,244] In a randomized, blinded, crossover pain study, Abbott and colleagues demonstrated an 80% success rate in excisional endometriosis surgery compared to an approximately 30% response to sham surgery. The effects were independent of extent of disease.[106] These reports substantiate a therapeutic role for laser laparoscopy in the management of endometriosis pain and indicate that the median time to pain recurrence after surgical treatment was 20 months. Future innovations in endoscopic surgery for endometriosis may include targeted energy absorption, such as photosensitization of the lesions using hematoporphyrin derivatives[245] or autofluorescence to detect nonpigmented endometriotic lesions.[246] Tissue staining with either indigo carmine or methylene blue at the time of laparoscopy can help accentuate the identification of subtle lesions.[247]

Treatment of ovarian endometriomas (chocolate cysts) can be particularly challenging. These respond very poorly to medical therapy, and hence, operative extirpation is the preferred approach. Jones and colleagues treated 73 consecutive cases of endometrioma larger than 2 cm by laser or bipolar diathermy. After 1 year, 16.4% of subjects had recurrences. This prognosis was noted to be particularly more likely if the cysts were bilateral.[248] According to a recent Cochrane Review, excision was better than drainage and ablation for (1) recurrence of the endometrioma, (2) recurrence of symptoms, and (3) fertility outcome.[249] Recurrence after surgical intervention can be decreased with the use of postoperative suppressive medical therapy such as hormonal contraceptives.[250]

Definitive treatment for endometriosis consisting of a hysterectomy with removal of both ovaries leads to the dilemma of postoperative hormonal therapy (for hypo-estrogenemia) in the face of exacerbating residual endometriosis.[251] The recurrence rate with hormonal therapy is 3.5% or 0.9% per year and there is no greater risk of recurrence of pain if estrogen is started immediately after surgery compared with a 6-month delay.[252] If hysterectomy with ovarian cryopreservation has been performed in a premenopausal woman, no postoperative suppressive medical therapy is warranted as the recurrence rate is small.[253]

Medical Therapy for Pain Associated with Endometriosis

Hormonal treatments approved for endometriosis treatment by the U.S. Food and Drug Administration (FDA) include gonadotropin-releasing hormone analogue (GnRHa), depot medroxyprogesterone acetate, and the androgen danazol. In addition, continuous oral contraceptives have been evaluated and are widely used.[254] An alternate progestin approved for endometriosis use in Europe, dienogest, is actually a component of the FDA-approved oral contraceptive Natazia™.[255] When used continuously following surgery for endometriomas, oral contraceptives significantly reduced recurrence when compared to nonusers.[256,257] The majority of evidence supporting medical therapy for endometriosis is observational; however, some randomized clinical trials have been evaluated.[258] An evidence-based assessment of treatment of endometriosis-associated pelvic pain in two randomized, placebo-controlled trials indicated that medroxyprogesterone acetate, danazol, or GnRHa were all more effective than placebo. Pain relief of more than 6 months' duration was noted in 40% to 70% of women.[259]

Although highly effective in the management of chronic pain with endometriosis, GnRHa treatment has been limited to 6 months by the FDA because of the hypoestrogenic effects induced by ovarian suppression. The previously mentioned principle of an estrogen threshold (Fig. 26.4)[260] has been used to overcome this limitation. Suppression of the hypothalamic-pituitary axis with GnRHa is now accompanied with "add-back" of exogenous ovarian steroids. Dose-seeking trials have identified "add-back" regimens that reverse vasomotor symptoms and bone mineral loss. Add-back regimens consisting of norethindrone acetate combined with low-dose estrogen can safely extend pain relief and bone preservation for at least 1 year, and one trial with a limited number of participants found no ill effect after 10 years of add-back therapy.[261,262] Three months of the nasal spray formulation of GnRHa seemed to be as effective as a 6-month course.[263] Another option is subcutaneous depot medroxy-progesterone. Two randomized, clinical trials demonstrated that the 6 months of subcutaneous progestin was as effective as leuprolide acetate in diminishing endometriosis-associated pain.[264,265] Drop-out rates were significant for both groups, however. The purported benefits of the subcutaneous progestin are decreased cost, easier administration, and more favorable effect on bone mineral density.

In a double-blind, placebo-controlled, crossover study, a nonsteroidal anti-inflammatory drug (naproxen) was twice as effective as placebo in relief of secondary dysmenorrhea associated with endometriosis.[266] High levels of cyclooxygenase-2 (COX-2) are expressed in endometriotic lesions,[267] implying that selective COX-2 inhibitors might also be of clinical benefit. Although this class of drugs is effective in the treatment of primary dysmenorrhea,[268] the limited data available from endometriosis trials were inconclusive.[269]

Experimental Medical Therapy

Like COX-2 inhibitors, a variety of evolving new medical treatments hold theoretical promise for the treatment of endometriosis-associated pain and inflammation. GnRH antagonists, which induce an immediate suppression of gonadotropin production without the initial "flare" effect of the GnRH superagonists,[270] may be advantageous in this setting.[271] Selective estrogen receptor modulators (SERMs) also may be of theoretical benefit. Due to its partial agonist activity within the endometrium, tamoxifen can exacerbate endometriosis.[272] Although raloxifene was shown to reduce endometriosis in murine and rhesus models of this disease,[273] a preliminary clinical trial unexpectedly showed exacerbation of this SERM on pain symptoms.[274] Through their effects on estrogen production and action, aromatase inhibitors[137] have been proposed as new therapeutics in endometriosis.[275] Aromatase inhibitors have been proposed

Table 26.3 Effects of Medicinal Herbs Used for Treatment of Endometriosis

Medicinal Herbs	Anti-Proliferative	Anti-Nociceptive	Anti-Oxidant	COX-2↓	Cytokines↓	NF-κB↓
Bupleurum	+				+	+
Chinese angelica	+	+	+		+	
Dahurian angelica	+	+		+	+	+
Cattail pollen					+	
Cinnamon twigs		+		+		
Cnidium fruit	+	+				
Corydalis		+			+	
Curcuma	+	+	+	+	+	+
Cyperus	+		+			
Frankincense	+	+			+	+
Licorice root	+	+		+	+	+
Myrrh		+	+		+	
Persica					+	
Poria	+	+		+	+	
Red peony root			+			
Rhubarb	+	+			+	+
Salvia root		+	+			+
Scutellaria	+			+	+	
Sparganium		+			+	
Tortoise shell						+
White peony root				+	+	

From Wieser F, Cohen M, Gaeddert A, et al. Evolution of medical treatment for endometriosis: back to the roots? Hum Reprod Update 13:487-499, 2007.

as new therapeutics in endometriosis.[276] Acceptance must await larger clinical trials. Although there is no randomized controlled trial, an early open-label, nonrandomized proof-of-concept study suggests a benefit in the treatment of rectovaginal endometriosis.[277] Endometriosis in this anatomical location also expresses the aromatase enzyme (Fig. 26.9).[138]

Progesterone receptor modulators have also been investigated.[278] Early experiences with high and low doses of the antiprogestin mifepristone showed an improvement in pelvic pain without ostensible regression of peritoneal implants.[279,280] The antiglucocorticoid effects of this class of compounds, and their ability to create a hyperestrogenic endometrial milieu,[281] are likely to limit clinical applicability. Interesting recent *in vitro* studies suggest that part of the effectiveness of mifepristone might be due to its antioxidant properties.[282] Ligands for another nuclear receptor, peroxisome proliferator-activated receptor (PPAR)-γ, were shown to reduce RANTES and IL-8 secretion and the proliferation of endometriotic stromal cells; inhibit macrophage activation and migration in *in vitro* and murine models of endometriosis[283-285]; and to reduce endometriotic lesion volume in rat and baboon models of the disease.[286-289]

Therapeutic agents that target cytokine production and action also are under investigation as possible clinical adjuncts for endometriosis treatment. Using a rat model of endometriosis there was efficacy shown with imiquimod (toll-like receptor agonist), recombinant human INF-α-2b, leflunomide, thiazolidinedione, and levamisole.[288,290-292] Administration of thiazolidediones or recombinant TNF-binding protein-1 or anti-TNF-α monoclonal antibody c5N inhibited the development of endometriosis in a baboon model[293-295] and blockade of VEGF action in mouse endometrium can be achieved using soluble VEGF-R1 (sflt-1).[296] Future applications of the same strategies may possibly be useful in clinical endometriosis.

A novel approach to the treatment of pain associated with deep, rectovaginal endometriosis was introduced[297] in a prospective, self-controlled trial of 11 symptomatic patients with rectovaginal endometriosis. Use of a levonorgestrel-releasing intrauterine device for 12 months resulted in significant improvement in dysmenorrhea, pelvic pain, and deep dyspareunia, and objective resolution of the size of rectovaginal endometriotic lesions by ultrasonography was noted. Another trial found similar pain relief after 6 months of therapy comparing the levonorgestrel intrauterine device with GnRHa.[298] Other agents that may have some potential if confirmed in larger clinical trials include, etonogestrel nonbiodegradable contraceptive implant, bufalin, BAY 11-7085, (a soluble inhibitor of NK-κB activation), dopamine agonists, French maritime pine bark extract, yiweining, rapamycin, metformin, oral gonadotropin releasing hormone antagonist, human chorionic gonadotropins, doxycycline, melatonin, genistein, atorvastatin, trichostatin A, and vaginal danazol.[299-310]

Evidence is accumulating to suggest that medicinal botanicals with anti-inflammatory and pain-alleviating properties hold promise for treatment of endometriosis.[311] Although their safety remains untested formally, in general these additives are not thought to impair ovulation or fertilization and hence may be taken by women attempting conception. Medicinal herbs and their active components exhibit cytokine-suppressive, COX-2-inhibiting, antioxidant, sedative, and pain-alleviating properties, which would be predicted to have salutary effects in endometriosis. Although controlled, randomized clinical trials are rare, phytotherapies with a long tradition of safe use (e.g., curcumin and its analogs) have multiple beneficial characteristics and should be evaluated more thoroughly (Table 26.3).[312]

Surgical Therapy for Infertility Associated with Endometriosis

The role of laparoscopic surgery in the amelioration of infertility in women with minimal to mild endometriosis has long

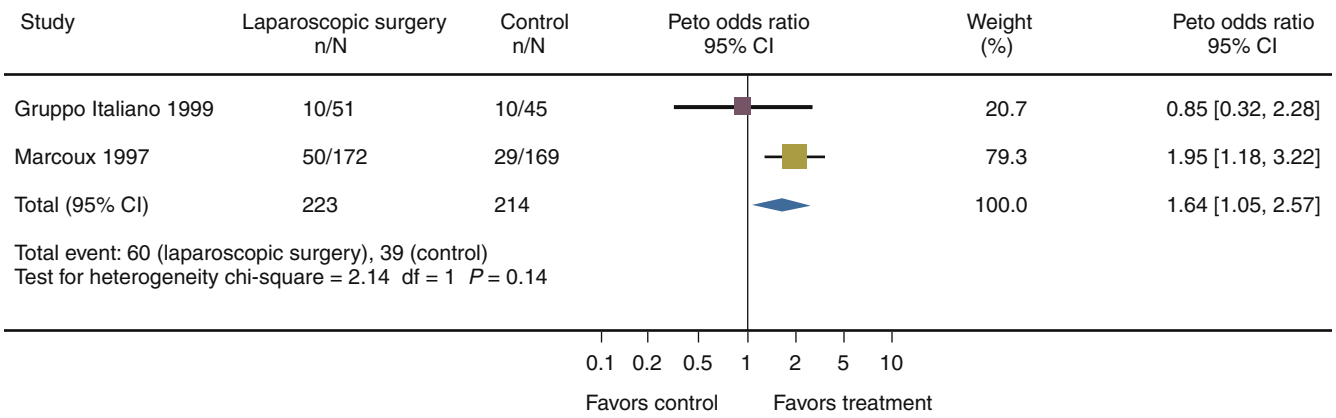


FIGURE 26.16 Meta-analysis of the two randomized trials assessing the efficacy of laparoscopic surgery in the treatment of subfertility associated with minimal-to-mild endometriosis. Combining live birth and ongoing pregnancy data from the two studies shows an improvement with laparoscopic surgical treatment (OR 1.64, 95%; CI 1.05-2.57). df, degrees of freedom *(From Jacobson TZ, Barlow DH, Koninckx PR, et al. Laparoscopic surgery for subfertility associated with endometriosis.* Cochrane Database Syst Rev *4:CD001398, 2002.)*

Table 26.4 Cytokines and Growth Factors in Peritoneal Fluid

Endometriosis Prevalence in Subfertile Women	Number Needed to Treat
10%*	120
20%	60
30%	40
40%	30
50%	24
100%†	12

*Assumes 10% of patients undergoing laparoscopy have evidence of minimal-to-mild endometriosis.
†Assumes all patients have laparoscopic evidence of minimal-to-mild endometriosis.
From Senapati S, Barnhart K. Managing endometriosis-associated infertility. *Clin Obstet Gynecol* 54:720-726, 2011.

been an accepted dictum. This question was objectively addressed in a recent meta-analysis of two randomized controlled trials: the multicenter Canadian trial[313] and a smaller Italian study.[314] In this analysis, destruction of lesions—even in early-stage disease with infertility—led to improved pregnancy rates in women attempting spontaneous conception. One of the criticisms of the Canadian study is that adhesiolysis was performed preferentially in the surgical treatment group and that this intervention, rather than lesion ablation per se, may have accounted for the higher pregnancy rate. Based upon the larger (and more positive) of the two studies,[313] an additional pregnancy was achieved for every eight operative laparoscopies performed (Fig. 26.16). Given an estimated prevalence of 30% endometriosis in an otherwise undiagnosed infertile population, the total number of laparoscopic cases to yield a single additional pregnancy would jump to 4 (Table 26.4). Hence, although a place exists for surgical treatment of endometriosis-associated infertility, it carries a relatively high cost. There is some evidence that ablation of lesions in minimal-to-mild disease prior to IVF results in a higher live-birth rate.[315]

No published controlled trial compares surgical treatment versus no treatment in women with moderate to severe endometriosis; however, many publications cite a near-zero pregnancy rate for women with extensive disease. Uncontrolled trials show increased pregnancy rates for these women if normal pelvic anatomy is restored.[316]

When considering surgical extirpation of endometriomas, the data are limited and conflicting. Some studies indicate that cystectomy may be deleterious to residual oocyte and hormone function,[317-319] whereas another showed no differences in fertility outcomes between those women with resected endometriomas, those with endometriosis, and those with tubal infertility.[320] Ovarian cystectomy for endometriomas has been associated with significantly diminished AMH after surgery.[321,322] Beretta and colleagues[323] demonstrated better pain and spontaneous fertility outcomes after cystectomy compared with drainage and coagulation. A recent Cochrane review of the published literature supports this recommendation.[249] Further studies will be needed to clarify the risk-to-benefit ratio of endometrioma excision in women with endometriosis-associated infertility.[324] Prospective trials are needed prior to recommending transvaginal ultrasound needle-guided aspiration of endometriomas prior to IVF.[325]

In cases of infertility due to moderate-to-severe endometriosis, some authors have advocated combined medical and surgical therapy.[326] The current medical treatments are described later in this section, but due to their anovulatory side effects, these treatments impair fertility in the short term.

Medical Therapy for Infertility Associated with Endometriosis

Most established medical therapies are aimed at inhibition of ovulation as a means of interrupting hormonal stimulation of endometriotic implants. Therefore, these approaches are used only for short periods of time to decrease the size or activation state of endometriotic lesions prior to attempted conception. Five trials using six different treatments were compared with placebo or no treatment. Another eight randomized trials were performed in which danazol was compared with a second medication. A meta-analysis[327] of several trials showed no differences between the treatment and control arms with respect to fertility outcomes

(odds ratio = 0.85; 95% confidence interval, 0.45-1.22). However, when one considers the time from diagnosis, commencement of medical treatment, and interruption of ovulation versus no treatment, patients undergoing medical treatment had a substantially lower monthly fecundity rate than control subjects because of the loss of opportunities for conception.[314] Conventional hormonal regimens, therefore, have no place in the treatment of endometriosis-associated infertility.

Superovulation has been studied in three randomized trials and led to an improved clinical pregnancy rate in women with endometriosis.[328-330] Whether or not intrauterine insemination is of any added benefit remains to be determined.

Use of gonadotropin releasing hormone agonist, or possibly oral contraceptives, for 3 months prior to IVF increases the clinical pregnancy rate by fourfold.[331,332]

Assisted Reproductive Technology

Three randomized trials have been done comparing ovulation induction[330] and ovulation induction combined with intrauterine insemination in women with endometriosis.[328] It was clearly shown in these studies that these treatments enhanced fertility relative to timed coitus alone.

A single randomized controlled IVF trial was reported, but only 15 patients underwent IVF and 6 had no treatment. These numbers are too small to derive meaningful conclusions.[333] In a retrospective study, no differences were found in women undergoing IVF versus expectant management over a 3-year period.[334] Although it seems that IVF may reduce the time to pregnancy, it is unknown whether it actually increases the absolute pregnancy rate. Further trials will be needed before recommendations can be made. It is comforting to note that IVF is not a risk factor for endometriosis recurrence.[335]

In a meta-analysis of 22 independent studies, Barnhart and colleagues[235] determined that the overall likelihood of achieving pregnancy was significantly lower for stage III-IV endometriosis patients (adjusted odds ratio, 0.56; 95% confidence interval, 0.44-0.70) compared with tubal factor control subjects. Multivariate analysis demonstrated a decrease in fertilization and implantation rates and fewer oocytes in the endometriosis subjects. This would be consistent with the lower AMH levels in moderate-to-severe endometriosis.[215]

Summary

Although recognized for centuries, the etiology and pathogenesis of endometriosis remain topics of intense debate and investigation. The classical theories of histogenesis have been replaced by a neoclassical concept that this is a complex, multigenic disorder influenced by epigenetic and environmental factors. Definitive establishment of a clinical diagnosis requires direct observation, and ideally histopathological confirmation of ectopic endometriotic implants. However, a presumptive clinical impression (supported by physical findings and confirmatory radiological imaging) is adequate for most clinicians to initiate therapy.[336] Future confirmatory biochemical tests, based on endometrial biopsy findings, and possibly by serum analytes may reduce the necessity of laparoscopic evaluation for diagnosis. However, surgical treatment of endometriosis, perhaps with improved means of endoscopic lesion ablation, will likely retain a prominent place in endometriosis therapy.

The development of new medical treatments for endometriosis will parallel our further understanding of its underlying pathophysiology. Increased concentrations of activated pelvic macrophages and lymphocytes and elevated levels of specific cytokines and growth factors support the hypothesis that the immune response is activated in endometriosis. Angiogenesis inhibitors may play a more prominent therapeutic role in the future, but care must be taken to avoid the potential teratogenic side effects of this drug class. Medicinal herbs, despite millennia of clinical use, remain untested formally and potentially harbor untoward effects on ovulation, fertilization, or embryonic development. Whether components of the innate and adaptive immune systems play primary, causative roles, or merely reflect a reaction to the presence of ectopic implants, is unknown at present. A complex network of locally produced chemokines, cytokines, and prostaglandins is proposed to modulate the growth and inflammatory behavior of endometriosis—affecting implant proliferation and invasion, the recruitment of capillaries to the growing lesions, and further attraction of leukocytes to foci of peritoneal inflammation. Future endometriosis therapeutics will need to exploit our identification of relatively specific targets within pathways that regulate the growth and invasion of endometriotic lesions, ideally sparing or correcting the function of a woman's eutopic endometrium.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 27

Benign Uterine Diseases

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Elizabeth A. Stewart

The traditional concept of the uterus is that the endometrium is the dynamic tissue, providing an intricate set of functions throughout the menstrual cycle that rarely culminates in implantation and pregnancy. The myometrium has been viewed as an inert tissue, chiefly important during pregnancy and when abnormal, providing the surgical livelihood of clinical gynecologists.

However, to understand both the physiology of menstruation and the pathophysiology of abnormal uterine bleeding, both the myometrial and the endometrial layers of the uterus are important. First, both myometrial processes (adenomyosis and leiomyomas) and endometrial processes (polyps) can result in abnormal uterine bleeding. Secondly, on the molecular level, since the mass of the myometrial layer is so much greater than that of the endometrial layer, the myometrium can act as a reservoir of growth factors or immune cells that then may act on the endometrium in a paracrine or local endocrine fashion.

The study of uterine molecular mechanisms is in its infancy. Study of the human uterus is difficult since all of the surgical specimens available for *in vitro* study are by definition abnormal. Additionally, many of the disorders that cause abnormal uterine bleeding (such as leiomyomas) appear to be heterogeneous in their molecular mechanisms. Just as the clinical phenotype of polycystic ovaries can result from various molecular defects, leiomyomas likely have many underlying genetic etiologies and environmental stimuli.

Nevertheless, basic investigation is beginning to reveal that a number of common molecular mechanisms are shared by phenotypically different diseases such as leiomyomas, adenomyosis, and endometrial polyps. We can speculate that in the future these diseases may be classified on the basis of molecular defects rather than on microscopy. This would allow us to understand the genotype-phenotype relationships that we currently find puzzling: for example, why some women have profound heavy menstrual bleeding with these diseases and others are asymptomatic. Understanding new elements of the biology of these diseases will lead to innovative therapy.[1]

Our lack of understanding of molecular physiology and pathophysiology has left us with extirpative surgical treatment as our therapeutic mainstay. While we have become more elegant in our surgical approach, the high risk of recurrent disease following conservative surgeries indicates that understanding the underlying mechanism and moving toward prevention is likely to be more successful.

The fact that modern women spend a larger percentage of their lifespan menstruating compared with women in previous centuries and females of other species may also contribute to many pathological conditions. Many genes are differentially regulated at specific parts of the menstrual cycle. Thus, constantly turning the same molecular switches on and off may have the same effect as constantly turning a light switch on and off: the system becomes disrupted.[2] This fact, as well as species differences, has made it difficult to use animal models to study the function of uterine tissue.

Finally, the economic implications of these diseases are significant. The cost of leiomyomas alone in the U.S. has been estimated at $5.9 to 34.4 billion annually.[3] Lost productivity due to clinically significant leiomyomas are also a substantial cost, accounting for over 40% of total costs which average in excess of $4800 per woman per year in a commercially-insured population.[4] Neither of these figures takes into account the cost of sanitary protection, alternative and complementary remedies or the costs of women who are symptomatic but not seeking care.

Uterine Leiomyomata

Epidemiology

Uterine leiomyomas, frequently termed myomas or fibroids, are benign clonal smooth muscle-cell tumors ranging in size from several millimeters to many centimeters (Fig. 27.1). Clinically, fibroids are appreciated in approximately 25% of all women and in African American women they appear to have a threefold increased incidence and relative risk.[5] Careful pathological study of surgical specimens suggests more than 80% of African American and 70% of Caucasian women have detectable leiomyomas which parallels the lifetime incidence of the clinical disease.[6,7] Thus, in African American women, it appears there is little occult disease. This suggests that in this group, growth acceleration of transformed myocytes into clinical fibroids may be ubiquitous.[7,8] African American women are not only significantly more likely to have leiomyomas than Caucasian women but to be younger at the time of diagnosis and hysterectomy.[5,9-11] They also have more severe diseases,[9,11,12] and are two to three times more likely to have hysterectomy for leiomyomas and six-fold more likely to have a myomectomy (Table 27.1).[13,14]

Known risk factors don't adequately explain this racial disparity.[15,16] Newly discovered genetic polymorphisms

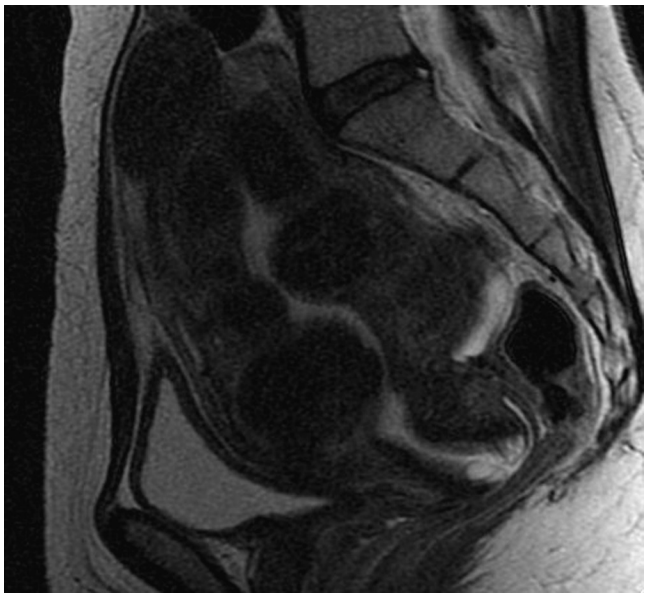


FIGURE 27.1 A T_2-weighted fast-spin echo (FSE) sagittal image of a leiomyomatous uterus with multiple type 2 submucosal fibroids. This sagittal image allows the endometrial cavity to be visualized from the fundus to the cervix, indicating that these fibroids are not displacing the cavity laterally. This series of images is typically best to visualize clinically important submucous fibroids. Visualization of the sacrum and coccyx, bladder and rectum allows assessment of bulk effects of the fibroid in the pelvis.

Table 27.1 Table Depicting Increased Burden of Fibroid Disease in African American Population

Fibroid Characteristic	African American vs. Caucasian Women	Reference Number
Incidence of uterine fibroids	Threefold increase	5
Relative risk	Threefold increase	5
Age at diagnosis	3-5 years younger	11
Severity of disease	Fivefold increase	11
Fibroid growth at older age (≥45 years)	Sevenfold-eightfold increase	53
Myomectomy risk	Sixfold increase	13
Hysterectomy risk	Twofold-threefold increase	14

that include abnormal transcriptional factors, increased aromatase activity and signal transduction genes may dictate a more severe phenotype of the disease in African American women.[17,18] Polymorphism of *catechol-O-methyltransferase (COMT)*, an essential enzyme for estrogen metabolism, has been shown to be linked to fibroid formation and is more common in African American women.[19] Vitamin D has been shown to inhibit fibroid proliferation via COMT pathway[20] and also reduces fibrosis caused by *transforming growth factor-β3 (TGFβ3)*.[21] Role of vitamin D has been reproduced in the Eker mouse model,[22] where supplementation with 1,25 dihydroxyvitamin D3 at 0.5 μg/kg per day for 3 weeks, lead to reduced myoma size compared to controls.[22] There have seen studies on African American women that correlate fibroid risk to polycystic ovarian syndrome[23] and self-reported experience of racism.[24] Understanding the unique genetic and environmental factors leading to increased risk for African American women is a key research agenda for leiomyomas. There is mixed data regarding fibroid risk in Latina women.[5,14,25]

Reproductive factors affect the risk of leiomyomas. Numerous studies have shown that parity is associated with decreased fibroid risk.[16,26,27] One hypothesis is that the remodeling of the postpartum uterus can clear nascent fibroids.[28] Support for this hypothesis came from recent studies.[29,30] In one of these reports of women beginning with pregnancy, 36% of them had no identifiable lesion on ultrasonography postpartum and 79% of the remaining fibroids had decreased in size.[31] Progestin use in the postpartum period was the only significant risk factor for limited regression of fibroids postpartum.[30] The fact that this study contrasts with previous reports, that progestin only injectable contraceptives are associated with decreased fibroid risk[32,33] and progestin only contraceptives are widely used in breastfeeding women make it critical to further explore this area. Although clinical dogma traditionally suggested that oral contraceptive pills (OCPs) were contraindicated for women with myomas, OCPs instead appear to protect against clinically evident fibroids with the caveat that timing of use may be important.[16,26,27] Exposure to OCPs between ages 13 and 16 for women in the Nurses' Health Study led to an increased relative risk of leiomyomas, while later OCP use was protective in direct proportion to duration of use.[16] Postmenopausal hormone replacement therapy (HRT) has been associated with a higher likelihood of having fibroids on pathology after surgery in one report, although this may have been due to bias, since HRT may have inhibited normal postmenopausal fibroid regression.[25] Early menarche is also associated with increased risk of developing fibroids,[11] and may explain earlier disease in African American women in whom menarche is earlier than Caucasian women.[34]

Environmental and dietary habits also appear to influence risk of myoma formation. Decreased vegetable and fruit intake, especially citrus fruits[35] and significant consumption of red meats was associated with an increased relative risk of fibroids and consumption of green vegetables with a decreased risk of myomas.[36] Reduced dairy consumption,[37] use of hair relaxer,[38] and increased intake of alcohol, especially beer, appears to increase risk in African American women.[39] Association of fibroid risk with high alcohol intake has also been reported in Japanese women.[40] No one has however, demonstrated that dietary intervention leads to changes in fibroid incidence, symptomatology, or fibroid regression. Major life events and stress,[41] as well as a history of abuse in childhood[42] have been linked to presence of uterine fibroids. *In utero* exposure to diethylstilbestrol (DES) in animals[43] and humans[44] as well as consumption of soy formula in infancy, low childhood socioeconomic status, early gestational age at birth and maternal pre-pregnancy or gestational diabetes status have all been associated to increase fibroid development risk.[15,44] Caffeine consumption was not noted to be a risk factor[39] and smoking is considered protective through an unknown mechanism.[26,45] Dietary Vitamin A from animal sources appears protective.[35]

Increased body mass index (BMI) or weight gain since age 18 also appears to influence myoma risk in some cohorts.[10,46-48] High dietary glycemic index and glycemic load[49] and decreased physical activity[50] are also risk factors. Finally, women with leiomyomas appear to be more

likely to have hypertensive disease than control women.[51] It is unclear whether this commonality is due to a common underlying mechanism since leiomyomas have been shown to share pathogenic features with development of metabolical syndrome.[52]

Pathophysiology

Leiomyomas have a median of 9% change in volume in a 6-month period.[53] Growth appears to be race related. African American and Caucasian women had similar growth till 35 years of age, after which growth rates declined for Caucasian women.[53] It is also shown that growth in fibroids is not dependent on position of the fibroid in the uterus.[54] Size of fibroids may be important as greater than 5 cm diameter lesions have less short-term change.[54]

Gonadal Steroids: Estrogen and Progesterone

There are substantial *in vitro* data supporting major roles for both estrogen and progesterone in the biology of uterine leiomyomas. The role of progesterone on myoma growth has moved beyond the simplistic concept of increasing mitosis to include inhibiting the apoptosis pathway via *B-cell lymphoma 2 (Bcl 2)* induction.[55-57] The apoptotic inhibitor *Bcl2* is present in leiomyomas but is largely undetectable in myometrium. Recently the role of *Kruppel-like transcription factor 11 (KLF11)* in integrating progesterone mediated myoma cell signaling and proliferation has been shown.[58,59] Likewise, regarding estrogen action, local action is likely key via an up-regulation of the enzyme *aromatase P450* and its gene *CYP19*.[60-62] Other elements of estrogenic response in myomas can also come into play as myoma cells have a modest increase in type I isotype of *17β hydroxysteroid dehydrogenase*.[63]

Modulation of steroid receptors is also important. Leiomyomas have increased amounts of both estrogen and progesterone receptor (ER and PR) messenger ribonucleic acid (mRNA) compared with normal myometrial tissue.[64-66] Both the A- and the N-terminally truncated B-isoforms of the PR appear to be present in both leiomyomas and myometrium, however, the A-isoform predominates.[67] Similarly, ER-α rather than ER-β appears to be the predominant form in leiomyomas.[68] In addition to the direct action of ovarian steroids on the uterus, it is possible that the reproductive axis may also influence uterine metabolism through the direct action of pituitary gonadotropins on the uterus. Gonadotropin-releasing hormone (GnRH), which is clinically used to reduce myoma size, abolishes gene expression differences between normal myometrium and myomas.[69] The placental glycoprotein chorionic gonadotropin (hCG) has been shown by several laboratories to have direct actions on myometrial metabolism.[70-72] Additionally, work has shown that follicle stimulating hormone (FSH), luteinizing hormone (LH), thyroid stimulating hormone (TSH), and their common α-subunit can all have stimulatory effects on uterine prolactin production.[71,73] There appears to be a variant LH/hCG receptor present in human uterine tissue that may modulate this action.[74,75] LH has also been associated with myoma formation but not growth independent of the patient's age.[76] Finally genome wide microarray studies have shown a strong role of glucocorticoids in pathogenesis of fibroids.[77]

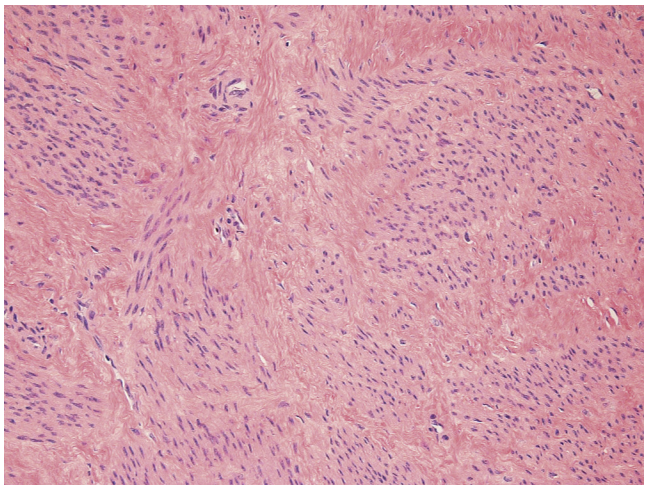


FIGURE 27.2 Histological evaluation of fibroid using hematoxylin and eosin staining highlights both cellular bundles and the acellular extracellular matrix. The extracellular matrix not only contains significant amounts of collagen type I and III but can also act as a reservoir for growth factors such as basic fibroblast growth factor (bFGF).

Fibrotic Factors

Leiomyomas can also be viewed as fibrotic tumors with a dynamic extracellular matrix (ECM) playing an important role in pathophysiology (Fig. 27.2).[78] This hypothesis dates back to the 1990s where experiments demonstrated that the ECM characterizing fibroids contains significant amounts of collagen types I and III protein, and up-regulation of mRNA levels occurs during the proliferative phase of the menstrual cycle in leiomyomas but not myometrium.[79] Other matrix components including *matrix metalloprotease stromelysin 3 (MMP 11)* and *dermatopontin* (a collagen binding protein, also having decreased expression in keloid scars) have also been shown to be dysregulated in leiomyomas.[80,81] Morphological arrangement of extracellular proteins is also abnormal in myomas.[82]

The *transforming growth factor-β (TGF-β)* system also appears to be involved in the pathophysiology of leiomyomas, as in other fibrotic processes. A complete review of this topic is beyond the scope of this chapter. Leiomyomas appear to have higher levels of *TGF-β* and particularly *TGF-β3* mRNA and protein and this in turn affects cellular proliferation.[1,83,84] Vitamin D supplementation has recently shown reversal of *TGF-β3* induced fibrosis in fibroids.[85] Additionally, *Granulocyte macrophage colony-stimulating factor (GM-CSF), connective tissue growth factor (CTGF), TGF-β4* (also known as *lefty* or *ebaf, endometrial bleeding-associated factor*), the sma and mad related (SMAD) family of transcriptions factors and the mitogen-activated protein kinase (MAPK) signaling pathway appear to be part of the fibrotic pathway dysregulated in myomas, or the myometrium or endometrium of the uterus in women with leiomyomas or abnormal uterine bleeding.[86-92] Alterations in ECM can modify mechanical stress on cells, leading to activation of Rho-dependent signaling. Activation of this solid state signaling and altered state of stress may also contribute to fibroid growth.[93,94]

Angiogenesis

Angiogenesis, the formation of new blood vessels, is physiological in the female reproductive tract as opposed to most other tissues where it is pathological. Abnormalities in uterine

blood vessels and angiogenic growth factors also appear to play a role in the pathobiology of myomas. The myomatous uterus shows increased numbers of arterioles and venules as well as venule ectasia.[95] These changes are not confined to the leiomyoma itself but also involve the myometrium and the endometrium.[96,97] Although such venous abnormalities were originally postulated to be the result of physical compression of the vascular structures by bulky myomas,[1] it is likely that molecular alterations are actually responsible for increased vessel number or abnormal function.[1,98]

The process of angiogenesis involves interactions with specific components of the ECM that are dysregulated in fibroids such as collagens type I and III.[99] There is also conflicting data regarding whether the resident immune cells (especially mast cells) contribute to myoma physiology by modulating angiogenesis.[100]

The *basic fibroblast growth factor (bFGF)* receptor-ligand system appears to be a significant factor in leiomyoma pathophysiology. In addition to promoting angiogenesis, *bFGF* is a smooth muscle-cell mitogen and acts similarly to estradiol on leiomyoma smooth-muscle cells.[101-103] Leiomyomas have increased levels of *bFGF* mRNA compared to matched myometrium, a reservoir of *bFGF* protein in the ECM and dysregulation of the endometrial type I *bFGF* receptor.[104,105]

Genetic Influences: Clinical

There are several lines of evidence that suggest that fibroids have a genetic component. First, monozygotic twins have twice the rate of concordance for hysterectomy of dizygotic twins.[106,107] There is also familial clustering; with a two-fold to sixfold increased risk of a woman having fibroids if a woman has an affected first-degree relative.[108,109] Finally, there are also specific syndromes that have a demonstrated genetic component and whose phenotype includes uterine leiomyomas in association with other specific lesions:

*Hereditary Leiomyomatosis and Renal Cell Cancer (HLRCC, MIM 605839)**

This syndrome is autosomal dominant, and affected families manifest cutaneous leiomyomas and papillary renal cell carcinoma (RCC).[110-112] Affected women can have uterine leiomyomas as well as uterine leiomyosarcomas.[110] Both malignancies (sarcomas and RCC) are atypical in their presentation compared to their sporadic counterparts; uterine sarcomas can appear in young premenopausal women, and the papillary RCC is often metastatic at presentation and more likely to be seen in women.[110] Two other syndromes had described only the association of cutaneous and uterine leiomyomas, but lessons form molecular genetics suggest these are incomplete forms of the HLRCC syndrome and should be of historical interest only (Reed Syndrome or Multiple Cutaneous and Uterine Leiomyomas [MCUL], Mendelian Inheritance in Man [MIM] 150800).[113,114]

Fumarate hydratase (FH), an enzyme that is part of the Krebs tricarboxylic acid cycle, is the gene mutation at 1q 42-43 responsible for hereditary leiomyomatosis and renal cell cancer (HLRCC) syndrome.[110,111,115,116] Germline mutations appear to result in absent or nonfunctional proteins, and thus, *FH* appears to act as a tumor suppressor.[112,117,118] *FH* appears to play a role in a small percentage of nonsyndromic leiomyomas and in Caucasian women.[119,120]

Although work on elucidating the pathogenesis of HLRCC syndrome continues, *FH* mutations appear to induce a change toward a hypoxic phenotype.[120,121] Thus, the hypothesized relationship between hypoxia and myoma pathogenesis appear linked for this subset of leiomyomas.[122]

Currently identifying women at higher risk of malignancy due to HLRCC syndrome is an important clinical task, just as identifying women whose families carry the breast cancer gene (BRCA) mutations.[123] However, in the future, individualized therapy will likely be possible based on genotype and underlying predisposition genes.[123]

Cowden Disease (MIM 158350)

This disease is a type of hamartomatous polyposis syndrome characterized by leiomyomas as well as other benign tumors, including lipomas and hamartomas. It is autosomal dominant in inheritance and has involvement of the candidate gene, *phosphatase and tensin homologue (PTEN)*.[124-128] Patients with Cowden disease have increased risk of endometrial, thyroid, kidney, and colorectal cancers. Around 40% of women with Cowden disease are reported to have fibroids.[129]

Cytogenetic and Molecular Genetics

There is also cytogenetic and molecular genetic evidence for the role of genetics in leiomyomas. Leiomyomas are monoclonal, and each tumor is an independent clonal event. Although this fact was originally investigated using G6PD polymorphisms, androgen receptor polymorphism studies concur.[130,131]

Secondly, certain cytogenetic rearrangements characterize leiomyomas. Although 40% of fibroids are 46,XX,[132] there are specific karyotypic abnormalities that have been consistent in a number of studies: t(12;14)—Translocations between chromosomes 12 and 14, Trisomy 12, rearrangements of 6p, 10q, and 13q, and Deletion of 3q and 7q.[123,132,133] There is evidence that karyotypic evolution is a late event in the pathogenesis of leiomyomas.[131] There is also some evidence that genotype is related to both fibroid size and location and that specific karyotypic groups have specific gene expression profiles.[132,134,135] Thus, many of the characteristics we attribute to submucous fibroids, as an example, may be related to genotype and thus, the clinical heterogeneity we see may be more intelligible when genotypic information is available.[136]

Many candidate genes identified for uterine fibroids map to the regions involved in these karyotypic groups. *High mobility group protein A2* (*HMGA2*, formerly called *HMGI-C*) is an architectural transcription factor located on chromosome 12 that is involved in the pathogenesis of fibroids with t(12;14).[137,138] The *HMGA2* gene is very large (13 kb with 5 exons) and most translocations involving leiomyomas map to the 5′ region of the gene.[137,139] However, recent evidence suggests that unique transcripts from the opposite deoxyribonucleic acid (DNA) strand may also play a role in pathogenesis.[140] Diminished stature, early age at menarche and higher risk of formation of fibroids has been linked to *HMGA2*.[141] *HMGA1 (HMGI[Y])* codes for a related gene on chromosome 6p.[142,143] Interestingly, abnormalities in expression of *HMGA2* in a

*The Web site: http://www3.ncbi.nlm.nih.gov/omim/contains the full online catalog of these genetic disorders.

murine model produce abnormalities of fat deposition and metabolism.[144,145] Abnormal expression of *HMGA2* could therefore potentially explain the correlation of metabolical syndrome in patients with fibroids.[52]

A genome wide association study (GWAS) from Japan, determined significant associations with uterine fibroids in three chromosomes 10q24.33, 22q13.1, and 11p15.5.[146] Subgroup analyses revealed strong association of marker single-nucleotide polymorphisms (SNPs) with uterine fibroids regardless of presence or absence of heavy or painful menstrual bleeding suggesting these SNPs are associated with predisposition genes.[146]

Finally the role of *mediator complex subunit 12 (MED12)* transcriptional factor, a mediator of both global and specific gene transcription located on chromosome Xq13.1 has also been described in the pathogenesis of uterine fibroids.[147] *MED12* was altered in 70% of tumors from 80 patients studied in this report from Scandinavia and pathway analysis suggested that ECM receptor interaction, Wingless family *(Wnt)* signaling and focal adhesion pathways were altered by this change. [147] Whole genome sequencing has recently shown *MED12* mutations to be present frequently in fibroids in racially and ethnically diverse American women confirming its importance as a key molecule in fibroid pathobiology.[148]

Rad51L1, (hREC2) encodes an enzyme which repairs double-stranded DNA breaks and is the only gene where a fusion transcript appears to play a role in fibroid biology. *Rad51L1* is on chromosome 14, and there are reports that in rare myomas with t(12;14) it forms a fusion transcript with 5′ *HMGA2*.[149-151] Inactivation of a tumor suppressor gene, *cut-like homeobox gene (CUTL1)*, acts in some myomas to suppress transcription of the C-*Myc* oncogene.[152]

Finally, the Eker Rat model for leiomyomas has a germline defect in the *tuberous sclerosis complex 2 (Tsc-2)* tumor suppressor gene.[153] In this model, recent work suggests that there is a developmental window where the expression of disease is modulated by the effect of interaction of the tumor suppressor and the steroidal milieu.[153] This animal model and especially cell lines that have been created from it have been a major asset for fibroid research. However, there are some facets of the Eker rat model that suggest it may be a better model for syndromic fibroids such as those seen in HLRCC syndrome rather than sporadic fibroids. New murine models are under development that may also aid researchers and also give insights into fibroid pathogenesis.[154] The high incidence of myomas may be explained in part by the predominance of tumor suppressor mechanisms.

A significant limitation of genetic studies is that they have largely been conducted in areas where Caucasians predominate and may not accurately reflect the karyotypes seen in African American women. Given the different clinical behavior of myomas in African American women, it is reasonable to believe that unique genes may be contributing to this risk. Association of a polymorphism in the *catechol-O-methyltransferase (COMT)* gene seen more frequently in African-American women was linked to leiomyoma risk.[19] *In vitro* work also suggests there may be differences in growth factor regulation in leiomyomas from African American women.[155] Linkage analysis for *FH* in non-syndromic leiomyomas demonstrated a significant effect of race with linkage only seen in Caucasian women and a negative relation in African American patients.[120]

Other Influences

Epidermal growth factor (EFG) is a growth factor mitogenic for smooth-muscle cells and EGF mRNA is up-regulated in leiomyomas only in the secretory phase of the cycle.[156,157] Receptor levels appear to be similar in leiomyomas and myometrium.[158] Latest work concentrates on the role of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase derived *reactive oxygen species (ROS)* for signaling EGF and platelet derived growth factor (PDGF) signaling pathway, leading to myoma cell proliferation.[159]

Heparin-binding growth factors are important biological regulators in leiomyomas since they can be secreted and bound to the reservoir of heparin sulfate proteoglycans filling the leiomyomatous ECM. *Heparin-binding epidermal growth factor (HBEGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), hepatoma-derived growth factor (HDGF),* and the previously described *basic FGF* are all found in myomas.[104,160] Many have also been documented to be stored in the ECM.

Insulin-like growth factors (IGFs) can act as smooth muscle-cell mitogens and were originally shown to have increased binding to leiomyomas compared to myometrium.[158] However, assessment of mRNA levels suggests that gene expression differed among studies.[161-163] Later studies suggested specific modulation of the IGF-binding proteins.[164] Recent work has shown the role of activation of tyrosine kinases and especially the *IGF-1* signaling pathway in fibroids.[165] Regulation of these factors following GnRH-agonist treatment has also been reported.[166] There may also be increased prevalence of leiomyomas in women with acromegaly.[167]

Prolactin also appears to play an important role in myoma pathogenesis. *In vitro* studies suggest that it is mitogenic for leiomyoma and myometrial smooth-muscle cells and that the prolactin receptor is present in these tissues, setting up an autocrine or local endocrine system.[168] Additionally, agents that appear to cause clinical regression of uterine leiomyoma also appear to decrease prolactin production *in vitro*.[67,73]

The resident immune cells also appear to influence leiomyoma biology. Mast cells have been implicated in leiomyoma pathobiology given that they are generally uniformly distributed in myometrium but highly variable in leiomyomas.[73] Recent work has suggested a correlation of mast cell number with vasculature.[169] A number of cytokines have also been shown to be differentially regulated in leiomyomas and myometrium. *Interleukin 8 (IL8)* has decreased expression of both the ligand and its receptor in myometrium compared to leiomyomas.[170] The functional significance of this is shown by the fact that neutralizing antibody to *IL8* decreases cellular proliferation *in vitro*.[170] *Monocyte chemotactic protein-1 (mcp1)* is largely undetectable in normal samples of leiomyoma and myometrium but is increased significantly following GnRH-agonist therapy.[171]

Wnt 7a, the human homolog of the wingless Drosophila genes involved in anteroposterior (AP) axis formation and smooth muscle-cell patterning, appears to be suppressed in leiomyomas compared to normal myometrium and to be inversely related to ER-α expression.[172] In contrast, *secreted*

frizzled related protein 1 (sFRP1), a modulator of *Wnt* signaling, is increased in leiomyomas (particularly in the late proliferative phase) and increased by estradiol treatment and hypoxia.[173] *HOX* gene expression does not appear to differ between leiomyomas and myometrium.[174] The mRNA for proto-oncogenes *cfos* and *cjun* are also overexpressed in leiomyomas compared to normal myometrium.[175]

Parathyroid hormone-related peptide (PTHrP) mRNA is also overexpressed in leiomyomas compared with normal myometrium.[176] Serum overexpression of this protein originating from a fibroid simulating the hypercalcemia of malignancy has been reported in the literature.[177-180]

Micro RNAs (miRNAs) are small noncoding RNAs which generally inhibit gene expression and appear to have a key role in leiomyoma pathogenesis. While early studies showed there was differential expression of specific miRNAs between leiomyomas and normal myometrium and association of key miRNAs with leiomyoma size and patient race, more recent studies have started to define the key regulatory pathways they influence.[181-184] In particular, dysregulated miRNAs appear to be involved in multiple adhesion pathways and multiple signally pathways including MAPK, calcium, and insulin.[183]

Principles of Treatment

Uterine leiomyomas do not always necessitate treatment. Generally, expectant management is appropriate until the woman develops enough symptoms that she requests treatment. The U.S. Agency for Healthcare Research and Quality on comparative management of fibroids has noted the lack of published data that examines the effectiveness of treatment strategies.[185] There are two important caveats to this generalization. First, although bleeding symptoms are usually evident, bulk symptoms can be insidious in their onset and often attributed to other processes such as aging. An initial assessment of whether bleeding, bulk-related symptoms, or both are prompting therapy helps to guide appropriate therapeutic options.[2] Women not electing therapy cannot reflexively be termed asymptomatic; they may have substantial symptoms but view the therapies they are offered as worse than the disease.[2] As a second step, assessing the patient's desire regarding reproduction helps refine the available options. In general, women with complaints of heavy menstrual bleeding alone tend to have more options for therapy (e.g., endometrial ablation, hysteroscopic myomectomy and hormonal therapy including progestin containing intrauterine device (IUD) than women with concurrent bulk-related symptomatology.

Finally, menopause can be a cure for women with myomas. Clearly, heavy menstrual bleeding ceases with the onset of menopause. However, not all women have enough volume reduction to alleviate their symptoms. In addition, bleeding symptoms may continue for women who elect postmenopausal hormone replacement therapy (HRT), and studies have suggested growth of myomas in women who take HRT.[25,186]

Surgical Therapies

Hysterectomy provides the only cure for fibroids and will remain a viable treatment option for the near term. Additionally, short-term outcome studies suggest that women with myomas experience improved quality of life following hysterectomy, and hysterectomy also eliminates concomitant conditions including adenomyosis, endometrial polyps, and abnormal pap smears.[2,187,188] Unlike the case in hysterectomy for endometriosis, the ovaries can be retained without losing therapeutic efficacy. Generally, women weigh the risk of menopausal symptoms against the risk of ovarian tumors in making this decision. The attention paid to the ovary's production of androgens postmenopausally and their possible importance in mood and libido appears to be leading to an increase in number of women who retain their ovaries even if they are perimenopausal.[189] The fact that hysterectomy, even without oophorectomy, decreases the risk of ovarian cancer may also affect decision-making on this issue.[190,191] Laparoscopic, rather than open hysterectomy if possible should be a goal if this treatment modality is chosen. As robot-assistance has shown reduction in likelihood of conversion to laparotomy at time of hysterectomy, there may be hidden potential in this new modality compared to traditional aggressive surgery.[192] Despite definitive treatment, hysterectomy alone (without oophorectomy) has been linked to increased incidence of cardiovascular morbidity,[193,194] prolapse,[195] and worsening cognitive function including Alzheimer and Parkinson disease.[196] These long-term adverse outcomes should be kept in mind when recommending hysterectomy as a surgical therapy.

Finally, the use of supracervical or subtotal hysterectomy in women with leiomyomas is debated. The fact that 7% of women in an unselected population have cyclic bleeding following this type of hysterectomy deserves further study to see whether these women had bleeding complaints or fibroids prior to hysterectomy.[197] Women may also run the theoretical risk of the formation of cervical fibroids following supracervical hysterectomy. Finally, accumulating data suggests that, at least in the short term, sexual functioning is not improved with supracervical hysterectomy.[198,199]

Nonetheless, as women seek less invasive and the healthcare system seeks less costly options, alternatives to hysterectomy will become more widely used. All surgical alternatives to hysterectomy, however, share the risk of new myoma formation what is commonly but incorrectly termed fibroid "recurrence." Unlike the similar phenomenon after surgery in malignant disease, this is unlikely to be persistence of the same tumor but instead growth of additional tumors that may have been missed, not treated, resistant to treatment, or not yet present at the time of initial therapy. Thus, following a variety of techniques, including abdominal myomectomy and uterine artery embolization, the risk of subsequent procedures is significant.[200,201]

Since the 1930s, abdominal myomectomy has been the traditional alternative to hysterectomy, because it preserves the uterus and allows childbearing.[202] However, open myomectomy does have morbidity similar to that of hysterectomy and has significant risk of subsequent surgery.[203-205] Myomectomies are now increasingly being performed laparoscopically, with or without robot-assistance. Rates of conversion to laparotomy have been reported as low as 2% after laparoscopic myomectomy[206] and it has shown fewer complications when compared to open myomectomy.[207,208] Patients undergoing traditional myomectomy via laparotomy, when compared to robot-assisted myomectomy, had more estimated blood loss and a longer length of stay in

the hospital.[209,210] When outcomes from robot-assisted laparoscopic myomectomy, standard laparoscopic myomectomy and open myomectomy were compared, patients with laparoscopic and robot-assisted procedures had similar blood loss and length of stay, both of which were reduced compared to the open procedure.[211] Short-term surgical outcomes are similar after robot-assisted myomectomy and standard laparoscopic myomectomy.[212] Reports have shown increased operating time with robotic procedures, however rate of complications are low compared to open surgery.[211,213,214] Using single port laparoscopy or laparoendoscopic single-site surgery (LESS) has led to success for myomas; however, since this is the newest form of laparoscopic innovation, data is limited.[215-217]

Abdominal myomectomy permits healthy pregnancies after surgery and pregnancy rates have been reported to be in the 50% to 60% range.[218] Uterine rupture following myomectomy is very rare at 0.5% to 1% and is suggested that it may be related to surgical technique.[219] The common clinical practice of counseling women who have had a myomectomy with a transmural uterine incision to undergo an elective cesarean section is based on this risk of uterine rupture following classical cesarean delivery. However, there is no evidence they are analogous situations.[220] There is enough evidence to question the rationale for the conventional practice and recommendation of cesarean delivery after myomectomy, even if the endometrial cavity has been breached.[221-225]

Though laparoscopic myomectomy involves much smaller incisions and a quicker recovery time, it does require a surgeon skilled in laparoscopic suturing and not all women have the size and number of fibroids amenable to this technique. While updated series suggest that the risk of uterine rupture is low following laparoscopic myomectomy, rare cases continue to be reported.[226-230] Because these uterine ruptures typically occur remote from term, appropriate counseling for patients contemplating pregnancy is important, especially if devascularization with cautery occurs intraoperatively. Data is very limited on obstetrical outcomes following robot-assisted myomectomy, but the few reports have been reassuring.[231,232]

Myolysis is a variation on the technique of laparoscopic myomectomy in which the leiomyoma tissue is coagulated rather than removed.[233] While this technique is easier to master than laparoscopic morcellation or suturing, localized destruction without repair may also increase the chance of uterine rupture and adhesion formation.[234]

For women with submucous myomas, the use of hysteroscopic myomectomy has distinct advantages. With their accessible location, type 0 and I (European Society of Hysteroscopy Classification) myomas can be resected with an intrauterine operative endoscope with good long-term results.[235] Although this procedure requires highly skilled practitioners, it can be done as outpatient surgery, often with a regional or local anesthetic and sedation that eases recuperation. Symptomatic relief is good with fewer than 16% of women in one large series who were treated for menorrhagia reporting second surgeries after 9 years.[235-237] Fertility rates appear excellent after hysteroscopic myomectomy, and there have been no case reports of uterine rupture after uncomplicated hysteroscopic myomectomy.[238]

For women who have completed childbearing and for whom bleeding is the primary problem, endometrial ablation, either alone or in combination with hysteroscopic myomectomy, may give relief with minimal invasiveness.[239] Increasingly, a levonorgestrel IUD can be used for a "reversible endometrial ablation."[240,241] In addition to providing effective control of bleeding, it provides contraception for women in this premenopausal age group, in contrast to surgical endometrial ablation which leaves women at risk for tubal and cervical and cervical ectopic pregnancies.

Uterine Artery Embolization (UAE)

Transcatheter arterial embolization has long been an effective percutaneous technique for controlling bleeding in a wide variety of disorders. Its use for the treatment of leiomyomas was first reported in 1995.[242] Although initially used as an alternative for patients who were felt to be poor surgical candidates, the resolution of symptoms in the initial cohort encouraged the use of this technique as a primary therapy.

UAE is increasingly the first line alternative to hysterectomy for women with bulk-related symptoms and no desire for future pregnancy,[243,244] and has been recommended by the American Congress of Obstetrics & Gynecology (ACOG) as a safe and effective form of uterine preserving treatment for fibroids.[220] It provides a decrease in heavy menstrual bleeding and bulk-related symptoms in 75% to 85% of women and a volume reduction of 30% to 46% over up to 5 years of follow-up.[244] Need for a second form of intervention after UAE is noted to be somewhere between 9% and 32%.[245-248] Previously thought of as a contraindication, fibroids greater than 10 cm diameter in size, have been shown to be treated successfully with UAE.[249,250]

A series of randomized clinical trials (RCT), mostly conducted in Europe, have compared outcomes of up to 5 years for women undergoing UAE and surgery (which included hysterectomy and/or myomectomy). These studies show women undergoing UAE have less pain, shorter hospital stay, a quicker return to work, equal post treatment symptom scores (based on standardized and validated questionnaires), no difference in health-related quality of life years and less healthcare costs when compared to surgery.[245,246,248,251-253]

It is relatively common for submucous myomas to be expelled vaginally after treatment[244] and thus, most hysteroscopically-resectable fibroids are still approached surgically. Similarly, the presence of pedunculated subserosal fibroids has historically been considered a relative contraindication to UAE therapy although no cases have been reported of intraperitoneal expulsion. There is some data that suggests that UAE can still be performed in these cases of pedunculated fibroids.[254,255] A laparoscopic approach can also be considered in such patients. Studies also suggest that high T2 signals predict greater volume reduction and complete devascularization predicts outcome at 5 years.[256-258]

Most patients develop significant pain and some vaginal discharge following the procedure and usually require intravenous narcotics for pain control. "Postembolization syndrome," defined as the combination of diffuse abdominal pain, mild fever, and mild leukocytosis is common and can occur in 30% to 40% of patients and gradually improves over a week.

It is important to assess the effects of UAE on the ability of women to become pregnant subsequently or to carry a pregnancy to term. Thus far, there are a number of reported patients who became pregnant following UAE.[259-265] Although no difference in intrapartum adverse outcomes was noted when patients after UAE were compared to patients after myomectomy, cesarean delivery rate was increased in both groups.[260,263] Pregnancy clearly can and does occur following UAE with success rates reported from 20% to 60%,[263,265] however, the risk of spontaneous miscarriage in these women has been noted to be higher compared to women with fibroids that have not undergone UAE treatment.[266] There are two major areas of caution for women wishing to optimize their fertility potential: effects on ovarian function and myometrial wall integrity.

Due to increased placental implantation issues after UAE, close monitoring of the placental status has been recommended.[264] Around 13% of women in one series, who were nulliparous and had no other risk factors, had some form of placenta previa or accreta in one series.[264] The early data on ovarian damage used amenorrhea as the indicator of perturbed ovarian function.[267,268] It is clear that amenorrhea risk is age related with women under 40 having a 3% risk and women over 50, a 41% risk.[267] Newer studies evaluate follicle stimulating hormone (FSH) and antimüllerian hormone (AMH) levels to detect more subtle damage.[269-272] While studies with short-term outcome may not show impact,[270] most have shown an age dependent risk, with risk increasing more so after 45 years of age.[269,271] Compared to hysterectomy, UAE causes more of a decrement in AMH levels after a 2-year follow-up following UAE treatment.[272] However, it is often overlooked that surgery also has an adverse impact on ovarian reserve. A putative mechanism is suggested in another report that indicated significantly increased FSH levels following UAE in patients with utero-ovarian vascular anastomoses.[271]

Focused Ultrasound: Non-Invasive Treatment

MRI guided focused ultrasound surgery (MRgFUS) provides a noninvasive ablation method that is FDA-approved for the treatment of uterine fibroids since 2004. While pioneered for the treatment of uterine fibroids, this modality can be used to treat multiple diseases and may prove to be the next step in surgical innovation from open to minimally invasive to noninvasive approaches.

Just as a laser amplifies and collates light into a therapeutic modality, FUS can deliver a large amount of energy to target tissues in a noninvasive way. Treatment is accomplished by placing a transducer against the abdomen, targeting an intraabdominal myoma without breaching the skin. The intensity of FUS used for treatment is significantly higher than that used in diagnostic ultrasound and can rapidly increase temperature at the focal point in excess of 70° C. At this temperature, coagulative necrosis will occur.[273] The procedure is performed under conscious sedation. After T2-weighted images are obtained (to develop a treatment plan), FUS sonications are targeted at the fibroid while MRI provides continuous thermal feedback.

The strongest predictor of MRgFUS success is the nonperfused volume (NPV) or the area devascularized by treatment that is nonperfusing in post treatment gadolinium imaging in the fibroid achieved after treatment.[274-280] Seventy one percent of women reached a target symptom reduction score on the uterine fibroid symptom and quality of life (UFS-QOL) questionnaire[281] at 6 months and 51% maintained this at 12 months in one of the pivotal trials.[276] In newer studies as higher NPV rates were achieved, quality of life improved.[282] Volume reduction is again proportional to NPV achieved after treatment. Up to 40% reduction in volume has been noted in fibroids after MRgFUS treatment.[280] When low NPV (of around 26%) was achieved, 28% of patients ended up with an alternative form of treatment at 12 months after MRgFUS.[282] Newer reports with higher NPV achieved (around 60%) led to failure of treatment in only 8% of women.[283]

Complications from MRgFUS are very rare. The most common complication is skin burns. These can occur because of poor coupling or abdominal scars on the patient that are encountered within the FUS bean pathway. An early trial reported around 5% risk of burns.[276] Only one case of extensive skin burns has been reported.[284] Use of acoustic reflectors (like cork or foam) and an energy-blocking patch on scars has shown success in prevention of burns.[285,286]

A parallel enrollment trial compared MRgFUS to abdominal hysterectomy, and recorded significant clinical complications and short form health survey (SF-36) at 1, 3, and 6 months. Clinically significant complications were lower in MRgFUS group, with SF-36 scores improved in both arms at 6 months, however, they were significantly better in the hysterectomy group.[287] There is currently an NIH funded randomized clinical trial comparing MRgFUS to UAE (NCT00995878, clinicaltials.gov) which should provide important information. Studies have also shown MRgFUS to be in the range of currently accepted criteria for cost effectiveness.[288,289]

Subsequent pregnancy related complications after MRgFUS treatment are minimal. Forty five pregnancies in 51 women were reported in the only published series.[290] Live birth rate of 41%, mean birth weight of 3.3 Kg, spontaneous abortion rate of 28%, and term delivery rate of 93% was noted.[290] At least one patient with a fibroid impinging on the cavity and unexplained infertility, conceived spontaneously after MRgFUS treatment.[291]

Ultrasound-guided focused ultrasound (typically referred to as high intensity focused ultrasound, HIFU) has been used to treat several solid tumors outside the United States.[292] Since feasibility studies showed promising results for use of ultrasound guided-HIFU for uterine fibroids[293,294] larger studies have been reported. These reports have not only demonstrated safety and efficacy of ultrasound guided-HIFU for treatment of uterine fibroids,[295-297] but also shown safety of pregnancy within 1 year of treatment in one report.[298]

Medical Therapies

There is a lack of randomized trials to demonstrate effectiveness of medical management of fibroids.[299] Oral contraceptive pills, progestins, nonsteroidal antiinflammatory drugs, antifibrinolytic agents, androgenic compounds, and progestin loaded intrauterine devices, all of which are useful in the treatment of idiopathic heavy menstrual bleeding, have not been studied with leiomyoma–related bleeding. Nonetheless, they are widely used and are likely effective in at least a subset of women with fibroids. A systematic

review suggested that in trials when women were assigned to medical therapy at least 60% of them had undergone surgery by 2 years.[240]

GnRH-Agonists

Since the action of native GnRH depends on its pulsatile release, the effects of GnRH-agonists depend upon their continuous presence. They first cause a time-limited increase in gonadotropin release, termed the flare. This subsequently leads to receptor downregulation, followed 1 to 3 weeks later by a hypogonadotropic hypogonadal state. It is this down-regulated phase that is useful clinically in the treatment of myomas. Alterations of the GnRH molecule, typically at the two glycine (G) residue positions 6 and 10, produce a longer half-life and are more useful for clinical purposes.

Many studies have focused on the efficacy and benefits of GnRH-agonist treatment for women with fibroids. Most women experience a substantial reduction in mean uterine volume of 30% to 60% over 3 to 6 months of therapy.[300,301] However, there is a wide range of responsiveness with rare individuals achieving no volume reduction. Both the estradiol levels at week 12 and the weight of the woman are correlated with the degree of uterine shrinkage.[301]

The other primary benefit of GnRH-agonist treatment is the induction of amenorrhea. Menses typically return 4 to 10 weeks following the end of GnRH-agonist treatment. Fibroid and uterine volume usually returns to pretreatment size within 3 to 4 months. The rapid return of ovarian steroidogenesis, coupled with an increase in the concentration of estrogen receptors in fibroids recently treated with GnRH-agonists, may contribute to the rapid regrowth of these tumors.[302]

GnRH-agonists can have significant adverse effects, the most important of which is bone loss. Six months of GnRH-agonist treatment can cause a 6% loss in trabecular bone, not all of which is reversible on discontinuation of therapy.[300] Symptomatic side effects of GnRH-agonist therapy are common. Hot flashes are universal in women undergoing treatment. Other less common side effects include sleep disturbance, irregular vaginal bleeding, vaginal dryness, headache, depression, hair loss, and musculoskeletal symptoms.[300]

Because of the concerns regarding bone loss with GnRH-agonists, clinical use of these drugs is typically confined to use as preoperative therapy or in women for whom a short period of treatment will be effective. The GnRH-agonist, Lupron® is FDA-approved for the presurgical treatment of uterine fibroids to correct anemia in conjunction with iron administration.[303] This is the only medical treatment FDA approved for treatment of this disease.

Administration prior to either hysterectomy or myomectomy is the most common current use of these agents. Length of therapy varies from 1 to 6 months depending on the surgical and hematological goals and the planned procedure. The amenorrhea induced by GnRH therapy leads to improved hemoglobin concentrations, which permits women who are anemic to correct this problem and potentially to donate their own blood for transfusion. Preoperative GnRH therapy also has been shown to reduce intraoperative blood loss significantly.[303] Although current guidelines from ACOG suggest that use of GnRH-agonists is beneficial preoperatively, they also state that for each individual the benefit must be weighed against the cost and the side effects.[220]

GnRH-Agonist Therapy with Estrogen and Progestin Add-Back Regimens

For many women, 3 to 6 months of symptomatic relief from leiomyomas does not allow them to avoid surgery but does afford them the opportunity to prepare themselves optimally for an operation. Therefore, the concept of adding additional therapy to minimize the side effects of prolonged therapy was developed, the so-called add-back regimens.[304-306] The goal of add-back regimens was to achieve a window of therapeutic efficacy during which side effects would be lessened or eliminated, yet no regrowth of the myomas would occur.

Studies have utilized one of two treatment strategies: simultaneous and sequential administration. With simultaneous treatment regimens, both the GnRH-agonist and the add-back regimens are started at the same time. In sequential treatment regimens, the GnRH-agonist is given alone for up to 6 months before steroid hormone treatment is added reducing a period of hypoestrogenism before steroid add-back therapy is started. Studies have suggested that sequential treatment is superior for therapeutic efficacy in the treatment of fibroids.[305]

Innovative GnRH-Agonist Add-Back Therapies

The estrogen receptor antagonists, tamoxifen and raloxifene, have been used in randomized prospective 6-month studies in a simultaneous add-back study design with the GnRH agonist.[307,308] Results were conflicting. No change in myoma size was noted when 20 mg of tamoxifen was used;[308] whereas, when 60 mg of tamoxifen was used a reduction in myoma size, but not symptoms, was noted.[307] Studies using raloxifene (60 mg/day) therapy in postmenopausal women with leiomyomas were able to cause a reduction in size.[309] Tibolone, a synthetic steroid, has been used as a single agent for menopausal HRT for its combination of estrogenic and progestational actions in the same molecule.[310] This medication has been studied in premenopausal women receiving GnRH-agonists for the treatment of myomas.[311] There was no inhibition of uterine shrinkage with tibolone, and patients showed preservation of bone density as well as symptomatic improvement. Thus, tibolone may be used as a single-agent add-back in the future.[312] Ipriflavone, an isoflavone that is a weak estrogen modulator, has been studied in add-back regimens.[313] Although originally studied to determine its effect on bone, it appears effective in slowing bone loss and decreasing symptoms without impeding the volume reduction of GnRH-agonist therapy.

GnRH-Antagonists

GnRH-antagonists have also been studied in the treatment of uterine leiomyomas.[314-317] The lack of flare effect and rapid onset of action give them an advantage over GnRH-agonists. Although they are not Food and Drug Administration (FDA)-approved for this indication, they have several significant advantages over GnRH-agonists. However, in the United States, these agents are marketed for use of ovulation induction and long-term preparations are not available. This could make treatment of fibroids cumbersome.

Progesterone Modulators

Clinical data regarding the efficacy of progesterone modulators has conformed the importance of progesterone in myoma biology. A concern of paramount importance while using progesterone receptor modulators (PRMs) is the potential of increased risk of endometrial hyperplasia or cancer. Pathologists have however shown a unique histological pattern in patients on these medications.[318] These PRM-associated endometrial changes do not have typical molecular signatures of malignant progression,[319] however, long-term data is still lacking.

Mifepristone (RU486) is a steroidal derivative of norethindrone, which acts primarily as an antiprogestin. It has, in high doses (50 mg/day), shown reduction in myoma size comparable to GnRH-agonists.[319] Thus, the clinical benefit was equivalent to that seen with GnRH-agonists, yet follicular levels of estradiol were maintained to support bone mass and provide symptomatic relief. Identical results were found with a reduction in dose to 25 mg/day; however, with a 5 mg/day dose, although acyclicity was maintained, volume reduction was reduced to 30%.[319-325] More recent studies suggest that doses of 5 and 10 mg produce volume reduction equivalent to those elicited by the higher doses, but produced amenorrhea in only 60% to 65% of women but did result in decreased menstrual blood loss.[320,324] Nonetheless, this provides significant symptomatic improvement. Mifepristone has mild side effects compared with GnRH-agonists. Adverse effects included mild and infrequent hot flashes in approximately 20% of patients during the first month of treatment only with higher doses; however, more persistent symptoms appeared in another study. Mifepristone is not approved for use by the United States FDA. A major impediment to off-label use is that the current available dose of RU486 is not appropriate (200 mg once for pregnancy termination versus 5 to 10 mg/day for fibroid treatment for 6 months).[326]

Ulipristal acetate, another PRM, was recently compared with placebo at 5 or 10 mg once daily for 13 weeks in a randomized control trial.[327] The medication resulted in resolution of heavy menstrual bleeding and significant reduction in fibroid volume, in women with uteri of less than 16 weeks gestational size.[327] There were also no findings of endometrial hyperplasia with its use. In another noninferiority trial, ulipristal was compared to GnRH-agonists, at 5 or 10 mg a day for 13 weeks.[328] Both arms had comparable rates of resolution of heavy menstrual bleeding, however, ulipristal resulted in less myoma size reduction compared to GnRH-agonist but a more rapid induction of amenorrhea.[328] Data from the supplementary appendix of these studies suggests that PRMs provide more prolonged volume reduction after treatment is discontinued compared to GnRH agonists.[328] Endometrial biopsies from women on ulipristal revealed a carryover effect of this medication after 3 months of therapy that lasted up to 6 months.[327] Women with symptomatic fibroids thus may have the option of a unique intermittent therapy with this medication.[329] Like Mifepristone however, the available FDA approved formulation (30 mg tablet) makes off-label use for fibroids problematic.

Other PRMs are being studied for the treatment of uterine leiomyomas.[330] These drugs appear to have efficacy similar to mifepristone but decreased side effects and increased specificity when interacting with the progesterone receptor.

Aromatase Inhibitors

Aromatase inhibitors have shown to decrease symptoms from fibroids and shrinkage in size when given to women that were pre- or peri-menopausal.[331-334] A randomized trial compared letrozole at 2.5 mg/day to triptorelin (3.75 mg/month) for 12 weeks in 70 women with a single fibroid ≥5 cm size. A statistically significant volume reduction in myoma size was noted in the letrozole group vs. the triptorelin arm (45% versus 33%).[334] Serum hormone levels were also significantly reduced in the triptorelin arm vs. the letrozole group.[334] Extensive data regarding the safety, efficacy, and cost-effectiveness of this class of medications as medical therapy for uterine fibroids is still lacking.

Serum Estrogen Receptor Modulators

Selective estrogen-receptor modulators (SERMs), which exhibit tissue-specific agonist or antagonist activity, appear to work better in animal models of myomas than in clinical trials. Studies have examined both tamoxifen and raloxifene. Clomiphene has not been studied and has been reported to cause growth of a myoma in a single case report.[335] Despite promising results in animal models,[336,337] clinical studies have had less impressive results.[309] However, in premenopausal women, raloxifene alone or when combined with GnRH agonists demonstrated little efficacy despite use at three times the conventional dose.[307,338]

Androgens

Danazol, an androgenic steroid most commonly used for the medical treatment of endometriosis, can be used to induce amenorrhea in order to control anemia due to fibroid-related heavy menstrual bleeding. A second androgenic steroid, gestrinone, has been shown to cause volume reduction and amenorrhea in women with myomas.[339] A great advantage of this drug is that, after it is discontinued, there is a carryover effect like those of PRMs; in one study, 89% of the women maintained a decreased uterine volume 18 months after cessation of therapy.[339] Unfortunately, gestrinone is not available in the United States. However, androgenic side effects including acne, hirsutism, and irreversible deepening of the voice have limited its clinical use.

Growth Factor-Directed Treatments

Particular factors which appear to be relevant to leiomyoma biology include the angiogenic factor basic fibroblast growth factor (bFGF), the fibrotic growth factor transforming growth factor-beta (TGF-β), and insulin-like growth factors I and II (IGF I and II), which mediate the effects of growth hormone (GH).[1,122] These molecules, as well as other growth factors, are likely to be targets for leiomyoma treatment in the future.

GH-Directed and IGF-Directed Therapy

Both *growth hormone (GH)* and the *IGFs* appear to have metabolical effects on uterine leiomyomas and the surrounding myometrium.[164] Because women with acromegaly (an excess of growth hormone) have a high incidence of leiomyomas, researchers decided to test the hypothesis that interfering with the growth hormone axis might work as a treatment for leiomyomas.[167] Lanreotide (a long-acting somatostatin analogue) has been used in seven premenopausal women with uterine myomas in a pilot

study in Italy.[340] Over the 3 months of treatment, both uterine volume and the volume of the largest leiomyoma were significantly reduced by 24% and 42%, respectively. Three months following the discontinuation of therapy there was some regrowth, but a significant reduction in uterine volume persisted at 17% and 29%, respectively. Levels of estradiol were not affected by this treatment, though both plasma *GH* and *IGF-I* levels were significantly reduced and additional pathological modulators may be effective.[341]

Anti-Angiogenic Therapies

There is significant evidence that the angiogenic factor *bFGF* and its type I receptor are important in the pathogenesis of leiomyoma-related bleeding.[104,105] In a variety of systems, *interferons (INF) IFN-α* or *INF-β* antagonize the effects of *bFGF* and have proven clinically useful in the treatment of a variety of vascular tumors. *In vitro* studies of leiomyomas demonstrate that *IFN-α* is an effective inhibitor of serum-stimulated and *bFGF*-stimulated DNA synthesis in both leiomyoma and normal myometrial cells, as well as in endometrial cells.[103] A case report also raises the possibility that *IFNs* may provide effective treatment for fibroids. A premenopausal woman who was treated with *IFN-α* for hepatitis C was noted to have significant shrinkage of a leiomyoma after 7 months of interferon therapy.[342] Tranilast [N-(3′4′-dimethoxycinnamonyl) anthranilic acid (N-5′)], a drug currently used in the treatment of a variety of allergic conditions, has been shown *in vitro* to decrease leiomyoma cellular proliferation by arresting cells at the transition from G0 to G1 phase.[343] While it acts as an angiogenesis inhibitor, it also works as a mast-cell stabilizer and a fibrosis inhibitor, which may have relevance for leiomyomas.[343]

Current research also involves work on the role of retinoic acid,[344,345] Vitamin D,[346,347] and green tea components[348] in preventing fibroid formation.

Adenomyosis

Adenomyosis, formerly termed endometriosis interna, is another benign uterine disease characterized by the presence of ectopic endometrial glands and stroma within the myometrium (Fig. 27.3). Furthermore, the surrounding myometrium is usually altered to produce hypertrophy. Disease ranges from grossly visible nodules termed adenomyomas, which can clinically resemble leiomyomas, to disease that is only detectable by microscopy. Definitions vary for the abnormal presence of gland within the stroma, with most settling on a definition of glands found one to three low-power fields from the endomyometrial junction. Clearly, differences in definition will lead to differences in perceived rates.

Classically, an adenomyotic uterus is termed *boggy, globular,* and *symmetrically enlarged*. However, this disease coexists with many other uterine conditions. One study has argued that adenomyosis is not indeed a true disease but a variant of the norm as women had similar symptoms for hysterectomy with and without adenomyosis.[349] Most women in this study were perimenopausal which could have been a major selection bias.

Adenomyosis can affect around 20% to 65% of women,[350] though the accuracy of these numbers can be questioned since diagnosis can only be made with certainty by microscopic examination of the uterus typically after a hysterectomy. In another series of hysterectomies, adenomyosis appears in about one quarter of all uterine specimens but is no more likely to coexist with symptomatic leiomyomas (23.3%) than with endometrial cancer (28.2%) or ovarian cancer (28.1%).[351]

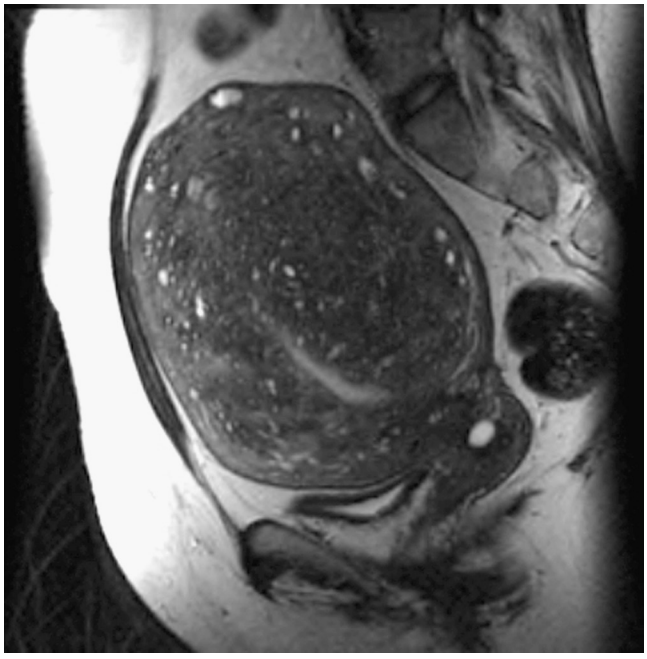


FIGURE 27.3 A T_2-weighted fast-spin echo (FSE) image of the adenomyotic uterus. Adenomyosis is characterized by proliferation of glandular elements of the uterus so that bright (white) areas similar in intensity to the endometrial cavity are seen deep into the uterine wall. The uterine walls can be asymmetric in this disease, and in this image the posterior wall is markedly thicker than the anterior wall. Thickening of the junctional zone, another characteristic of adenomyosis is not seen in this image.

Unlike leiomyomas, adenomyosis is associated with increasing parity.[351-356] It is estimated that at least 80% of women with this disorder are parous. However, this may be a confounding variable since women with a history of multiple pregnancies may simply have had more indications and/or inclination to proceed to hysterectomy during which the diagnosis could be made. Studies that suggest the presence of adenomyosis with imaging modalities rather than histopathology have suggested the presence of this disease process in adolescents as well.[357,358] The California Teachers Study noted clinical differences in women with endometriosis and adenomyosis.[354] Women with adenomyosis were older, had higher parity, early menarche, shorter menstrual cycles and were more obese compared to women with endometriosis.[354] Another study compared women with presence of fibroids and adenomyosis to women with fibroids only.[359] Women with both fibroids and adenomyosis had more pelvic pain and dysmenorrhea, higher parity, a history of previous uterine surgery and had more clinical depression compared to women with fibroids only.[359] Women with histopathology proven adenomyosis were more likely to have a history of previous uterine surgery in several reports.[355,359,360] Data regarding smoking as a risk factor for adenomyosis is controversial.[353,356]

Clinically, adenomyosis has similarities to leiomyomas in that its peak incidence is in women ages 40 to 50 years old with approximately 60% of women reporting abnormal uterine bleeding, chiefly heavy menstrual bleeding. Abnormal distribution of thick and dilated vessels in the endometrium, particularly in the secretory phase of the menstrual cycle, is one explanation for heavy menstrual flow in such women.[361,362] Dysmenorrhea is the other frequent symptom of adenomyosis, occurring in approximately one quarter of all cases.[350] Dysmenorrhea has been correlated with deep penetration and/or a high density of endometrial glands within the myometrium.[363] Abnormal uterine bleeding in the presence of adenomyosis is now classified as FIGO class AUB-A type of bleeding (see the section on abnormal bleeding later in this chapter).[364]

The most widely quoted hypothesis regarding the pathogenesis of adenomyosis is that invasion of the myometrium by the endometrium induces hypertrophy and hyperplasia of the myometrium. Proponents of this theory often quote the association of parity with adenomyosis to suggest that disruption of the layers of the uterus at the time of pregnancy and cesarean delivery may predispose to this condition. However, experimental evidence indicates instead that adenomyosis can be metaplastic process or a developmental defect. First, adenomyosis has been diagnosed in a woman with Rokitansky-Kuster-Hauser syndrome, who lacked eutopic endometrium.[365] Additionally, studies comparing the molecular expression of growth factors show distinct differences between ectopic and eutopic endometrium.[89,366-369] Factors that appear common to the pathogenesis of leiomyomas and adenomyosis include angiogenic factors such as *bFGF*, fibrotic factors including *GM-CSF*, the gonadotropin receptor LH, and resident immune cells.[89,368,370-374] The efficacy of some conventional and investigational therapies may be mediated through these systems.[375,376]

Gonadal steroid hormones also play a role in pathophysiology of adenomyosis. Adenomyotic implants express higher aromatase and estrone sulfatase activity,[377,378] and also have polymorphisms in estrogen receptors (ER).[379] *In vitro* studies have shown normalization of aromatase activity by gonadotropin releasing hormone agonists (GnRH) and danazol, but there is lack of data to show these effects *in vivo*.[375,377] The role of estrogen and ER in adenomyotic implants is further supported by the fact that endometrial hyperplasia was more prevalent in women with adenomyosis in one report.[380] A murine model of adenomyosis also supports this, as early tamoxifen exposure in these mice lead to development of adenomyosis and abnormal myometrium.[381]

Interestingly, another murine model of adenomyosis has been developed by placing a graft of pituitary tissue in a uterine horn.[382,383] Prolactin appears to be the key pathogenic agent in this model: the mice have elevated levels of plasma prolactin, and administration of bromocriptine prevents the development of adenomyosis.[382,384] In this model, there does appear to be endometrial cell invasion due to degeneration of myometrial cells.[383] Indirect exposure of the uterus due to hyperprolactinemia secondary to selective serotonin reuptake inhibitor (SSRI) medications can also induce adenomyosis.[385] This theory is further strengthened by recent work that showed both clinical depression and antidepressant use to be increased in women with adenomyosis.[359] A second model using the FORKO (follitropin receptor knockout mouse) suggests that the rising levels of FSH seen with aging may also play an important pathogenic role in this disease.[386]

Although definitive diagnosis of adenomyosis requires histology, imaging techniques are increasingly able to suggest the appropriate diagnosis. Both transvaginal ultrasonography (TVS) and magnetic resonance imaging (MRI) are used for this purpose. MRI is a better imaging modality for adenomyosis but is expensive. It can also differentiate very well, between an adenomyoma and a fibroid.[387] TVS is a less expensive imaging technique but is known to be operator dependent. A review of 23 articles comparing sensitivity and specificity MRI and TVS revealed both techniques to have similar sensitivity (0.72 for TVS and 0.77 for MRI) and specificity (0.81 for TVS and 0.89 for MRI).[388] Computed tomography has no role in diagnosis of adenomyosis[389] and needle biopsy should be reserved for cases where malignancy needs to be ruled out.[390]

The only definitive treatment for adenomyosis is total hysterectomy. GnRH-agonist treatment has been shown to produce amenorrhea, a transient decrease in uterine size and even in the ability to conceive.[391-393] Other medical therapies include use of levonorgestrel-releasing intrauterine device,[394-397] and a single case report of danazol containing intrauterine device.[398] Unfortunately, resumption of pretreatment uterine size and recurrence of symptoms are usually documented within 6 months of cessation of therapy.[396]

Data regarding conservative surgery (if adenomyoma present) are scarce. Adenomyomectomy has been reported to improve symptoms of adenomyosis,[399,400] and one study reports conservative surgery and GnRH medical therapy following treatment to be superior to surgery alone.[401] Other reported techniques include endomyometrial ablation and laparoscopic myometrial electrocoagulation which appear to decrease symptoms in more than half of patients with 3 years of follow-up data.[402,403]

Both UAE and MRgFUS have been reported for the treatment of adenomyosis. UAE can achieve success rates of approximately 50% over a 36-month follow-up.[404] In a recent report after a median follow-up of 58 months around 18% women ended with a hysterectomy, however, 73% of women were completely asymptomatic.[405] For MRgFUS, the largest study to date included 20 patients with a 6-month follow-up and indicated safe and effective MRgFUS therapy in all subjects enrolled.[406] Another MRgFUS case reports a spontaneous pregnancy with full term delivery after treatment.[407] Ultrasound-guided high-intensity focused ultrasound ablation has also been studied in one report.[297] Seventy eight patients with adenomyosis were enrolled and after a mean follow-up of 24 months around 90% of the patients had complete relief of symptoms.[297]

As imaging techniques get better, adenomyosis is being diagnosed with increasing frequency in women of reproductive age. Data on these women is limited to small case series. Indirect evidence shows a link between adenomyosis and infertility,[408] however, there is no direct link.[409] Increased risk of preterm birth and preterm premature rupture of membranes in women with adenomyosis (diagnosed with TVS or MRI) was noted in one epidemiological study.[410]

Endometrial Polyps

Endometrial polyps, as their name suggests, arise from the endometrial layer of the uterus. They are characterized by glandular proliferation surrounding a central core of prominent blood vessels in the stroma. Polyps are associated with abnormal uterine bleeding, particularly spotting and irregular bleeding, but the underlying mechanism has not been articulated. Several mechanisms are thought to play a role in development of endometrial polyps. These include overexpression of endometrial aromatase activity,[411,412] monoclonal endometrial hyperplasia,[413] genetic factors, particularly cytogenetic rearrangements of chromosome 6p,12q and 7q,[414,415] and alterations in endometrial levels of matrix metalloproteinases and cytokines.[416] Recent work has also shown the role of increased *TGF-β*, *VEGF*,[417] and *bcl-2*[418] in the pathogenesis of endometrial polyps. Historically polyps have been known to have estrogen receptors, though current literature suggests the presence of both estrogen and progesterone receptors (ER and PR).[419,420] It is suggested that both estrogen and progesterone contribute to the elongation of endometrial glands, stroma, and blood vessels to give them a characteristic appearance.[421] Progestins also have an antiproliferative function on polyps[422] as do androgens, however, data suggest that testosterone does not substitute for progestational activity for endometrial polyps.[423]

The estimated prevalence of polyps varies widely from 7.8% to 34.9% depending on the definition and diagnostic modality used.[424-427] The reported prevalence of polyps is as low as 0.9% in women less than 30 years old,[425] and increases with age. Risk factors for development of endometrial polyps include obesity,[428,429] hypertension,[428] diabetes,[428,430] and advancing age, especially postmenopausal status.[428] At least one paper has challenged these classical risk factors and has shown only age to be statistically significant risk factor.[431]

Tamoxifen use in postmenopausal women can lead to development of polyps in around 2% to 36% of women.[432] Tamoxifen induced polyps may be large in number and size and also show unique molecular alterations.[432-435] The levonorgestrel loaded IUD has shown to reduce tamoxifen induced polyp development in patients being treated for breast cancer when followed for a year.[436] Data regarding a relationship between endometrial polyps and postmenopausal hormone therapy are contradictory.[425,437-440]

The most frequent symptom in women with endometrial polyps is abnormal uterine bleeding (AUB-P) in the updated Federation Internationale de Gynecologie et d'Obstetrique (FIGO) classification[441] and is reported in 50% of symptomatic cases.[442] Conversely of women who have abnormal bleeding, approximately 30% have evidence of endometrial polyps.[443] Although abnormal bleeding is the most common clinical representation most polyps may remain symptom free and are an incidental finding on imaging.[444] The size, number, and location of polyps do not correlate with clinical symptomatology.[445]

Most polyps are benign and malignant transformation can occur in 0% to 12.9% of polyps.[446-448] Microsatellite instability has been noted in patients with multiple polyps or polyps with hyperplasia.[449] A recent meta-analysis revealed that the prevalence of malignant polyps was higher in postmenopausal women compared to women in their reproductive years (5.42% versus 1.7%) and that malignant polyps were more likely to bleed than non-malignant ones.[450] Risk factors associated with malignant endometrial polyps include an age greater than 60 years, polyp size greater than 1.5 cm in length, menopausal status, and abnormal bleeding.[432,451,452]

Endometrial polyps are still diagnosed following dilation and curettage (D&C) or hysterectomy, though new methods of diagnosis and treatment are being utilized more frequently.[453] Increasingly, the use of saline-infusion sonography (SIS), also termed sonohysterograms, or less commonly, office hysteroscopy diagnose polyps (Fig. 27.4). Polyps can also be diagnosed by hysterosalpingogram if the uterine cavity is also visualized. Newer 3-D ultrasound probes provide more accurate visualization between the endometrium and myometrium at the fundus and the cornual angles, hence improving the diagnostic accuracy compared to standard ultrasound.[454] A recent review reported a similar performance of transvaginal ultrasound (TVS), saline infusion sonography and hysteroscopy for detection of endometrial polyps.[455] Both hysteroscopy and SIS give a better sense of the endometrial cavity than TVS, however, SIS has an added advantage of providing the examiner with information regarding the adnexa and myometrium as well.

Medical management of polyps has limited role. There is some data regarding prevention of polyp formation in women being treated for breast cancer with tamoxifen,

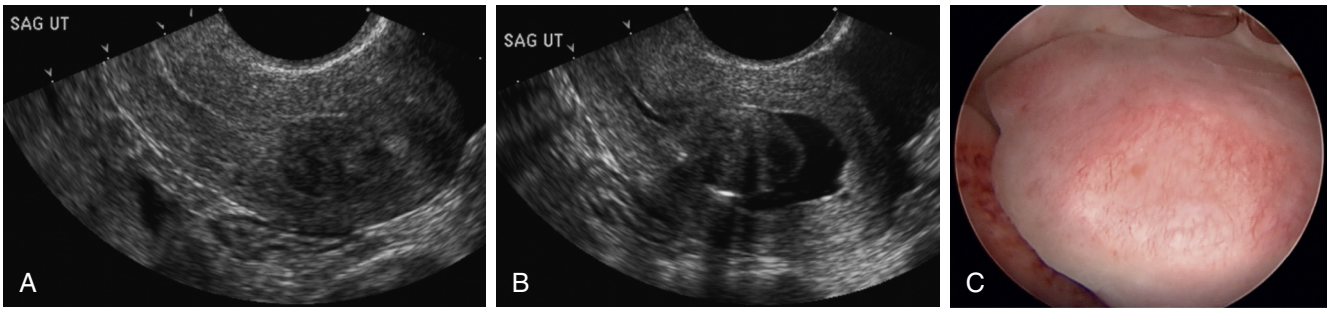


FIGURE 27.4 Comparison of imaging modalities for diagnosis of endometrial polyps. **A,** A sagittal view of a retroverted uterus obtained by a transvaginal ultrasound. A thickened endometrial stripe is appreciated, though the thickness is not well defined. **B,** A saline-infusion sonogram of the same patient. After infusion of saline into the endometrial cavity, the endometrial polyp is visualized protruding into the endometrial cavity and a thin endometrium is seen. **C,** A photograph taken during hysteroscopy reveals a well-defined polyp in the endometrial cavity. Polyps tend to blood vessels that are not as coarse as seen in submucosal fibroids and they have softer edges. *(Images courtesy of Dr. Mary Frates, Department of Radiology, Brigham and Women's Hospital and Harvard Medical School, Boston.)*

its use is limited to research protocols only.[436] Observing asymptomatic polyps can be an option in low risk individuals. When left untreated, asymptomatic polyps can regress spontaneously. In one trial, 27% of lesions resolved within 1 year of follow-up in premenopausal patients.[456] Polyps with mean length of 1.5 cm or more were less likely to resolve.[456] Other studies have shown complete resolution of polyps less than 1 cm.[426,457] The majority of cases of endometrial polyps are however, treated with hysteroscopic resection or hysteroscopically-guided D&C rather than the traditional D&C. Visualization and direct removal with this technique is reported to be more effective in reducing recurrence rates.[458] It has been reported that the volume of menstrual loss in women that underwent polypectomy did not differ from women who did not undergo polypectomy, but intermenstrual bleeding was significantly improved by polypectomy.[459]

There are little data on the effects of polyps on infertility. There are many theories of how polyps can effect implantation. These include mechanical obstruction hindering ostium function and affecting sperm migration[460] and decreased endometrial receptivity due to cytokines and cellular adhesion molecules.[461-464] The only randomized trial showed an improvement of pregnancy rates after polypectomy in women undergoing intrauterine insemination (IUI).[465] Other retrospective data have shown no difference in pregnancy rates, especially if the polyp was less than 1.5 cm in length.[466,467] In light of conflicting data, expert opinion recommends removal of polyps when diagnosed prior to *in vitro* fertilization (IVF) treatment. When lesions are seen during stimulation the treatment decision is made best on an individual basis.[468]

Abnormal Uterine Bleeding

Abnormal uterine bleeding (AUB) affects up to one third of reproductive age women and can occur in conjunction with the pathological processes we have discussed or in their absence. AUB also accounts for around one third of all outpatient gynecological visits.[469] In the United States, AUB (previously called dysfunctional uterine bleeding) is commonly equated with anovulatory bleeding, whereas in Europe it is a diagnosis of exclusion of excessive bleeding not due to demonstrable pelvic disease, complications of pregnancy, or systemic disease. Heavy menstrual bleeding (HMB) may occur in 10% to 30% of women and in up to 50% of women in the perimenopause.[470,471] However, the self-reporting of menstrual regularity and flow is highly variable. Change in pattern of flow is probably the most important sign of pathology. HMB is the type of abnormal bleeding frequently associated with benign uterine pathology, including leiomyomata and adenomyosis. Fragile, large thin-walled vessels and an aglandular endometrium appear to underlie the menorrhagia associated with some submucous myomas.[472] These vessels may arise from abnormalities in angiogenesis associated with growth factors released by myomata (e.g., basic fibroblast growth factor, vascular endothelial growth factor).[1]

There has been a recent push towards changing and standardizing terminology for AUB as confused terminology has made research collaborations and interpretation of clinical trials difficult.[473,474] Terms like *menorrhagia*, *metrorrhagia*, and *dysfunctional uterine bleeding* should be abolished.[474] To standardize communication, FIGO created a universal classification of causes of abnormal uterine bleeding for women in their reproductive years.[364,475-477] The "PALM-COEIN" nomenclature has four categories that are defined by structural criteria. These are: PALM; bleeding due to Polyps, Adenomyosis, Leiomyomas, and Malignancy or hyperplasia. The other four are unrelated to structural abnormalities and include: COEIN; bleeding due to Coagulopathy, Ovulatory disorders, Endometrial causes, Iatrogenic and pathologies Not classified. It is hoped that this classification system should facilitate collaborations to study AUB, however, the ease of use has to be demonstrated. The four structural causes of AUB, excluding bleeding due to malignancy have been discussed in the chapter. Previously classified true dysfunctional uterine bleeding equates to the four nonstructural causes of bleeding in the FIGO classification.

Around 13% of women with HMB have some form of coagulopathic disorder.[478] Systemic disease as a cause of HMB should be suspected in adolescents where coagulopathies including thrombocytopenia, von Willebrand disease, or other coagulopathies may be the underlying cause.[479]

Ovulatory disorders include both ovulatory and anovulatory dysfunction leading to AUB. Anovulatory dysfunctional uterine bleeding is characterized by irregular and prolonged bleeding secondary to disturbances in the hypothalamic-pituitary-ovarian axis. It is most common in the extremes of reproductive life[480] and in association with polycystic ovary syndrome. The unopposed action of estrogen on the uterus, resulting in dilated veins and the lack of suppression of spiral arteriole development, may represent the underlying pathophysiology.[470,471,481] Large, thin walled tortuous vessels can be demonstrated on the surface of the hyperplastic endometrium. Unopposed estrogen reduces vascular tone either through direct effects of estrogen on vascular smooth muscle cells or increased production of nitric oxide, leading to vasodilatation. The endometrium often breaks down unevenly in these circumstances. Scattered patches of thrombotic foci and necrotic degeneration are found adjacent to abnormally proliferated endometrium.

Ovulatory dysfunctional uterine bleeding is characterized by regular episodes of heavy menstrual flow, usually with the heaviest loss during the first 3 days of menstruation. Though many ovulatory disorders can be traced back to endocrinopathies, the underlying abnormality appears to be defects in processes that regulate loss of blood during menstruation, primarily angiogenesis, vasoconstriction, and hemostasis. In contrast to anovulatory dysfunctional uterine bleeding, the surface vessels of the endometrium appear to be grossly normal and only minor abnormalities have been described in endometrial and myometrial veins like venule ectasia.[1]

Endometrial causes of AUB are similar in pathogenesis to ovulatory disorders. In HMB the local endometrial hemostasis process is disrupted. This can be due to deficient production of vasoconstrictors like endothelin-1 and prostaglandin F2α, increased production of vasodilators such as prostaglandin E2 and prostacyclin, and excessive breakdown of clot in the endometrium by abnormal production of plasminogen activator.[482-484] In addition, vascular smooth muscle cell proliferation is reduced in the spiral arterioles in the midsecretory and late secretory stages in women

with menorrhagia, possibly contributing to vessel instability. *Endometrial bleeding associated factor (EBAF)* (a.k.a. *TGF-β4*) a member of the *TGF-β* family of growth factors suppresses production of collagen and promotes expression of collagenolytic and elastinolytic enzymes by antagonizing the normal signaling pathway activated by *TGF-β* growth factors. Abnormal expression of *EBAF*, which in a normal cycle occurs only in the late secretory and menstrual phases, has been reported in the endometrium of women with HMB.[86] *Angiopoietin 1 & 2* (*Ang-1* and *Ang-2*) may also be involved in the pathogenesis of HMB. *Ang-1* promotes vascular maturation, while *Ang-2* destabilizes vessels and initiates neovascularization.[485] An altered ratio of *Ang-1* to *Ang-2* in the endometrium due to down-regulation of Ang-1 expression is also associated with HMB.[485,486]

AUB due to iatrogenic causes (AUB-I) usually include the use of exogenous steroid therapy in form of combined estrogen and progestin pill, patch or ring, and levonorgestrel-releasing intrauterine device. The *not classified* category (AUB-N) includes a number of entities that do not fall into any group, for example AUB due to uterine arteriovenous malformation. Furthermore, this category has been left for new entities that may cause AUB that have not yet been discovered. It is important to note that a woman can have AUB due to more than one cause, for example a woman with HMB due to submucosal fibroid (AUB-L) can also have concomitant anovulation if she has PCOS and would have AUB-O type of bleeding as well.

These pathophysiological mechanisms proposed to underlie dysfunctional uterine bleeding are targets for novel therapeutic interventions (Fig. 27.5). The use of nonhormonal oral anti-fibrinolytic medication called tranexamic acid has shown a 66% response rate for HMB[487] and has shown to be extremely helpful in patients with AUB.[488,489] While tranexamic acid has been widely used internationally for many years, its recent FDA approval is leading to increasing use in the United States. Other options would include the use of antiprogestins or selective progesterone receptor modulators to suppress endometrial growth and stabilize the endometrial vasculature; MMP inhibitors to prevent extracellular matrix catabolism, including the matrix of the vessel walls; and non-steroidal anti-inflammatory drugs (NSAIDs) or selective cyclooxygenase-2 (COX-2) inhibitors to suppress prostanoid synthesis. Even though their use is not novel, combined estrogen and progestin formulation in form of patch, ring or pill, cyclical oral progestins, progestin-releasing intrauterine devices, are still unfortunately being used as the primary therapy for such patients.[490-493] Surgical destruction or removal of endometrium via endometrial ablation with newer nonresectoscopic, operator friendly devices has shown impressive success rates[239,494,495] and are also shown to be safe.[496] Hysterectomy is a definitive but less desirable option for AUB patients that are not concerned about fertility preservation.

Intrauterine Adhesions

The formation of intracavitary synechiae in an organ that routinely undergoes sloughing and regrowth without scarring is not well understood.[497] The clinical literature consistently reports that pregnancy frequently precedes the formation of intrauterine adhesions. The relationship between intrauterine adhesions and pregnancy is thought to be the result of defects in the regeneration of the

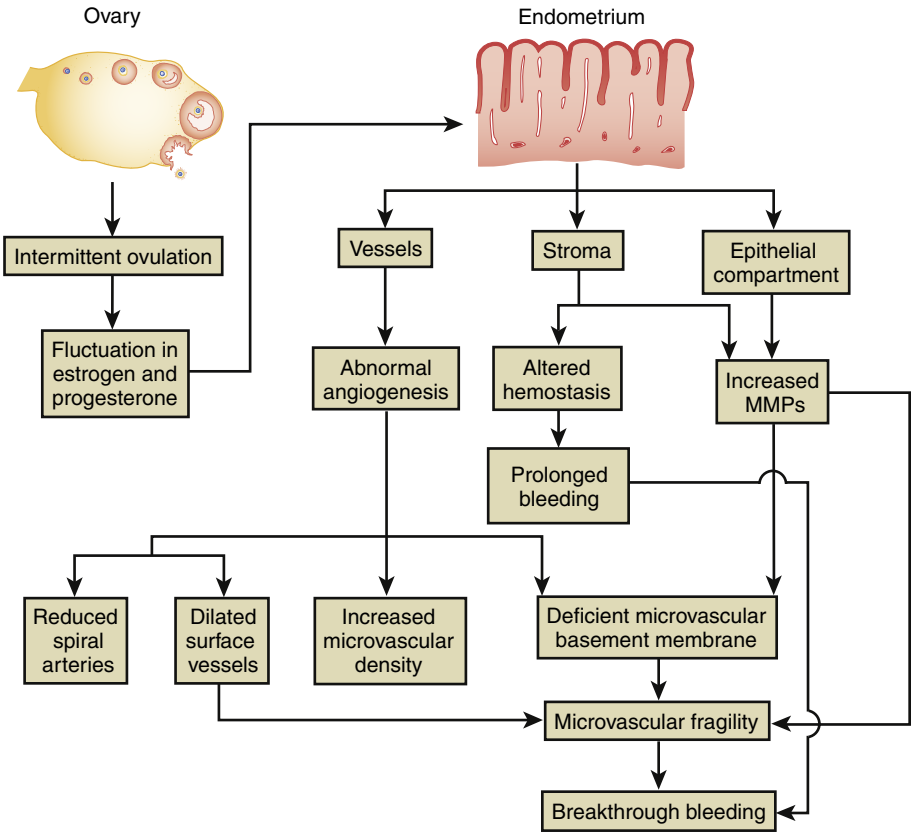


FIGURE 27.5 Ovarian and uterine mechanisms underlying breakthrough bleeding. A schematic drawing detailing the interaction between ovarian steroids and local endometrial factors leading to abnormal uterine bleeding. MMPs, matrix metalloproteinases.

endometrium after delivery, especially the area underlying the placenta. The placental site takes up to 6 weeks to repair with the thrombosed vessels and superficial necrotic tissue exfoliated as growing endometrium undermines the area. Trauma to the regenerating endometrium involved in restoring stroma and epithelium at this site, for example as a consequence of curettage performed 1 to 4 weeks after delivery and may result in a permanent scar with adhesions. This is considered the primary reason for intrauterine adhesions in the industrialized world. A review of 1856 women with intrauterine adhesions revealed 67% had undergone D&C for spontaneous or induced abortion and 22% had a postpartum D&C.[498] In another study, women were either randomized to nonsurgical treatment or D&C after incomplete abortion. Of the women that underwent D&C, 7.7% had some form of intrauterine adhesions compared to no adhesions in the nonsurgical group.[499] Curettage in the first 48 hours postpartum seems to cause fewer adhesions than when they are done later than that.[500] Not only D&C, but any sort of intrauterine instrumentation can lead to development of intrauterine synechiae. The average incidence of adhesion formation after hysteroscopic myomectomy for example has been reported around 10% at second look hysteroscopy after surgery.[501] In another study the rate of adhesion formation was noted at 6.7% after uterine septum take down, 31.3% after myoma resection, and as high as 45.5% after multiple myoma resection.[502] Even though there is need for larger randomized trials there is some evidence that highlights the use of adhesion barriers after hysteroscopic procedures.[503,504] The role of infection after abortion or delivery in formation of uterine synanche is controversial.[505,506] A comparison of uterine cavities of women that delivered via cesarean with and without endometritis at the time, did not show any differences when it came to intrauterine adhesions.[507]

In the developing world, infection of the endometrium particularly with mycobacterium tuberculi is an important cause of uterine synanche. With the human immunodeficiency virus-acquired immune deficiency syndrome (HIV-AIDS) epidemic and ease of air travel, genital tuberculosis should not be considered a threat to the developing world only. It is also important to note that most African studies have still attributed the major cause for intrauterine adhesions to be previous cavitary instrumentation.[508-510] Estimated prevalence of genital tuberculosis varies widely between 2% to 25% in infertile women, depending on diagnostic criteria used and the geographical location.[511,512] Tuberculous adhesions can be in as high as 35% of women with the infection[513] and have a poor prognosis for future fertility.[498,514] A characteristic *pipe stem* or *beaded* appearance on hysterosalpingogram and culture with acid fast stains on endometrial biopsy is helpful but, both tests have poor sensitivity. Newer tests like interferon-gamma release assays are more useful currently.[515]

Women with intrauterine adhesions may have no symptoms or a variety of menstrual disorders including hypomenorrhea, oligomenorrhea, amenorrhea, dysmenorrhea, and very rarely heavy menstrual bleeding.[498] Infertility, amenorrhea, and hypomenorrhea are the most common presenting complaints with infertility rates reported as high as 43%.[498] A number of classification systems have been proposed for women with intrauterine adhesions.[516] All systems require a thorough evaluation of the endometrial cavity to note the consistency, extent, and location of the adhesions. There is paucity of data from comparison of these classification systems.[516]

Hysterosalpingography is performed to view the cavity, and hysteroscopy reveals the intrauterine lesions. Ultrasonography, sonohysterography, and magnetic resonance[517,518] imaging can also be valuable in certain cases (Fig. 27.6). One group compared hysterosalpingography, sonohysterography, and transvaginal ultrasonography in patients with infertility and found both hysterosalpingography and sonohysterography imaging to have similar sensitivities of around 75% in detecting intrauterine adhesions. There was very limited use of transvaginal sonography in detection of synechiae in this study.[519] The gold standard for diagnosis of intrauterine adhesions is by diagnostic hysteroscopy.[520]

There is no consensus regarding the optimal methods to treat and prevent intrauterine adhesions. Since asymptomatic adhesions do not interfere with a woman's general well being, expectant management in such women has lead to resumption of regular menses in 1 to 7 years in up to 78% of women in one report.[498] There is no role of medical therapy for treatment of intrauterine adhesions. Before wide spread use of hysteroscope, blind D&C was used. Schenker and Margalioth reported an 84% rate of resumption of normal menses in women after D&C. Fifty one percent of these women conceived and 55% had term deliveries.[498] Surgical removal of adhesions hysteroscopically with blunt dissection, scissors, electrocautery, or laser ablation is now recommended. Return of normal menses after hysteroscopic resection varies between 92% to 96%.[521] The primary goal of surgery is to restore normal volume and shape of the uterine cavity.[521] In the nonindustrialized part of the world where hysteroscopic resection may not be feasible, there have been reports to show D&C to be equivalent to hysteroscopic resection, which is reassuring.[510] Post operative management is focused upon reducing the risk of reformation of adhesions, the rate of which can be as high as one in three women with mild to moderate and two in three women with severe adhesions.[516,522-525] Insertion of an intrauterine device or balloon catheter after lysis of adhesions is commonly employed to prevent recurrence.[526]

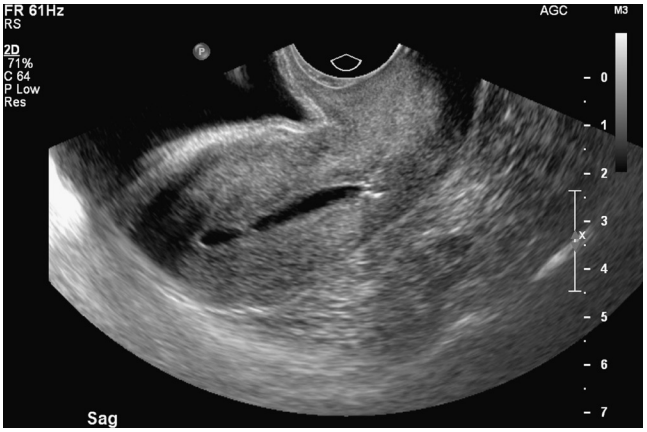


FIGURE 27.6 A sagittal view of an anteverted uterus obtained during saline-infusion sonography. An adhesion can be appreciated in this patient with a history of dilation and curettage (D&C) to treat a first trimester pregnancy loss.

Stimulation of endometrial proliferation with exogenous estrogens alone or in combination with a progestin has been advocated although there is debate as to the efficacy of this treatment as well as the administration of antibiotics and anti-inflammatory steroids.

Successful pregnancy rates after hysteroscopic resection of adhesion can be up to 63%,[521] with issues of placentation being most serious complication of pregnancy. Of 696 births reported in a recent review,[521]17 pregnancies had placenta accreta. Preterm delivery rate was 40% to 50% in this group of patients.[521]

Dysmenorrhea

Primary dysmenorrhea, menstrual pain not associated with recognizable pelvic disease, is due to intrinsic uterine dysfunction. Occurring only in ovulatory cycles, the symptoms of dysmenorrhea usually commence a few hours before the onset of the menstrual flow. Pain is greatest when the endometrium is shedding rapidly, approximately 12 hours after flow begins. The diagnosis of primary dysmenorrhea is established when secondary causes of dysmenorrhea are ruled out and based on medical history and normal findings on pelvic and rectovaginal examination and imaging.

The prevalence of dysmenorrhea in women varies between 50% to 90% in most studies.[527-536] Under 30 years old, smoking, irregular or heavy menstrual flow, body mass index (BMI) less than 20 kg/m^2, menarche before age 12, history of sexual assault, and a strong family history of dysmenorrhea are a few of the many risk factors known for primary dysmenorrhea.[537,538]

The painful cramps of dysmenorrhea are associated with uterine contractions; in women with dysmenorrhea, uterine contractile activity is heightened during menses, and basal myometrial tone and amplitude of contractions are increased.[539] During intense contractions, there is a reduction in blood flow to the endometrium suggesting that ischemia, in part, causes the pain of dysmenorrhea. There is also evidence from Doppler studies to show higher resistance in uterine vessels in women with primary dysmenorrhea, which can reduce blood flow to the endometrium.[540,541] The uterine contractions are prompted by prostaglandins, which are potent uterotonic agents both *in vitro* and *in vivo* acting through cell surface prostaglandin receptors.[542] Prostanoids may also directly sensitize uterine pain fibers. The notion that prostanoids are central to the pathogenesis of dysmenorrhea is supported by the observations that eicosanoids, most prominently prostaglandin F2-alpha ($PGF_2\alpha$), are found in high concentrations in menstrual fluid and that $PGF_2\alpha$ levels are higher in the endometrium and menstrual fluid of women complaining of dysmenorrhea than in women with pain-free menses.[543] This high concentration of prostaglandins has been shown not only in the endometrium but also in saliva of women during attacks of menstrual migraine associated with dysmenorrhea.[544] More recently patients with primary dysmenorrhea have also been hypothesized to have endothelial dysfunction.[545]

Treatment of primary dysmenorrhea is individualized to each patient's severity of symptoms. All drugs effective in inhibiting prostaglandin synthesis, including the potent nonsteroidal anti-inflammatory drugs (NSAIDs) ibuprofen, naproxen, and mefenamic acid alleviate symptoms of dysmenorrhea.[546] A Cochrane database review included 73 randomized trials to compare NSAID therapy to placebo and paracetamol. NSAIDs were significantly more effective in reducing pain symptoms compared to placebo or acetaminophen.[547] A combination of NSAID and paracetamol may also be helpful in relieving pain of primary dysmenorrhea.[548] Choosing an appropriate NSAID for treatment may be difficult. It is not clear whether some NSAIDs work better than others.[547] Some studies suggest that fenamates (class that includes mefenamic acid, flufenamic acid, tolfenamic acid, etc.) may have better pain relieving propensities than phenylpropionic acid derivatives (class that includes ibuprofen and naproxen).[549,550] Though there may not be sufficient data to prove the superiority of medications from the fenamates class, a reasonable option would be to start with NSAIDs from phenylpropionic acid derivative group and move onto fenamate class of medications if insufficient relief is noted.

Combined oral contraceptive pills (OCPs) are second line medications after NSAIDs for relief of pain from primary dysmenorrhea. No randomized trials have compared the efficacy of NSAIDs with OCPs. A systematic review compared OCPs to placebo and evaluated 10 randomized trials. A treatment benefit with OCPs was noted (pooled OR 2.99, 95% CI 1.76-5.07).[551] Continuous rather than cyclical administration of OCPs has shown more success in treating patients with primary dysmenorrhea.[552,553] Contraceptive ring users have similar results to OCP users for dysmenorrhea,[554] where as contraceptive patches have not shown comparable results to OCPs.[555] The use of levonorgestrel releasing intrauterine device for primary dysmenorrhea has been limited to case reports.[556] Commonly if treatment with NSAIDs is not successful after 3 months, OCPs are tried for another 3 months. If no relief is noted, reasons for secondary dysmenorrhea should be explored.

A long list of complementary and alternative medicines has been used for treatment of primary dysmenorrhea. Most of them have shown similar efficacy to the conventional nonsteroidal anti-inflammatory medications. Heat applied to the lower abdomen,[557,558] aerobic exercise,[559] yoga,[560,561] acupuncture,[562] aromatic essential oil massage,[563] and the use of far infrared emitting belts[564] are one of the few nonpharmacological agents tested for dysmenorrhea in randomized clinical trials and have shown promising results. Data on diet supplements are limited to a few small studies. A low fat vegetarian diet,[565] diet rich in dairy,[566] omega-3 fatty acids,[567] and vitamin E supplementation[568,569] have been effective in pain management from primary dysmenorrhea.

Newer medications that include calcium antagonists like nifedipine are also effective because they prevent uterine contraction.[570-572] Magnesium[573] and glyceryl trinitrate[574] have also been used for primary dysmenorrhea. In addition to prostanoids, other uterotonic substances including lipoxygenase products, vasopressin, and oxytocin may have a role in dysmenorrhea. Thus, antagonists of the V_1 receptor and oxytocin receptor have a therapeutic effect in dysmenorrheic subjects. Reductions in nitric oxide (NO), which relaxes uterine smooth muscle, may also contribute to the intensified contractions associated with dysmenorrhea.[575]

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 28

Endocrine Diseases of Pregnancy

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Errol R. Norwitz

Physiological and endocrine adaptations occur in the mother in response to the demands of pregnancy. These demands include support of the fetus (volume support, nutritional and oxygen supply, and clearance of fetal waste), protection of the fetus (from starvation, drugs, toxins), preparation of the uterus for labor, and protection of the mother from potential cardiovascular injury at delivery. The presence of a preexisting endocrine disorder is likely to affect the ability of the mother to adapt to the demands of pregnancy and, as a result, may influence fetal growth and development. Drugs used to treat such disorders may also affect perinatal outcome. The most common preexisting endocrine disorders that can complicate pregnancy are diabetes mellitus, thyroid dysfunction, and obesity. Less common preexisting maternal endocrine disorders include pituitary tumors, diabetes insipidus, and hyperparathyroidism.

The physiological and endocrine adaptations that characterize pregnancy can also lead to the development of pregnancy-specific diseases in previously healthy women, the most common of which are gestational diabetes and disorders of the endocrine and sympathetic nervous systems associated with preeclampsia and preterm labor. This chapter is designed to review in detail the underlying pathophysiology of these pregnancy-specific diseases, as well as the effects of pregnancy on pre-existing endocrine disorders. A better understanding of these conditions will improve the ability of clinicians to optimize maternal and perinatal outcome in such pregnancies.

Diabetes Mellitus

Effects of Pregnancy on Maternal Glucose Metabolism

Normal pregnancy can be regarded as a diabetogenic (prodiabetic) state with evidence of insulin resistance, maternal hyperinsulinism, and reduced peripheral uptake of glucose. These endocrine alterations, which result primarily from the production of anti-insulin hormones from the placenta (see Chapter 12), are designed to ensure a continuous supply of glucose for the developing and growing fetus. Therefore, in pregnancy, the control of maternal glucose metabolism is shared by the mother and the fetoplacental unit. The endocrine and molecular mechanisms by which the fetoplacental unit is able to reset the carbohydrate homeostatic equilibrium in the mother are not clear, but likely involve the action of several placental hormones. These hormones include growth hormone (GH), human chorionic somatomammotropins (hCS, placental lactogens), corticotropin-releasing hormone (CRH), cortisol, and progesterone.

Insulin Production and Action Changes during Pregnancy

Major functional changes occur in insulin production and action during pregnancy. β-cells in the islets of Langerhans within the pancreas—the cells responsible for insulin production—undergo hyperplasia, leading to insulin hypersecretion and an increase in circulating insulin levels throughout pregnancy.[1-3] It is this mechanism, along with the hemodilution of pregnancy and the initial enhanced responsiveness of cells to circulating levels of insulin,[4-6] that is likely responsible for the fasting hypoglycemia seen in early pregnancy. However, as pregnancy progresses, peripheral resistance to insulin increases.[4-7] In an attempt to overcome this resistance, the pancreas further increases insulin secretion. This compensatory response serves to return circulating maternal glucose levels to the normal range, but results also in chronically elevated insulin levels (both in the fasting and fed state), in the postprandial hyperglycemia that characterizes normal pregnancy,[8] and in islet cell hyperplasia.

Beta cell hyperplasia and expansion are controlled, in part, by prolactin and hCS, which both cause an increase in the number of pancreatic β-cells in pregnancy. Recent data from mice have provided some insights into the mechanisms regulating β-cell hyperplasia. Kim and colleagues found that serotonin acts downstream of lactogenic hormone signalling via the prolactin receptor to stimulate β-cell proliferation.[9] Karnik and colleagues reported that repression of menin—the protein product of the MEN1 gene—is necessary for normal β-cell proliferation, and that prolactin represses menin levels in pancreatic islets.[10]

Insulin resistance refers to a decrease in the ability of a fixed concentration of circulating insulin to stimulate peripheral glucose uptake in adipocytes and muscle cells. The condition can be demonstrated either by the insulin tolerance test or by glucose loading tests. An insulin tolerance test involves the injection of a standard dose of insulin followed by serial blood glucose measurements. The clearance

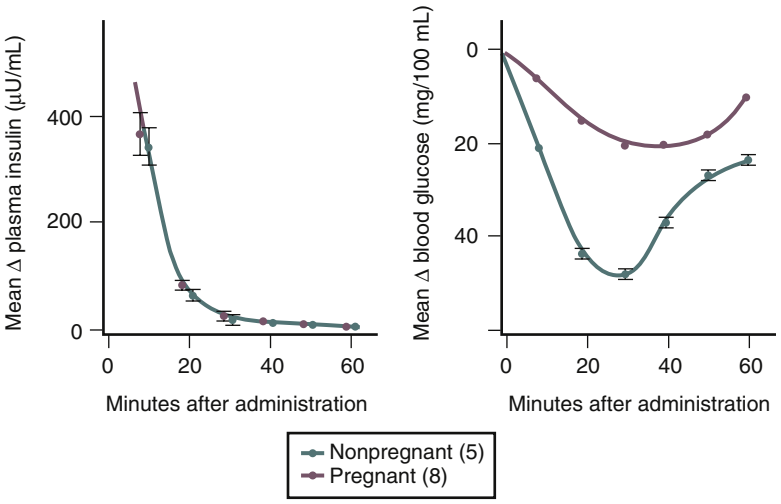


FIGURE 28.1 Effect of pregnancy on insulin clearance and insulin sensitivity. *Left,* Intravenous injection of insulin (0.1 unit/kg) results in identical clearance curves for circulating insulin in nonpregnant *(open circles)* and pregnant subjects *(closed circles). Right,* In pregnant subjects *(closed circles),* the intravenous injection of insulin results in a smaller decline in circulating glucose than that seen in nonpregnant subjects. The blunted biological effect of insulin in pregnancy suggests that pregnancy is a state of insulin resistance. *(From Burt RL, Davidson WF. Insulin half-life and utilization in normal pregnancy.* Obstet Gynecol *43:161, 1974, with permission from The American College of Obstetricians and Gynecologists.)*

of insulin from the circulation is not altered by pregnancy (Fig. 28.1).[11] The half-life of insulin is approximately 7 minutes both before and during pregnancy.[11] However, in pregnant subjects, the administration of insulin results in a smaller decline in circulating glucose than that seen in nonpregnant subjects (Fig. 28.1). Furthermore, in pregnancy, intravenous (Fig. 28.2) or oral (Fig. 28.3) administration of glucose causes significant hyperinsulinemia as compared with the nonpregnant state,[4] resulting in relative hyperinsulinemia after meals. Taken together, these data provide evidence in support of pregnancy being an insulin-resistant state.

The molecular mechanisms responsible for the insulin resistance in pregnancy are not well understood, but several factors are likely involved. Although insulin receptor kinase activity does not appear to be affected by pregnancy, the numbers of high-affinity insulin receptors on the surface of adipocytes are threefold lower in pregnancy than in nonpregnant women.[2,12] The glucose transport system also appears to be perturbed in pregnancy, with a threefold reduction in insulin-stimulated glucose transport as compared with nonpregnant controls.[12]

The movement of glucose into adipocytes and skeletal muscle cells is mediated by the glucose transport proteins, GLUT-1 and GLUT-4. GLUT-1 is responsible for basal glucose transport and is not responsive to insulin. Insulin increases glucose uptake in cells by stimulating the translocation of GLUT-4 from intracellular sites to the cell surface.[13,14] Up to 75% of insulin-dependent glucose disposal occurs in skeletal muscle, whereas adipose tissue accounts for only a small fraction.[15] In some pregnancies complicated by gestational diabetes, GLUT-4 is markedly reduced and fails to translocate to the cell surface with insulin stimulation, leading to a reduction in glucose transport in both the basal and insulin-stimulated states.[16] Taken together, these data suggest that the peripheral insulin resistance that characterizes pregnancy likely results from several integrated mechanisms, including a decrease in insulin receptor number, a "postreceptor" defect in insulin action, and alterations in glucose transport systems.[15-18] The postreceptor mechanisms that contribute to insulin resistance in late pregnancy occur in skeletal muscle at the β-subunit of the insulin receptor, at the level of insulin receptor substrate-1 (IRS-1), and in the cytoplasm, where increased free intracytoplasmic p85α regulatory subunit of phosphatidylinositol 3-kinase leading to decreased ability of insulin to stimulate the association of catalytic proteins with IRS-1.[19,20] Together, these alterations in insulin signaling likely result in less glucose uptake in skeletal muscle.

Impaired suppression of nonesterified fatty acids (NEFAs) in pregnant women suggests another mechanism for insulin resistance in pregnancy. NEFAs have been demonstrated to impair insulin-stimulated hepatic glucose uptake and whole body glucose disposal.[21] Impaired NEFA suppression to endogenous insulin has been observed in a pregnant population subjected to a hyperinsulinemic clamp.[22] Thus, inappropriately elevated NEFA levels may play a role in the insulin resistance observed in pregnancy. Dysregulation of NEFA has been implicated in both insulin resistance in normal pregnancy and decreased insulin secretion in gestational diabetes, and has been posited as a pathophysiological link between gestational diabetes and the subsequent development of type 2 diabetes (DM2).[23-25]

Fetoplacental Counterregulatory Hormones

Although the molecular mechanisms have yet to be fully elucidated, the fetoplacental unit is clearly responsible for the pregnancy-induced insulin resistance, exerting its effect largely through the production of counterregulatory (anti-insulin) hormones. Insulin promotes the uptake of glucose by adipocytes and muscle cells. Counterregulatory hormones inhibit insulin-mediated glucose uptake by adipocytes and muscle cells, acting largely at a postreceptor level. Such hormones include, among others, GH, hCS, cortisol, and progesterone.

Placental GH and hCS

Placental GH differs from pituitary GH by 13 amino acid (191 nucleotide) substitutions[26] and circulates in at least three isoforms: 22-, 24-, and 26-kDa.[27] Human chorionic somatomammotropins are single-chain proteins produced largely by the syncytiotrophoblast with a high degree of sequence homology to both GH and prolactin. In primates, hCS genes appear to have evolved from a precursor GH gene; in nonprimate species, on the other hand, the placental lactogens appear to have evolved from a precursor

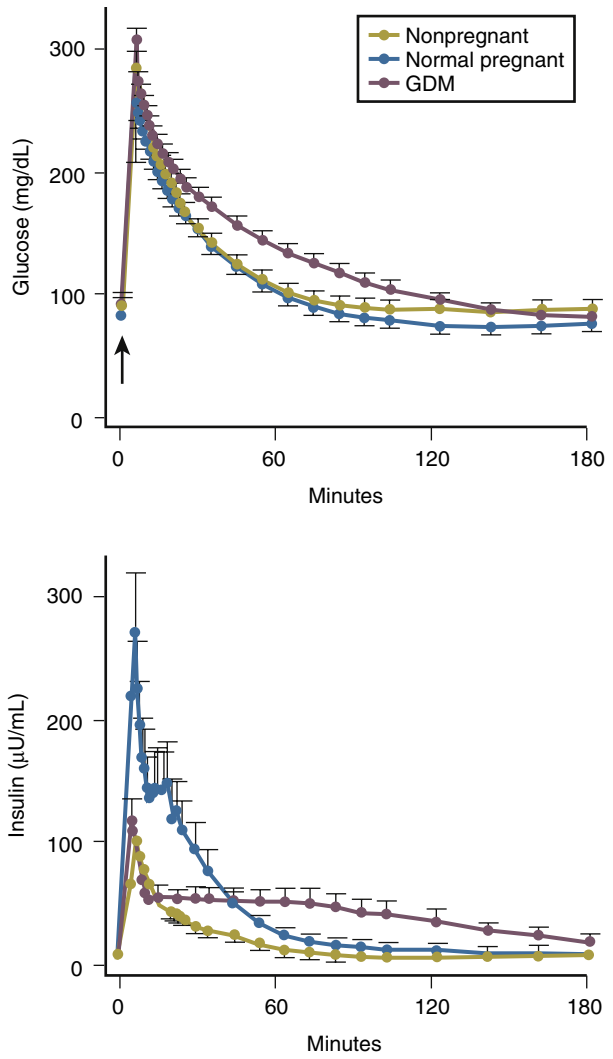


FIGURE 28.2 Insulin and glucose response to an intravenous glucose challenge. Comparison of insulin and glucose responses to a rapid intravenous infusion of glucose (300 mg/kg) in nonpregnant women, pregnant women, and pregnant women with gestational diabetes mellitus (GDM). In GDM, there are both elevated glucose and insulin concentrations, suggesting that the condition is an insulin-resistant state. The *arrow* indicates injection of glucose. *(From Buchanan TA, Metzger BE, Freinkel N, Bergman B. Insulin sensitivity and B-cell responsiveness to glucose during late pregnancy in lean and moderately obese women with normal glucose tolerance or mild gestational diabetes.* Am J Obstet Gynecol *162:1008, 1990.)*

prolactin gene. Because of these differences in evolution, we have chosen to use the term "human chorionic somatomammotropins" to refer to these genes collectively.[28] The dominant isoform of hCS is 191 amino acids in length, with a molecular mass of 23 kDa.[26,29] hCS binds with high affinity to prolactin (PRL) receptors, but with low affinity to GH receptors, suggesting that it functions largely as a lactogen rather than as a somatogen in pregnancy. Conversely, placental GH binds with high affinity to GH receptors but with low affinity to PRL receptors.[30] Thus, by midgestation, the endocrinological mileu is one of high levels of two lactogenic hormones (PRL and hCS), and placental GH, which is acting almost exclusively as a somatogen. hCS is also secreted directly into the fetal circulation, but is present in much lower levels than fetal PRL. Placental GH, however, is detectable only in maternal blood.[31,32]

The factors that regulate GH and hCS synthesis and secretion are not fully understood, but somatostatin and GH-releasing hormone produced by the cytotrophoblast may play inhibitory and stimulatory roles, respectively.[33-35] Additional regulation of hCS may be provided by insulin and angiotensin II, which both stimulate hCS release,[36,37] as well as by dynorphin (Fig. 28.4).[38] This placental endocrine and paracrine-autocrine regulatory system is similar to that observed in the hypothalamic-pituitary axis, which has led Dr. Samuel Yen to refer to the placenta as *the third brain*.[39]

The genes coding for GH and hCS are clustered together in a single region of chromosome 17 in the following order (5′ to 3′): *hGH-N* (pituitary GH gene), *hCSL*, *hCS-A*, *hGH-V* (placental GH gene), and *hCS-B*.[40] The pattern of expression of these genes is tissue-specific and changes throughout gestation. For example, the placenta does not express the *hGH-N* (pituitary GH) gene. Pituitary GH is secreted in a pulsatile fashion from the maternal anterior pituitary, and can be measured in the maternal serum throughout the first trimester of pregnancy.[41] Thereafter, however, pituitary GH secretion progressively declines. By the third trimester of pregnancy, pituitary GH secretion is effectively suppressed and cannot be rescued by induction of a hypoglycemic stimulus or by amino acid infusions (which will be discussed later in this chapter).[42,43] In contrast, circulating concentrations of placental GH—encoded by the *hGH-V* (placental GH) gene and expressed exclusively in the placenta—increase progressively throughout the second and third trimesters of pregnancy.[41,44] Similar differences are seen in the expression of the *hCS* genes. For example, at 8 weeks' gestation, the *hCS-A* and *hCS-B* genes are expressed equally in the placenta; however, at term, expression of *hCS-A* is five times greater than that of *hCS-B*. Results of radioreceptor assay studies suggest that the relative contributions to circulating GH-like activity in pregnancy at term are 85% from *hGH-V* (placental GH), 12% from hCS, and less than 3% from *hGH-N* (pituitary GH).[45] Multiple genes, multiple mRNA species (as in the *hCS-L* and *hGH-V* genes, which generate two distinct mRNA transcripts on the basis of alternative splice-acceptor sites for each gene[46]), and heterogeneity in posttranslational processing result in many isoforms of these key placental hormones. The potential teleological advantages of having multiple placental GH-like genes are to ensure that the placenta can generate sufficient quantities of GH-like hormone to regulate maternal and fetal metabolism, and to minimize the risk of pregnancy failure due to a functional "knockout" of any single gene.

During pregnancy, there is a major transition in the locus of control of the GH axis from the maternal hypothalamic-pituitary unit to the placenta. Circulating levels of placental GH and hCS increase throughout pregnancy. These proteins act through cell surface receptors (GH and prolactin receptors belong to a superfamily of cytokine receptors that share a high degree of sequence homology[28]) to stimulate the production of insulin-like growth factor-1 (IGF-1). Circulating concentrations of IGF-1 in the maternal serum increase throughout pregnancy, reaching a peak near term.[47,48] This increase in IGF-1 has also been observed in a pregnant dwarf with complete pituitary GH deficiency,[42] suggesting

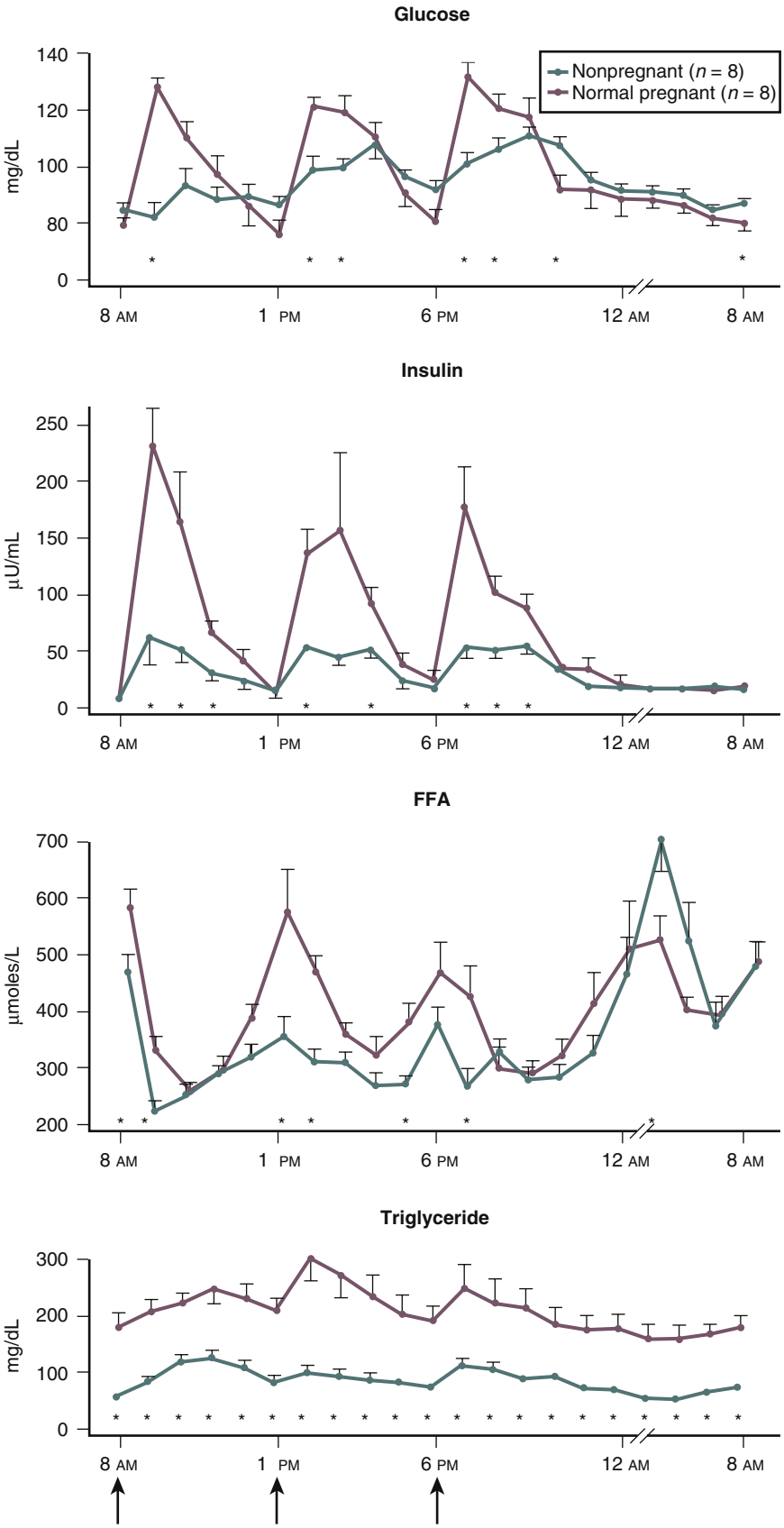


FIGURE 28.3 Effect of pregnancy on carbohydrate and lipid metabolism in the fed and fasting state. Comparison of glucose, insulin, free fatty acid, and triglyceride responses to 24 hours of feeding and fasting in nonpregnant *(open circles)* and normal pregnant women in the third trimester *(closed circles)*. In the fed state *(asterisk)*, pregnancy is associated with elevated levels of circulating glucose and insulin. In the fasting state, pregnancy is associated with decreases in glucose below those that are seen in the nonpregnant state. The *arrows* indicate timing of meals. FFA, free fatty acids. *(From Phelps RL, Metzger BE, Freinkel N. Carbohydrate metabolism in pregnancy.* Am J Obstet Gynecol *140:730, 1981.)*

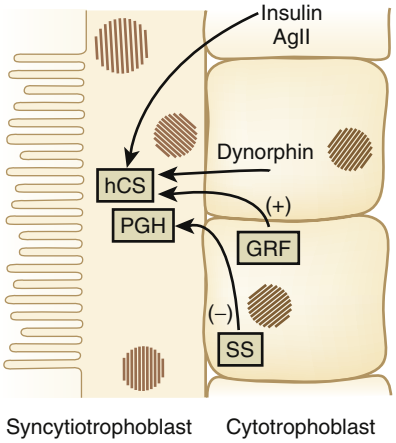


FIGURE 28.4 Regulation of placental counterregulatory hormones. Diagrammatic depiction of a potential placental regulatory system involving growth hormone-releasing factor (CRF), somatostatin (SS), human chorionic somatomammotropin (hCS), and placental growth hormone (PGH). The modulating roles of dynorphin, insulin, and angiotensin II (AgII) are also shown. The placenta has endocrine regulatory systems that parallel those in the maternal and fetal hypothalamic-pituitary units. Yen has proposed that the placenta is a "third brain."

that placental hormones may mediate this effect. Concentrations of IGF-binding protein-1 (IGFBP-1) rise in the first trimester, reach a peak at approximately 12 to 14 weeks of gestation, and thereafter remain stable for the remainder of pregnancy.[49] The level of unbound (bioavailable) IGF-1 therefore increases as pregnancy progresses, and likely contributes to the suppression of *hGH-N* (pituitary GH) gene expression in the latter half of gestation.

In the circulation, GH can exist in a free form or bound to a GH-binding protein (GHBP). Approximately 30% of circulating GH is bound to GHBP and therefore not biologically active.[50] Veldhuis and colleagues[51] proposed that GHBP may serve as a buffer to prevent the level of free (bioavailable) GH from falling too low between secretory pulses. GHBP is the ectodomain of a larger cellular GH receptor and is released into the circulation after proteolytic cleavage of the parent molecule. It is likely, therefore, that circulating concentrations of GHBP parallel that of the cellular GH receptor in important target organs like the liver. This relationship allows for a balance between GH action (mediated through the GH receptor) and inactivation (by binding to the GHBP). The greater the levels of circulating GHBP, the greater the concentration of cellular GH receptors and the greater the sensitivity of cells to the actions of GH. GHBP concentrations tend to decline as gestation advances,[52] although the reason for this change and its physiological significance remain unclear.

This system is not a prerequisite for pregnancy success, because a normal pregnancy is possible in women with Laron dwarfism in which both the GH receptor and GHBP are absent.[53] However, aberrations in this system may be associated with pregnancy-related complications. For example, GHBP levels have been shown to be significantly higher in women with gestational diabetes compared with nondiabetic pregnant women.[54] This observation suggests that the concentration of GH receptors is also increased in women with gestational diabetes, leading to a degree of sensitization to the effects of GH and hCS. An increased sensitivity to the effects of circulating GH could explain many of the endocrine changes observed in gestational diabetes, including insulin resistance, higher serum glucose levels, and an increased incidence of fetal macrosomia.

Differential levels of placental GH and hCS have been noted in normal and pathological pregnancies. Low maternal levels of placental GH and hCS have been noted in pregnancies complicated by hypertension, preeclampsia, and intrauterine growth retardation.[55,56] Conversely, high levels of hCS have been noted in the blood of pregnant mothers with gestational diabetes.[57,58] Placental gene expression profiling has demonstrated different mRNA expression profiles of placental GH and hCS in pregnancies affected by preeclampsia and GDM, compared to normal pregnancies.[59] The same investigators also demonstrated downregulation of the entire GH/hCS cluster in the placenta in pregnancies with small-for-gestational-age newborns, with significantly increased expression of hCS mRNA placental transcripts in pregnancies resulting in large-for-gestational-age newborns.[60] Uteroplacental insufficiency, as seen in preeclampsia and other maternal hypertensive disorders, may lead directly to decreased placental GH expression, resulting in decreased maternal lipolysis and decreased maternal IGF-I.[31]

Circulating levels of placental GH, IGF-1, and the IGFBPs appear to correlate with birth weight. For example, a decrease in both placental GH and IGF-1 levels has been associated with intrauterine growth restriction (IUGR).[61] The decrease in placental GH levels is due both to a decrease in placental mass and a decrease in the density of placental GH-secreting cells.[62] There is also a strong inverse correlation between maternal IGFBP-1 concentrations and birth weight in both term and preterm pregnancies.[63,64] The higher the IGFBP-1 concentration, the lower the circulating level of unbound (bioavailable) IGF-1 and the lower the birth weight. Moreover, several investigators have reported a positive correlation between birth weight and circulating concentrations of IGF-1 in the fetus and neonate.[65,66] Reece and colleagues[66] reported that IGF-1 concentrations were significantly lower in neonates below the mean birth weight for gestational age than levels in neonates above the mean (mean ± SEM: 40 ± 11 versus 86 ± 6 ng/mL, respectively), with no differences in IGF-2 concentrations. Similar findings were reported by Lassarre and colleagues.[65] Conversely, maternal hyperglycemia may increase placental and fetal weight via induction of IGF-2 and fetal hyperinsulinemia.[67] Increased maternal fat stores may reduce plasma adiponectin, and thus increase hCS expression via decreased adiponectin suppression.[68] Increased hCS in the fetal circulation may promote fetal hyperinsulinemia via induction of β-cell replication, increasing fetal weight gain.[31,69,70] Taken together, these data suggest that GH, hCS, IGF-1, as well as their binding proteins (GHBP and the IGFBPs) may play an important role in governing fetal growth and pregnancy outcome, and that these endocrine factors may be regulated by both the mother and the fetoplacental unit.

Cortisol

Cortisol is a potent diabetogenic hormone. It promotes lipolysis in adipocytes and protein breakdown in muscle, leading to an increase in circulating free fatty acids and amino

Table 28.1 Fasting Maternal Glucose, Insulin, Glucagon, Amino Acid, Alanine, Free Fatty Acid, and Cholesterol Concentrations in Late Pregnancy Compared with the Nonpregnant State

	Measurement (mean ± SEM)	
	Nonpregnant State	Late Pregnancy
Glucose (mg/dL)	79 ±2.4	68 ± 1.5*
Insulin (μ/mL)	9.8 ± 1.1	16.2 ± 2.0*
Glucagon (pg/mL)	126 ± 6.1	130 ± 5.2
Amino acids (μM)	3.82 ± 0.13	3.18 ± 0.11*
Alanine (μM)	286 ± 15	225 ± 9*
Free fatty acids (mg/dL)	76 ± 7	181 ± 10*
Cholesterol (mg/dL)	163 ± 8.7	205 ± 5.7*

*$P < .05$.

Data from Freinkel N, Metzger BE, Nitzan M, et al. Facilitated anabolism in late pregnancy: some novel maternal compensations for accelerated starvation. In Malaisse WJ, Pirart J (eds). Diabetes International Series 312. Amsterdam: Excerpta Medica, 1973, p 474.

acids.[71] Levels of adrenocorticotropic hormone (ACTH) and cortisol increase in pregnancy.[72] The increase in ACTH is due, at least in part, to an increase in CRH production by the placenta (discussed later in this chapter). Much of the increase in total cortisol concentration in pregnancy is due to the excessive production of corticosteroid-binding globulin by the liver under the influence of estrogen. However, there is also a significant increase in urinary free cortisol excretion during pregnancy, suggesting that circulating levels of free cortisol may also be increased.[73] The relative contribution of an increase in circulating free cortisol to the insulin resistance of pregnancy is unclear.

Progesterone

High concentrations of progesterone have been shown to cause insulin resistance in cells in culture and in laboratory animals by decreasing insulin receptor number and causing a postreceptor defect in insulin action, which has yet to be fully elucidated.[74-76] High circulating concentrations of progesterone may likely contribute to the pregnancy-related insulin resistance.

Fasting State

In nonpregnant women, there is a constant need to maintain circulating glucose concentrations for use by the brain. During an overnight fast, glucose is released from the liver by both glycogenolysis (breakdown of glycogen stores [75%]) and gluconeogenesis (production of glucose from circulating metabolical precursors [25%]). The precursors for gluconeogenesis include pyruvate, alanine (from muscle), glycerol (from the breakdown of triglycerides in adipose tissue), and lactate (from anaerobic metabolism).

Pregnancy is associated with an increased demand for glucose and alanine, both of which are required by the developing fetus. As such, the fasting state in pregnancy is characterized by a rapid and often severe decrease in maternal serum glucose and alanine concentrations. Associated with these changes is an increase in the circulating levels of free fatty acids (derived from triglyceride breakdown in adipose cells) and ketone bodies (Fig. 28.3; Table 28.1).[77,78] The hyperketonemia that characterizes late pregnancy is the result of enhanced lipolysis, which is likely due, in turn, to the insulin resistance in adipocytes caused primarily by the placental counterregulatory hormones.

In pregnancy, the acceleration of lipid catabolism during fasting helps the mother to rely on fat as a major energy source, thereby minimizing protein catabolism (preserving muscle mass) and allowing both glucose and amino acids to be used preferentially by the fetus.[8] These metabolical adaptations have been termed "accelerated starvation" by Freinkel.[79] Although a useful descriptive term, this characterization of pregnancy is largely inaccurate because fat mass is known to increase significantly during pregnancy.[80]

Fed State

The fetus is a thief! Many of the metabolical and endocrine adaptations associated with pregnancy are designed to maintain a preferential and uninterrupted supply of metabolical fuel from mother to fetus as dictated by the progressively increasing demands of the growing fetus. The placenta is relatively impermeable to fat, but readily transports glucose, amino acids, and ketone bodies from the maternal to the fetal circulation.

Pregnancy is associated with hyperlipemia, both in the fasting and the fed states. Total plasma lipid concentrations increase progressively after 24 weeks of gestation. Increases in triglycerides, cholesterol, and free fatty acids are significant (Fig. 28.5 and Fig. 28.6; Table 28.1).[8,78,81,82] High-density lipoprotein cholesterol levels rise during early pregnancy, and low-density lipoprotein cholesterol concentrations increase in later pregnancy.[81,82]

In pregnancy, an oral glucose load is associated with a greater increase in circulating glucose concentration, a smaller decline in free fatty acids, and a larger increase in serum triglycerides than that seen in the nonpregnant state (Fig. 28.5).[8,78] A similar effect has been observed in pregnancy after meals (Fig. 28.3).[8] These adaptations allow the mother to use primarily available triglycerides, glycerol, and free fatty acids for metabolical fuel after meals, and to preserve glucose and amino acids for preferential use by the fetus (Fig. 28.7). The cause of these metabolical changes is likely the lipolytic action of the placental counterregulatory hormones (GH, hCS, cortisol, and progesterone), which serve to promote lipolysis during fasting and hypertriglyceridemia in the fed state.

Pancreatic β-Cells: The Missing Link

To a point, pancreatic β-cells will respond to insulin resistance with increased insulin secretion. Bergman and colleagues first characterized a predictable hyperbolic relationship between the quantity of insulin produced by β-cells and tissue sensitivity to insulin.[83] The disposition index, or the "hyperbolic correction," is a measure of insulin secretion corrected for insulin resistance.[84] A left-shifted curve, representing decreased compensatory insulin secretion for a given level of insulin resistance, may be seen in women who develop both gestational diabetes (GDM) and type 2 diabetes mellitus (DM2). Buchanan posits that insulin resistance actually causes the β-cell dysfunction observed in GDM, and that chronic insulin resistance leading to β-cell failure may be the mechanism by which women with GDM progress to DM2.[85]

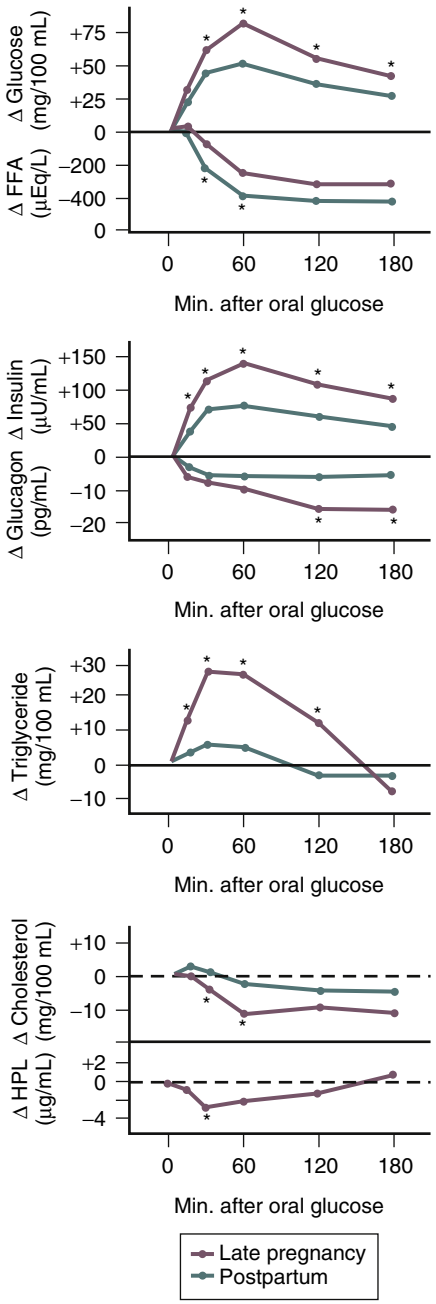


FIGURE 28.5 Effect of pregnancy on short-term carbohydrate and lipid metabolism following an oral glucose challenge. Differences in the central metabolical response to an oral glucose load in pregnant *(closed circles)* and non-pregnant *(open circles)* subjects. FFA, free fatty acids; HPL, human placental lactogen (chorionic somatomammotropin). *(From Freinkel N, Metzger BE, Nitzan M, et al. Facilitated anabolism in late pregnancy: some novel maternal compensations for accelerated starvation. In Malaisse W], Pirart J [eds]. Diabetes International Series 312. Amsterdam:* Excerpta Medico, *1973, p 474.)*

Gestational Diabetes

Depending on the patients screened and the diagnostic criteria used, GDM complicates approximately 7% of all pregnancies, resulting in at least 200,000 cases annually in the United States alone.[86] The American Diabetes Association (ADA) classification of diabetes mellitus is summarized in Box 28.1.[86] Gestational diabetes mellitus (GDM)

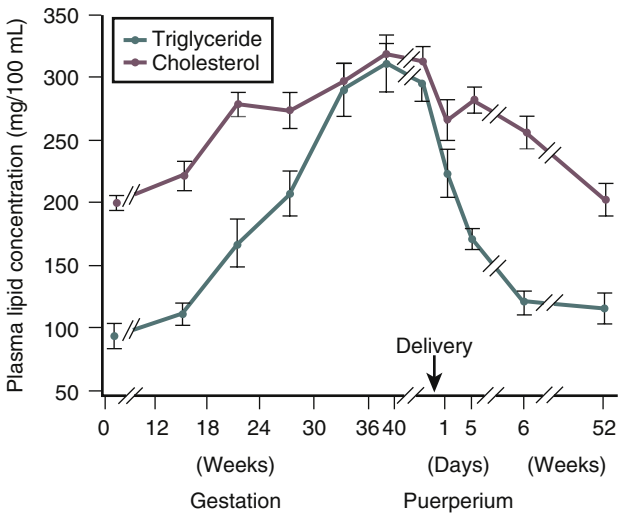


FIGURE 28.6 Changes in plasma cholesterol and triglyceride concentrations during pregnancy and in the puerperium. Fasting lipid concentrations were measured serially throughout pregnancy, at delivery, in the puerperium, and at 12 months. The results are the mean + SEM. *(From Potter JM, Nestel PJ. The hyperlipidemia of pregnancy in normal and complicated pregnancies.* Am J Obstet Gynecol *133:165, 1979.)*

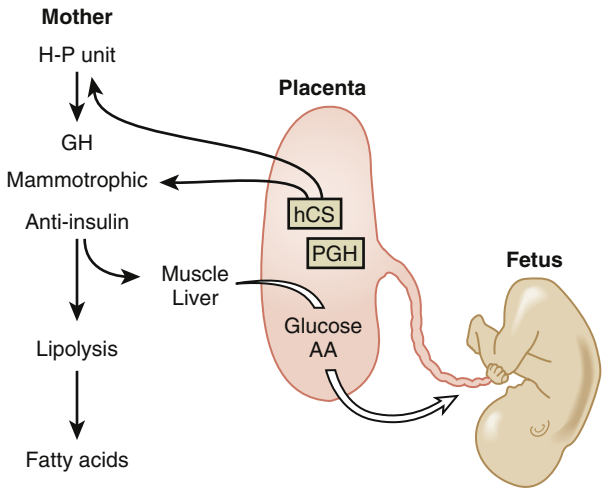


FIGURE 28.7 Effect of pregnancy on maternal carbohydrate metabolism. The proposed functional role of human chorionic somatomammotropin (hCS) and placental growth hormone (PCH) in the adjustment of maternal metabolical homeostasis with preferential transfer of amino acid (AA) and glucose to the fetus. Maternal metabolism relies on triglycerides and fatty acids.

has previously been defined as any degree of carbohydrate intolerance with the onset of pregnancy or first recognized during pregnancy.[19] The American Congress of Obstetricians and Gynecologists (ACOG) still endorses this terminology.[87] Due in part to the increasing prevalence of obesity among young women, an increasing proportion of women will have unrecognized type 2 diabetes at the time of screening for gestational diabetes. To address this increased potential for prepregnancy diabetes, in 2010 the International Association of Diabetes and Pregnancy Study Group (IADPSG) recommended changing the classification of diabetes diagnosed during pregnancy to overt or gestational.[88]

Box 28.1 American Diabetes Association (ADA) Classification of Diabetes Mellitus

TYPE 1 DIABETES MELLITUS
Caused by β-cell dysfunction, usually leading to absolute insulin deficiency

TYPE 2 DIABETES MELLITUS
Typically associated with peripheral insulin resistance and varying degrees of abnormalities in insulin secretion

GESTATIONAL DIABETES MELLITUS
Characterized by peripheral insulin resistance resulting from counterregulatory (anti-insulin) hormones produced by the placenta

OTHER TYPES
Genetic defects in carbohydrate metabolism
Diseases of the exocrine pancreas
Endocrinopathies
Drug-induced or chemical-induced diabetes
Infections
Uncommon forms of immune-mediated diabetes
Genetic syndromes sometimes associated with diabetes

The American Diabetes Association (ADA) endorsed this recommendation.[86] Overt diabetes would be diagnosed at the initial prenatal visit in women who met any of the following criteria: (1) fasting plasma glucose ≥126 mg/dL [7.0 mmol/L], (2) hemoglobin A1C ≥6.5%, or (3) random plasma glucose ≥200 mg/dL [11.1 mmol/L], followed by a confirmatory elevated fasting plasma glucose or A1C as previously described.

Screening for Gestational Diabetes

Patients with GDM are typically asymptomatic. Some experts and organizations have recommended screening all pregnant women for GDM.[89-93] The World Health Organization has recommended universal screening and utilizing a 75-g, 2-hour oral glucose tolerance test (OGTT) for screening and diagnosis.[91] The United Kingdom has also adopted the 2-hour OGTT for screening and diagnosis, but does not recommend universal screening.[94] ACOG has endorsed a selective risk-based approach to screening, utilizing a 50-g 1-hour glucose challenge test (GCT), followed by a 100-g 3-hour OGTT for diagnosis if GCT is positive.[87,95] The United States Preventive Services Task Force and the Canadian Task Force on Preventive Health Care both concluded there is insufficient evidence to recommend universal screening for gestational diabetes.[96,97] The most recent IADPSG study group recommendations recommend universal screening at 24 to 28 weeks' gestation with the 75-g, 2-hour OGTT, and screening high-risk women for pregestational diabetes with fasting or random plasma glucose, or hemoglobin A1C at the first prenatal visit.[19] The low-risk category that some recommend excluding from GDM screening includes women under age 25 who have normal body mass index (BMI), who have no first-degree relatives with the condition, and who are not members of ethnic or racial groups with a high prevalence of diabetes (Hispanic, Native American, Asian, or African American). Identifying and deferring screening in these low-risk women will decrease the overall number of patients screened by approximately 10% and will only overlook 3% of all women with GDM.[98]

Table 28.2 Glucose Screening for Gestational Diabetes with the 50-g Oral Glucose Load Test

Glucose Cutoff	Proportion of Women with a Positive Test	Sensitivity for Gestational Diabetes Mellitus
≥140 mg/dL (≥7.8 mmol/L)	14%-18%	≈80%
≥130 mg/dL (≥7.2 mmol/L)	20%-25%	≈90%

Data from Kjos SL, Buchanan TA. Gestational diabetes mellitus. *N Engl J Med* 341:1749, 1999.

Screening traditionally has been performed at 24 to 28 weeks of gestation.[99,100] For women at risk for undiagnosed type 2 diabetes (women with a history of gestational diabetes or polycystic ovarian syndrome, a high BMI, persistent glycosuria, a strong family history of diabetes, a prior macrosomic infant, or a prior unexplained late fetal demise), early screening for GDM should be performed at the first prenatal visit.[86] If the early screen is negative, the screen should be repeated at 24 to 28 weeks.

The Two-Step Approach

At this time, there is no universal accepted standard for screening and diagnosis of diabetes in pregnancy. Screening for diabetes in pregnancy has traditionally been performed as a two-step approach. The first step utilizes the *glucose load test* (GLT), also known as the *glucose challenge test* (GCT). First proposed as a screening test for GDM by O'Sullivan and colleagues in 1973,[101] the GLT is a nonfasting 50-g oral glucose challenge followed by a venous plasma glucose measurement at 1 hour.[99,102-104] The GLT is considered positive if the 1-hour glucose measurement is greater than a previously agreed threshold. Both 130 mg/dL and 140 mg/dL have been suggested.[99,105] Use of a lower cutoff will increase the detection rate of women with GDM, but will result in a substantial increase in the false-positive rate (Table 28.2).[105,106] There is no absolute GLT cutoff that should be regarded as diagnostic of GDM.[99,105-108]

The second step of the two-step approach requires a three-hour *glucose tolerance test* (GTT), which is only performed if the GLT is positive. A fasting glucose measurement of at least 105 mg/dL in the setting of a positive GLT is highly predictive of an abnormal GTT.[108] In pregnancy, the GTT involves an overnight fast and subsequent 100-g oral glucose challenge. Venous plasma glucose is measured fasting and at 1 hour, 2 hours, and 3 hours after the glucose load.

Although there is general agreement that two or more abnormal values are required to confirm the diagnosis,[99,105] there is little consensus about the glucose values that define the upper range of normal in pregnancy and no threshold that perfectly predicts adverse pregnancy outcome. Even one elevated value has been associated with an increased incidence of macrosomia and birth injury. A secondary analysis of a treatment trial for mild gestational diabetes found that elevations in fasting glucose and 3-hour GTT levels were associated with adverse pregnancy and neonatal

Table 28.3 Diagnostic Thresholds for Gestational Diabetes During 100-g Glucose Tolerance Test

	Plasma Glucose Values mg/dL (mmol/L)		
	National Diabetes Data Group*	Sacks et al#	Carpenter and Coustan§
Fasting	105 (5.8)	96 (5.3)	95 (5.2)
1-hour	190 (10.6)	172 (9.4)	180 (9.9)
2-hour	165 (9.2)	152 (8.3)	155 (8.6)
3-hour	145 (8.1)	131 (7.2)	140 (7.7)

*National Diabetes Data Group. Classification and diagnosis of diabetes and other categories of glucose intolerance. *Diabetes* 28:1039, 1979; #Sacks DA, Abu-Fadil S, Greenspoon JS, Fotheringham N. Do the current standards for glucose tolerance testing in pregnancy represent a valid conversion of O'Sullivan's original criteria? *Am J Obstet Gynecol* 161:638, 1989; §Carpenter MW, Coustan DR. Criteria for screening tests for gestational diabetes. *Am J Obstet Gynecol* 144:768, 1982.

outcomes.[109] The same study found that fasting glucose of 90 mg/dL or greater and 1-hour value of 165 mg/dL or greater were associated with an increased risk for adverse neonatal outcomes, while a 1-hour value of 150 mg/dL or greater was associated with an increased risk for large for gestational age neonates. Two- and 3-hour glucose levels were not associated with an increased risk for adverse outcomes until far beyond current diagnostic thresholds, however, suggesting a need to reevaluate current diagnostic cutoffs for the 3-hour GTT (Table 28.3).

The One-Step Approach

In 2010, the one-step approach to diagnosing diabetes in pregnancy was proposed by IADPSG.[88] This approach was subsequently endorsed by the ADA,[86] but not by ACOG.[87] The IADPSG approach advocates diagnosing gestational diabetes in women who meet either of the following criteria: (1) fasting plasma glucose ≥92 mg/dL (5.1 mmol/L), but less than 126 mg/dL (7.0 mmol/L) at any gestational age, or (2) at least one abnormal result on a 75 gram 2-hour oral glucose tolerance test, which could be administered at 24 to 28 weeks of gestation. Abnormal values are defined as: (1) fasting plasma glucose ≥92 mg/dL (5.1 mmol/L), but less than 126 mg/dL (7.0 mmol/L) or (2) 1-hour value ≥180 mg/dL (10.0 mmol/L) or (3) 2-hour value ≥153 mg/dL (8.5 mmol/L) (Box 28.2).[88] This interpretation of the 2-hour OGTT results marks a departure from both the WHO criteria (in which only an abnormal fasting or 2-hour plasma glucose can be used for diagnosis), and the current ADA criteria (which require two abnormal values for the diagnosis of gestational diabetes). To reflect the continuous association between maternal glycemic concentrations and adverse perinatal outcomes seen in the Hyperglycemia and Adverse Pregnancy Outcomes (HAPO) study data, the IADPSG-selected thresholds for the 2-hour OGTT represent the average glucose values at which there were 1.75 times the odds of infant birth weight, cord blood C-peptide (a proxy for fetal hyperinsulinemia), and percent neonatal body fat greater than the 90th percentile.[110] Women with one or more values above the thresholds above had a two-fold higher frequency of preeclampsia and large for gestational age infants, and more than a 45% increase in preterm delivery and primary cesarean delivery. The algorithm for screening and diagnosis of GDM proposed by IADPSG is outlined in Box 28.2.

Box 28.2 Strategy for the Detection and Diagnosis of Hyperglycemic Disorders in Pregnancy

FIRST PRENATAL VISIT

- Measure FPG, A1C, or random plasma glucose on all or only high-risk women†
- If results indicate overt diabetes (FPG ≥ 7.0 mmol/L [126 mg/dL], A1C ≥ 6.5%, random plasma glucose ≥11.1 mmol/L [200 mg/dL] with confirmation):
  - Treat and follow-up as for preexisting diabetes
- If results not diagnostic of overt diabetes and FPG ≥ 5.1 mmol/L (92 mg/dL) but <7.0 mmol/L (126 mg/dL):
  - Diagnose as GDM
- If results not diagnostic of overt diabetes and FPG < 5.1 mmol/L (92 mg/dL):
  - Test for GDM from 24 to 28 weeks' gestation with a 75-g OGTT‡

24-28 WEEKS' GESTATION: DIAGNOSIS OF GDM

- 2-hour, 75-gram OGTT: perform after overnight fast on all women not previously found to have overt diabetes or GDM during testing earlier in this pregnancy:
  - Diagnose overt diabetes if FPG ≥ 7.0 mmol/L (126 mg/dL)
  - Diagnose GDM if one or more value equals or exceeds the following thresholds:
    - FPG ≥5.1 mmol/L or 92 mg/dL
    - 1-hour plasma glucose ≥10.0 mmol/L or 180 mg/dL
    - 2-hour plasma glucose ≥8.5 mmol/L or 153 mg/dL
- OGTT is normal if all values are less than above thresholds

Data and format adapted from Metzger, BE et al. for the International Association of Diabetes and Pregnancy Study Groups Consensus Panel. International Association of Diabetes and Pregnancy Study Groups Recommendations on the Diagnosis and Classification of Hyperglycemia in Pregnancy. *Diabetes Care* 2010;33(3):676-682.[88]

Guidelines are to be applied to women without known diabetes antedating pregnancy. Postpartum glucose testing should be performed for all women diagnosed with overt diabetes during pregnancy or GDM.

†Decision to perform evaluation for glycemia on all pregnant women at first prenatal visit or only on women with characteristics indicating a high risk for diabetes is to be made on the basis of the background frequency of abnormal glucose metabolism in the population and on local circumstances.

‡The IADPSG panel concluded there is insufficient evidence to know whether there is a benefit of generalized testing to diagnose and treat GDM before the usual 24-28 weeks' gestation.

FPG, fasting plasma glucose; GDM, gestational diabetes mellitus; OGTT, oral glucose tolerance test.

The utilization of IADPSG diagnostic criteria for overt and gestational diabetes would result in 18% of women being diagnosed with diabetes in pregnancy.[88] The potential long-term economic impact of implementing the IADPSG guidelines is unknown at this time, but in the short-term, healthcare costs would likely be increased. Including the costs of long-term health intervention with diet and exercise, a recent cost-effectiveness analysis estimated that for every 100,000 pregnancies, the IADPSG approach would increase costs by over $125,600,000.[111] This analysis concluded that the IADPSG screening recommendations are cost effective only if post-delivery care reduces diabetes incidence.

Identification of overt diabetes early in pregnancy is important due to the associated increased risk of congenital

anomalies, and maternal complications such as nephropathy and retinopathy. In contrast, GDM poses little immediate risk to the mother. Such women are not at risk of diabetic ketoacidosis (DKA), which is primarily a disease of absolute insulin deficiency, but are at increased risk for developing hypertensive disorders of pregnancy compared to women without GDM.[112,113] GDM has been associated with a variety of perinatal and neonatal complications, including increased risk of macrosomia, operative delivery, shoulder dystocia, birth trauma, hypoglycemia, hyperbilirubinemia, hypocalcemia, and perinatal mortality.[99,114] In 2008, the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) Study Cooperative Research Group reported the results of a multinational, multicenter study that examined the relationship between maternal hyperglycemia and adverse pregnancy outcomes.[110] The study demonstrated a continuous relationship between maternal hyperglycemia and large-for-gestational-age infants, cord blood C-peptide, neonatal hypoglycemia, and cesarean delivery. The risk of these adverse outcomes increased continuously as fasting plasma glucose levels increased from 75-mg/dL range, and as the 1- and 2-hour oral GTT values increased.

Transplacental glucose transport is a facilitated process that is mediated by the glucose transporter isoform GLUT-1.[115,116] Some researchers have hypothesized that maternal hyperglycemia in diabetes increases placental glucose transfer, resulting in fetal hyperglycemia and increased fetal insulin concentration that in turn stimulates fetal growth.[117] However, even in diabetic pregnancies with evidence of strict glycemic control, fetal macrosomia (defined as an estimated fetal weight of at least 4500 g[118]) is not uncommon,[119-121] which suggests a complex relationship between metabolical derangement and fetal growth in diabetes.[122] Other factors that may contribute to fetal macrosomia include obesity and high circulating levels of amino acids and lipids.[105]

Many of the complications of GDM are due to fetal macrosomia. Increased birth weight is associated with an increased risk of cesarean delivery, operative vaginal delivery, and birth injury to both the mother (vaginal, perineal, and rectal trauma) and fetus (including orthopedic and neurological injury).[99,101,105,114,123-125] Shoulder dystocia with resultant brachial plexus injury are serious consequences of fetal macrosomia, and are further increased in the setting of GDM because the macrosomia of diabetes is associated with increased diameters in the upper thorax of the fetus. Recent data also suggest that offspring of mothers with both gestational and prepregnancy diabetes may be predisposed to develop obesity, diabetes, and hypertension, via intrauterine programming/epigenetic modification.[126-130] For example, a study of 9439 children aged 5 to 7 years old evaluated the prevalence of childhood obesity relative to maternal glycemia in pregnancy.[130] This study found that maternal fasting glucose level of at least 95 mg/dL on a 3-hour 100-gram oral glucose tolerance test, gestational diabetes by Carpenter and Coustan criteria, and a top quartile 50-g, 1-hour glucose challenge test result were all associated with childhood obesity. Treatment of GDM reduced the rates of childhood obesity to rates similar to that of offspring born to women with normal glucose challenge tests, but this effect was only observed among offspring with birthweight of 4000 g or less. The authors concluded that maternal hyperglycemia resulted in metabolical imprinting for childhood obesity in offspring.

Interventions that definitively decrease the risk of macrosomia and subsequent adverse outcomes are limited. Two multicenter randomized clinical trials have demonstrated that the treatment of mild hyperglycemia in pregnancy reduces neonatal morbidity and macrosomia.[131,132] The Australian Carbohydrate Intolerance Study in Pregnant Women Trial (ACHOIS) demonstrated that treating pregnant women with impaired glucose tolerance on a 75-g OGTT resulted in a significant reduction in a composite perinatal outcome including perinatal death, shoulder dystocia, orthopedic injury and nerve palsy.[131] In the ACHOIS trial, glycemic control was achieved through a combination of dietary counseling, four times daily home blood glucose monitoring (maintaining fasting glucose levels less than 99 mg/dL and 2-hour postprandial levels less than 126 mg/dL), and insulin for persistent hyperglycemia. There was a reduction in the diagnosis of macrosomia (21% to 10%), but an increase in intensive care unit (ICU) admissions (61% to 71%). Induction of labor was increased in the intervention groups (from 29% to 39%) with no increase in the cesarean delivery rate, which was stable at 31% to 32%. In 2009, a second randomized controlled trial also demonstrated improved outcomes in women with glucose intolerance on a 100-g OGTT.[132] Landon and colleagues reported that glycemic control achieved through a combination of dietary counseling, four times daily home blood glucose monitoring (maintaining fasting glucose levels less than 95 mg/dL and 2-hour postprandial levels less than120 mg/dL), and insulin for persistent hyperglycemia did not result in a significant reduction in combined neonatal morbidity and mortality—a composite outcome including stillbirth, perinatal death, neonatal hyperbilirubinemia, hypoglycemia, hyperinsulinemia, and birth trauma. Treatment of glucose intolerance did, however, result in significant reductions in mean neonatal birth weight and fat mass, reduced frequency of large for gestational age neonates (14.5% to 7%), birth weight greater than 4000 g (14% to 6%), shoulder dystocia (4% to 1.5%) cesarean delivery (34% to 27%), and pregnancy-induced hypertension (14% to 9%), compared to usual care.

GDM cannot be prevented. The goal of antepartum management is to prevent fetal macrosomia and its resultant complications by maintaining maternal blood glucose at desirable levels throughout gestation (fasting, below 95 mg/dL; 1-hour postprandial, below 140 mg/dL or 2-hours postprandial, below 120 mg/dL).[99] Initial recommendations should include a diabetic diet consisting of 30 to 35 kcal/kg of ideal body weight given as 40% to 50% carbohydrate, 20% protein, and 30% to 40% fat to avoid protein catabolism. Daily home glucose monitoring and weekly antepartum visits to monitor glycemic control should also be instituted. If diet alone does not maintain blood glucose at desirable levels, insulin administration may be required.[133] If initial fasting glucose levels are consistently greater than 95 mg/dL and 2-hour postprandial values over 120 mg/dL, insulin therapy can be started right away with every effort made to avoid iatrogenic hypoglycemia.[134,135] The Fifth International Workshop on Gestational Diabetes[19] recommended exercise as an adjunct to diet for the treatment of GDM, although randomized studies have failed to demonstrate a beneficial effect of exercise on glycemic control in women with GDM.[136]

In the United States, oral hypoglycemic agents (sulfonylureas in particular) have not traditionally been recommended for use during pregnancy because of the possibility of fetal teratogenesis and prolonged neonatal hypoglycemia. This class of drugs works by stimulating pancreatic β-cells to synthesize and release insulin. Because the adverse fetal consequences of GDM are likely related to fetal hyperinsulinemia, any agent that could cross the placenta and increase fetal insulin production should be used with caution in pregnancy. First-generation sulfonylureas have been shown to cross the placenta and, as such, are contraindicated in pregnancy. There are conflicting data regarding the transplacental passage of second-generation sulfonylurea agents (glyburide and glipizide). The majority of human studies to date have demonstrated minimal fetal exposure,[137-139] likely secondary to high protein binding[140] and possibly active transport of glyburide from the fetal to the maternal circulation.[141,142] One study reported umbilical cord blood concentrations of glyburide as high as 70% of maternal serum concentrations,[143] but the majority of studies have found that cord blood concentrations of glyburide range from undetectable,[144] to 1% to 2% of maternal serum concentrations.[145,146]

Congenital malformations associated with the use of oral sulfonylurea drugs in pregnancy have been described,[147] but most of these reports failed to take into account maternal glycemic control. More recent studies have shown that the risk of malformations correlates strongly with the degree of glycemic control at the time of conception and is unrelated to the type of anti-diabetic therapy.[148] Moreover, such reports refer to oral hypoglycemic treatment in early pregnancy, whereas treatment for GDM only begins after the period of fetal organogenesis thereby eliminating any concern regarding malformations due to treatment alone.

Between 1974 and 1983, Coetzee and Jackson[149] treated 423 women with a new diagnosis of diabetes in pregnancy with oral hypoglycemic agents, and found no cases of serious neonatal hypoglycemia and no increase in perinatal mortality. A clinical trial by Langer and colleagues[144] randomized 404 women with singleton pregnancies and GDM requiring treatment to either glyburide or insulin therapy. Results showed no difference in glycemic control or neonatal outcome (including congenital malformations, macrosomia, neonatal hypoglycemia, or admission to neonatal intensive care). Eight women (4%) in the glyburide group required insulin therapy. Glyburide was not detected in the cord blood of any of the infants in the glyburide group. Jacobsen and colleagues performed a retrospective analysis comparing 236 women taking glyburide to 268 women taking insulin for control of gestational diabetes unresponsive to diet therapy.[150] Women in the insulin group had a higher mean BMI and higher mean fasting value on GTT, suggesting these women may have had a greater predisposition to poor glycemic control. The study reported no significant differences between groups in birth weight, macrosomia, or cesarean delivery. More women in the glyburide group achieved mean fasting and postprandial goals (86% versus 63%), and their neonates were less likely to be admitted to the neonatal intensive care unit (NICU) compared to women taking insulin (15% versus 24%). Women treated with glyburide, however, had a higher incidence of preeclampsia (12% versus 65%) and their neonates were more likely to receive phototherapy (9% versus 5%). From the standpoint of maternal safety, hypoglycemia is the most commonly reported maternal side effect. Langer and colleagues reported that 2% of patients taking glyburide had blood glucose measurements less than 40 mg/dL, compared with 20% of patients taking insulin.[144] Other studies reported no significant difference in rates of maternal hypoglycemia between women taking glyburide compared to insulin.[151,152] While the limited data currently available do not permit firm conclusions to be drawn about the efficacy and safety of oral hypoglycemic drugs in pregnancy, these agents are being used more frequently by obstetric care providers.[153]

Unlike glyburide, metformin is known to cross the human placenta,[154] which has reduced enthusiasm for its use during pregnancy. Metformin is a biguanide agent that acts by reducing peripheral insulin resistance and inhibiting gluconeogenesis. Since insulin acts as a potent growth factor, there is a theoretical concern that metformin passage across the placenta may lead to excessive fetal growth. Despite these concerns, metformin is now commonly being used by reproductive endocrinologists to achieve pregnancy in women with polycystic ovarian syndrome (PCOS).[155] Although *in vitro* models[156,157] and *in vivo* studies[154] have shown that metformin does cross from the maternal to the fetal compartment, multiple small retrospective studies have failed to show any significant adverse outcome with first trimester metformin exposure.[155,158,159]

The largest randomized clinical trial to date investigating the efficacy of metformin compared to insulin for treatment of gestational diabetes is the Metformin in Gestational Diabetes Trial (MiG).[160] In this randomized, open-label trial, 363 women were randomized to metformin and 370 to insulin, with 46% of women on metformin requiring supplemental insulin. There was no significant difference in perinatal complications between treatment groups. The primary composite outcome of neonatal hypoglycemia, respiratory distress, need for phototherapy, birth trauma, 5-minute Apgar score less than 7, or prematurity was 32% in both the metformin and the insulin group. Women preferred metformin to insulin therapy (77% versus 27%), and there were no serious adverse events associated with the use of metformin. Rowan and colleagues also followed the infants from the MiG study to the age of 2 years old in the offspring follow-up (TOFU) study, and found that children born to women taking metformin had larger measures of subcutaneous fat, but overall body fat was the same as in children whose mothers were treated with insulin alone.[161] The authors concluded that further follow-up is needed to determine whether children exposed to metformin will develop less visceral fat and therefore be more insulin sensitive than children exposed to insulin alone. A smaller open-label study randomized 100 women with GDM to therapy with insulin or metformin, finding no significant differences in the incidence of large for gestational age, mean birthweight, or neonatal morbidity.[162] Thirty-two percent of women randomized to metformin required supplemental insulin; these women tended to have higher BMIs, higher fasting blood glucose levels, and required medical therapy for GDM earlier than those women who achieved adequate glycemic control with metformin alone. Thus, metformin may provide a reasonable alternative to insulin in the

Table 28.4 White Classification of Diabetes in Pregnancy

White Class	Age of Onset (yr)	Duration (yr)	Vascular Disease	Therapy
A	Only in pregnancy	Only in pregnancy	No	A1: diet controlled A2: insulin requiring
B	>20 or	<10	No	Insulin
C	10-19 or	10-19	No	Insulin
D	<10 or	>20	Benign retinopathy hypertension	Insulin
F	Any	Any	Nephropathy	Insulin
R	Any	Any	Proliferative retinopathy	Insulin
H	Any	Any	Atherosclerotic heart disease	Insulin
T	Any	Any	Renal transplant	Insulin

treatment of gestational diabetes, particularly in lean or moderately overweight women who develop GDM later in gestation.

A recent randomized trial comparing metformin to glyburide in 149 patients with GDM found that use of glyburide was more likely to result in adequate glycemic control.[163] Insulin was required to achieve adequate glycemic control in 35% of patients taking metformin but only 16% of those randomized to glyburide. A systematic review concluded that maternal glucose levels did not differ substantially between pregnant women treated with oral diabetes agents versus insulin, and that the use of glyburide or metformin was not associated with an increase in adverse maternal or neonatal outcomes compared to the use of insulin.[164] The ADA and ACOG currently do not endorse the use of oral anti-hyperglycemic agents during pregnancy.[95,165]

The risk of stillbirth is increased in women with poorly-controlled GDM,[99,105,125] although it is not clear whether this is true also of pregnancies with mild disease.[166,167] For this reason, many centers recommend weekly fetal testing starting at 32 weeks and early delivery (typically at 38 to 40 weeks) for women with GDM who require oral or insulin therapy and those with a pregnancy complication (macrosomia, polyhydramnios, or hypertension). Whether weekly fetal testing and early delivery is necessary in pregnancies complicated only by diet-controlled GDM is not clear. Sonographic estimation of fetal weight should be considered at 36 to 38 weeks' gestation. The use of prophylactic elective cesarean delivery to reduce the risk of maternal and fetal birth injury in the setting of fetal macrosomia remains controversial.[99] It is clear, however, that induction of labor for so-called "impending macrosomia" does not decrease the risk of cesarean delivery or intrapartum complications.[168,169] In labor, maternal glucose levels in pregnancies complicated by GDM should be maintained at 100 to 120 mg/dL to minimize the risk of fetal hypoxic injury. For the same reason, neonatal blood glucose levels should be measured within 1 hour of birth, and early feeding should be encouraged. Delivery of the fetus and placenta effectively removes the source of the anti-insulin hormones that cause GDM. As such, no further management is required in the immediate postpartum period.

GDM frequently indicates underlying insulin resistance. Fifty percent of women with GDM will experience GDM in subsequent pregnancies, and 30% to 65% will develop type 2 diabetes later in life.[105,170-172] All women with GDM should, therefore, have a standard (nonpregnant) 75-g GTT approximately 6 weeks postpartum and should consider preventative and early diagnostic strategies like weight reduction, increased exercise, and regular screening for diabetes.

Pregnancy in Women with Type 1 or Type 2 Diabetes Mellitus

Pregestational diabetes, which affects approximately 1% of women of childbearing age, can be due either to absolute insulin deficiency (type 1, insulin-dependent diabetes mellitus [IDDM]) or to increased peripheral resistance to insulin action (type 2, noninsulin-dependent diabetes mellitus [NIDDM]), as shown in Box 28.1. The fasting glucose cutoff for diagnosing pregestational diabetes was reduced from 140 to 126 mg/dL in 1997.[173] On the basis of this change, there are approximately 21 million people with pregestational diabetes in the United States alone.[174]

The White classification of diabetes in pregnancy (Table 28.4) was developed by Dr. Priscilla White at the Joslin Diabetic Center in Boston, Massachusetts, in an attempt to correlate severity of diabetes with pregnancy outcome.[175] Although this classification is commonly used, any direct correlation between White class and prognosis remains unclear. Features known to be associated with poor pregnancy outcome include DKA, poor compliance, hypertension, pyelonephritis, and vasculopathy.

In contrast to GDM, pregestational diabetes is associated with significant maternal and perinatal mortality and morbidity (Box 28.3).[176,177] One of the more important complications is the increased risk of congenital malformations associated with hyperglycemia at the time of fertilization and embryo development. The incidence of congenital anomalies and spontaneous abortions in such patients correlates directly with the degree of glycemic control at conception, as measured by circulating maternal glycosylated hemoglobin levels.[178-180] Overall, approximately 30% to 50% of the perinatal mortality in diabetic pregnancy is due to fetal malformations.[181] Structural anomalies commonly seen in association with diabetes include cardiac defects (ventricular septal defects, transposition of the great vessels), renal agenesis, and neural tube defects (anencephaly, open spina bifida).[182] Some congenital defects—specifically sacral agenesis and caudal dysplasia—are as much as 400 times more common in the offspring of women with diabetes than in women with normal glucose metabolism[183] and, as such, are considered pathognomonic. The overall prevalence of these anomalies, however, is low.

Box 28.3 Pregnancy-Related Complications of Pregestational Diabetes

MATERNAL
- Spontaneous abortion
- Diabetic ketoacidosis
- Hypertension
- Preeclampsia/eclampsia
- Preterm birth
- Cesarean delivery
- Severe perineal injury
- Infectious morbidity (chorioamnionitis, endometritis, wound infection)

FETAL
- Congenital anomalies
- Macrosomia
- Intrauterine growth restriction
- Late fetal demise
- Non-reassuring fetal testing (previously referred to as "fetal distress")

NEONATAL
- Birth trauma (e.g., hypoxic ischemic cerebral injury; skull, clavicular, and long bone fractures; shoulder dystocia and brachial plexus injury)
- Hypoglycemia
- Neonatal sepsis
- Delayed organ maturation (respiratory distress syndrome, hyperbilirubinemia)

The factors responsible for diabetic embryopathy are not well defined, but glucose[184,185] and ketone bodies such as β-hydroxybutyrate[186,187] have both been implicated. Recently, oxidative stress[188,189] and apoptosis dysregulation[190,191] have been identified as potential mechanisms for diabetic embryopathy in animal models. Moreover, in a rat model of oxidative stress-induced diabetic embryopathy, the abnormal phenotype can be rescued by the use of antioxidants.[192,193] Several prospective randomized studies have shown that strict glycemic control around the time of conception is effective in reducing the risk of congenital malformations in women with established diabetes.[177,182,194-198] In one trial, intensive preconception management of diabetic women with vascular disease reduced the malformation rate from 19% to 8.5% [198] Unfortunately, most women with diabetes do not seek care prior to conception.[199,200] Maternal serum α-fetoprotein estimation at 15 to 20 weeks' gestation and a detailed sonographic fetal anatomical survey (with or without a fetal echocardiogram) at 18 to 22 weeks can be useful in screening for fetal malformations.

Intensive antepartum management should be initiated as early as possible and continued throughout gestation with a view to maintaining maternal blood glucose at desirable levels (fasting, below 95 mg/dL; premeal, below 100 mg/dL; 1 hour postprandial, below 140 mg/dL or 2 hours postprandial, below 120 mg/dL; during the night, not below 60 mg/dL; with an overall goal of mean capillary glucose levels of 100 mg/dL, corresponding to a glycated A1C ≤6%). Although the frequency of self-glucose monitoring recommended in pregnancy varies by organization, glucose should be monitored a minimum of four times daily (fasting, and 1 or 2 hours postprandial), and could be monitored as many as nine times daily (fasting, premeal, 1 or 2 hours postprandial, before bedtime, and at 3 AM if nocturnal hypoglycemia is suspected).[201] ACOG and the ADA provide recommendations for not only postprandial but premeal blood glucose levels.[176,202] If compliance with frequent fingersticks is in question, postprandial blood glucose levels have been shown to correlate more closely with adverse pregnancy and neonatal outcomes than do fasting or premeal blood glucose levels.[135,203,204] Initial recommendations for management of pregestational diabetes include a strict diabetic diet, regular exercise, daily home glucose monitoring, insulin treatment, and weekly antepartum visits to monitor glycemic control.[176] Such an approach has been shown to decrease perinatal mortality from a baseline of 20% to 30% to approximately 3% to 5%.[120,177,194,196,198]

Insulin, rather than oral agents, has been the mainstay of therapy in pregestational diabetics. To date, there are no randomized clinical trials to establish efficacy of oral agents in the management of pregestational diabetes in pregnancy. Insulin should be administered subcutaneously at 0.7 to 1.0 units/kg per day in divided doses. Traditional recommendations have been to administer two-thirds of the total daily dose in the morning (60% Neutral Protamine Hagedorn [NPH], 40% regular or rapid acting) and one-third in the evening (50% NPH, 50% regular/rapid acting). Rapid-acting insulin, namely lispro or aspart, achieves better glycemic control with fewer hypoglycemic episodes compared to regular insulin.[205-207] Lispro or aspart has the additional benefit of convenience, as these rapid-acting insulins can be administered immediately premeal, while regular insulin has to be administered 20 to 30 minutes before a meal. Alternative regimens may include dividing the total daily insulin requirement into 50% basal and 50% prandial, with either half or two-thirds of the basal administered as NPH in the morning, and the other half or one-third of basal insulin administered as NPH before bedtime. The remaining 50% of total daily insulin requirement may be divided in thirds and administered as lispro or aspart before meals. Insulin doses should be adjusted by approximately 20% up or down in response to the results of capillary blood glucose monitoring. Care should be taken to avoid iatrogenic hypoglycemia due to excessive insulin administration.[134]

Assessment of thyroid function is recommended (6% of diabetic women have coexisting thyroid disease), and baseline liver and renal function tests (including 24-hour urinary protein quantification and creatinine clearance determination) should be performed at the first prenatal visit. An ophthalmological examination should also be carried out every trimester. Glycosylated hemoglobin levels should be determined every 4 to 6 weeks throughout gestation.[176,178,179] At any one time, approximately 5% of maternal hemoglobin is glycosylated, known as hemoglobin Al (HbA1). HbAlc refers to the 80% to 85% of HbA1 that is irreversibly glycosylated and is therefore a more accurate measure of glycemic control. Because red blood cells have a life span of around 120 days, HbAlc measurements reflect the degree of glycemic control over the past 3 months.

Hypertension, prematurity, and late fetal demise are the most common complications of pregnancy in diabetic women.[120,176,181,208] Approximately 30% of diabetic women will develop hypertension in the third trimester. Pregnancy-induced hypertension often results in labor induction and is a major contributor to premature delivery in diabetic

women. Sustained maternal hyperglycemia results in fetal hyperglycemia that leads, in turn, to fetal hyperinsulinemia and increased oxygen demand. As such, fetuses of diabetic mothers are at increased risk of antepartum hypoxic ischemic cerebral injury and late fetal demise.[208,209] For this reason, weekly antepartum fetal testing (fetal cardiotocography) is usually recommended starting at 32 weeks' gestation.[210] After 36 weeks, testing is usually performed twice weekly. If the fetal heart rate tracing is abnormal, further testing (either a biophysical profile or contraction stress test) is mandatory.

A major issue in the care of the pregnant diabetic woman is the proper timing of delivery. No pregnant diabetic woman should be delivered after 40 weeks because of the increased risk of late fetal demise. If metabolical control is good, spontaneous labor at term should be awaited. Women with poorly controlled diabetes or with complications (worsening hypertension, intrauterine growth restriction [IUGR], oligohydramnios), on the other hand, should probably be delivered at around 37 to 38 weeks. Early delivery in pregnancies complicated by pregestational diabetes is associated with an increased risk of fetal respiratory distress syndrome, and consideration should be given to validation of fetal lung maturity prior to elective induction. Fetal lung maturation is inhibited by insulin and testosterone, and enhanced by endogenous cortisol, thyroxine, prolactin, and estradiol-17β. In infants of diabetic mothers, hyperinsulinemia and hyperandrogenemia are common findings and may contribute to the delay in lung maturation observed in diabetic pregnancies.[211-213] The increased testosterone observed in male infants of diabetic mothers may be due to elevated concentrations of human chorionic gonadotropin (hCG), which stimulates testosterone synthesis in fetal Leydig cells.

As many as 25% of infants of diabetic mothers are macrosomic.[176,214] If the estimated fetal weight is at least 4500 g, many authorities would recommend elective cesarean delivery at or beyond 39 weeks to minimize the risk of birth trauma, primarily shoulder dystocia and resultant brachial plexus injury. Elective cesarean delivery in women with pregestational diabetes should always be scheduled early in the morning, the patient's morning prandial insulin dose should be withheld and basal insulin dose can be halved.

During labor, patients are starved. Intravenous glucose should therefore be administered (typically 5% dextrose at a rate of 75 to 100 mL/hour) to all women with pregestational diabetes, and blood glucose levels should be checked every 1 to 2 hours. Regular insulin should be given as needed either by intravenous infusion (starting at 0.5 to 1 units/hour) or subcutaneous injection to maintain maternal glucose levels at around 100 to 120 mg/dL. Strict maternal glycemic control in labor is critical to preventing fetal hyperglycemia and hyperinsulinemia, both of which increase fetal oxygen demand and thereby predispose to fetal cerebral hypoxic ischemic injury.[208]

During the first 48 hours postpartum, women may have a "honeymoon period" during which their insulin requirement is decreased. Moreover, the need for strict glycemic control is reduced, and circulating glucose levels of 150 to 200 mg/dL can be comfortably tolerated during this period pending discharge from the hospital and regulation of glucose levels in the home environment. Once a woman is able to eat, she can return to her prepregnancy insulin regimen.

Obesity and Pregnancy

Obesity is one of the greatest public health challenges in the United States.[215] The prevalence of obesity has steadily risen since the 1980s.[216] In 2003 to 2004, 61.8% of U.S. women were overweight and 33.2% were obese.[217] From 2000 to 2005, the prevalence of obesity increased by 24%, with the prevalence of morbid obesity increasing by 50%.[218] Even more concerning, U.S. female children and adolescents (younger than 20 years old) had a 16% overweight prevalence suggesting that this public health issue will worsen rather than improve in the near future. As expected, the increased obesity prevalence is now evident also in the obstetric population.[219,220]

The preferred method of weight assessment is the body mass index (BMI). BMI is calculated as the body weight in kilograms divided by the square of the height in meters. Normal weight is defined as a BMI between 18.5 and 24.9 kg/m^2. Overweight refers to a BMI between 25 and 29.9 kg/m^2. Obesity is a BMI greater than 30 kg/m^2. Obesity is further divided into class I (30 to 34.9 kg/m^2), class II (35 to 39.9 kg/m^2), and class III (BMI greater than 40 kg/m^2) also known as *morbid obesity* or *extreme obesity*. The advantage of using BMI for defining obesity is that no adjustments need to be made for gender or height, and no tables are required for determining the normal range.[221]

Obesity is a complex neuroendocrine and metabolical disorder that has been implicated in a large number of fetal and maternal complications, including spontaneous abortion, congenital malformations, stillbirth, preeclampsia, GDM, fetal macrosomia, cesarean delivery, venous thromboembolic disease, surgical complications, and urinary tract infections.

Congenital malformations classically associated with obesity include neural tube defects, ventral wall defects, and abnormalities of the great vessels.[222-224] There is some evidence suggesting an increased risk for other anomalies in offspring of obese women, including hypospadias, isolated hydrocephalus, and orofacial clefts.[225-229] In one study, obese women weighing more than 110 kg had a four-fold increased risk of having a fetus with a neural tube defect as compared with a control population weighing 50 to 59 kg. For women weighing 80 to 89 kg, the risk was increased 1.9-fold.[224] Interestingly, folic acid supplementation (0.4 mg daily) did not appear to reduce the risk of neural tube defects in this cohort of obese women.[224] These data are consistent with other studies showing that a BMI greater than 29 kg/m^2 is associated with a 1.9-fold increase in the risk for neural tube defects.[222] The mechanism by which obesity causes congenital anomalies is not known. It is conceivable that, in obese women, subtle abnormalities in glucose metabolism contribute to the increased risk of congenital malformations, similar to that of pregestational diabetes.

Perinatal mortality is increased with progressive obesity.[230-232] In a cohort of 167,750 Swedish women,[230-232] Cnattingius and colleagues (1998) demonstrated a 1.7-fold increased risk in late fetal death for overweight women (BMI 25 to 29.9 kg/m^2) and a 2.7-fold increase for obese

women (BMI greater than 30 kg/m^2) when compared to women with a prepregnancy BMI less than 20 kg/m^2.[231,233]

Obesity has consistently been associated with hypertensive disorders of pregnancy.[234,235] For example, one large prospective multicenter cohort study of more than 20,000 women demonstrated that obese women with a BMI between 30 to 34.9 kg/m^2 had a 2.5- and 1.6-fold relative risk for gestational non-proteinuric hypertension and preeclampsia, respectively. For obese women with a BMI greater than 35 kg/m^2, a similar association was found with a relative risk of 3.0- and 3.3-fold, respectively.[236] In a systematic review of 13 studies including over 1.4 million women, O'Brien colleagues (2003) calculated a twofold increased risk of developing preeclampsia with every 5-7 kg/m^2 increase in BMI.[237]

Other well-documented risks of obesity include an increased incidence of GDM and fetal macrosomia,[220,236,238] birth injury, maternal perineal trauma, and cesarean delivery.[220,236,238] In a study of 20,130 births, a BMI greater than 39 kg/m^2 was associated with a 46% cesarean delivery rate compared with 20% in a control group of women with a BMI less than 29 kg/m^2.[239] In obese women, limiting total pregnancy weight gain to no more than 15 to 25 pounds appears to decrease the risk of fetal macrosomia without increasing the risk of low birth weight or IUGR.[240] Randomized controlled trials seeking to establish the optimum gestational weight gain in obese women are ongoing.[241,242] The increased cesarean delivery rate is associated also with increased surgical morbidity in obese women, including anesthestic complications, wound separation and infection, and venous thromboembolic events.[243-245] Maternal obesity also appears to be protective against spontaneous preterm birth. The risk of spontaneous preterm birth decreases with increasing maternal BMI, perhaps due to decreased uterine activity in these women compared to normal BMI controls.[246-248]

Obstetric management of the obese patient should include calculation of BMI, careful attention to blood pressure, a nutrition consultation, institution of a daily exercise program, and early screening for GDM. A careful sonographic anatomy survey (with or without fetal echocardiogram) at 18 to 22 weeks' gestation is indicated in obese women given the increased risk of fetal structural anomalies, although body habitus may result in suboptimal imaging. Serial growth scans should also be considered given the limitations of other methods of fetal growth assessment. Anesthesia consultation in the third trimester should also be considered prior to the onset of labor. If cesarean section is required, every effort should be made to reduce the risk of wound separation and infection, including prophylactic antibiotics, and closure of the subcutaneous layer.[249]

For women in developed countries, obesity has become one of the leading endocrine causes of morbidity and mortality. Effective treatments for obesity have so far eluded medical science. Bariatric surgery, which includes a multitude of procedures to reduce gastric capacity or bypass the stomach, has been shown to promote weight loss and reduce long-term mortality.[250,251] Its use as a weight loss tool has increased in popularity,[252] and the resultant weight loss often leads to improved fertility. The safety of pregnancy after these procedures is unclear. Early case reports suggested an increased risk of adverse pregnancy outcomes.[253-255] However, more recent studies have suggested no significant increased risks during pregnancy, but a number of potential benefits, including reduced risks of diabetes and hypertension.[256-259] Such women should all be given vitamin and mineral supplementation, especially iron, folate, and vitamin B12.[249]

Leptin is a hormone secreted primarily by adipocytes and acts to suppress appetite while increasing energy expenditure, thereby regulating body weight. Mice lacking the leptin gene (*ob/ob* mice) are obese and anovulatory. Administration of human leptin to *ob/ob* mice increases energy expenditure, reduces weight and fat mass, and restores ovulation and fertility.[260,261] In humans, absolute leptin deficiency is rare and supplemental recombinant leptin has not been shown to promote weight loss. During pregnancy, leptin is produced by maternal and fetal adipocytes as well as syncytiotrophoblast cells.[262] Circulating leptin levels rise rapidly in the first trimester, maintain high levels throughout pregnancy, and drop precipitously after delivery, suggesting a major contribution from the placenta.[263,264] Leptin levels in the maternal circulation correlate with maternal body mass, but not with levels in umbilical cord blood at birth or birth weight. However, leptin levels in umbilical cord blood do correlate directly with both birth weight[265,266] and fetal adiposity.[267] The significance of the increased leptin levels in the maternal circulation during pregnancy are unclear, although it has been suggested that leptin may act to mobilize maternal fat stores and increase the availability of substrate to the fetus.[268] In diabetic pregnancies, alterations in maternal and fetal leptin levels have not been particularly informative.[267] In pregnancies complicated by preeclampsia, umbilical cord leptin levels are decreased and reflect the reduction in fetal growth and fat stores.[269] Interestingly, maternal leptin levels are increased in preeclampsia and appear to correlate with the severity of the disease.[270,271] The significance of this observation remains unclear. Although the regulation and mechanisms of action of leptin are not fully understood, it is possible that manipulation of the leptin-leptin receptor system may ultimately prove to be a safe and effective treatment for adult obesity and fetal macrosomia.

The developmental origins of health and disease hypothesis posits that the *in-utero* environment induces fetal adaptive responses to facilitate offspring health and survival.[272] The metabolical and hormonal changes that result from these responses lead to altered homeostatic set points, which ultimately may prove maladaptive in other environments. Fetal adaptation is likely mediated by epigenetic phenomena, including DNA methylation and histone modification.[273] There is growing evidence that maternal obesity and overnutrition may induce fetal adaptive responses that predispose to obesity, diabetes, metabolical derangements, and increased cardiovascular risk later in life.[274-278] Maternal hyperglycemia, an inflammatory intrauterine mileu, and leptin dysregulation all may contribute to the increased cardiometabolical risk for offspring born to obese gravidas.[130,279-282] Sheep and rat models suggest that *in-utero* overnutrition may predispose to childhood and adult obesity via increased leptin exposure *in utero*, with subsequent inhibited leptin receptor development in the hypothalamus, and thus decreased sensitivity to leptin-mediated appetite suppression.[283-285] Indeed, increased leptin and insulin concentrations have been described in human offspring of obese

mothers, suggesting that fetal programming of appetite and satiety pathways may be an important determinant of adult disease states.[286,287]

Hypothalamic-Pituitary Diseases

Pregnancy-Associated Changes in Pituitary Structure and Function

The pituitary gland is composed of three parts: the anterior lobe (adenohypophysis), the intermediate lobe (prominent in the fetus but attenuated in the adult), and a posterior lobe (neurohypophysis). During pregnancy, the structure and function of the pituitary gland are significantly altered.[288] In the nonpregnant state, the pituitary gland weighs between 0.5 and 1.0 g. One autopsy study of 118 pregnant women demonstrated a 30% increase in the weight of the pituitary gland compared with nonpregnant controls (1070 mg versus 820 mg, respectively).[289] This increase in weight is associated also with an increase in volume (Table 28.5)[290,291] and a change in shape. In pregnancy, the pituitary gland develops a convex, dome-shaped superior surface,[292] which may impinge on the optic chiasm and account, in part, for the bitemporal hemianopia observed in some apparently healthy pregnant women.[293,294] Pregnancy is *not* associated with an increased incidence of pituitary adenoma.

On the basis of the hormones they produce, the adenohypophysis contains at least five different cell types: lactotropes (that primarily secrete prolactin), corticotropes (adrenocorticotropic hormone [ACTH]), somatotropes (GH), gonadotropes (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]), and thyrotropes (thyroid-stimulating hormone [TSH]). The cellular composition of the adenohypophysis, however, changes throughout pregnancy. This is especially true of the lactotrope cell population. Immunohistochemical studies have shown that, in the nonpregnant state, approximately 20% of cells in the adenohypophysis are lactotropes.[295,296] This number increases in pregnancy such that, by the third trimester, approximately 60% are lactotropes. Moreover, the increase in lactotropes is most pronounced in the lateral portions of the adenohypophysis. By 1 month postpartum, the number of lactotropes in the anterior pituitary of nonlactating women is decreased. However, postpartum resolution of lactotrope hyperplasia is incomplete, and nonpregnant multiparas have on average more lactotrope cells than do nulligravid women.

In contrast to lactotropes, the numbers of somatotropes, gonadotropes, and α-subunit—secreting cells in the adenohypophysis decrease in pregnancy and the number of thyrotropes do not change. These changes in cellular composition are associated with changes in circulating hormone levels.

Table 28.5 Volume of the Pituitary Gland Throughout Pregnancy as Determined by Magnetic Resonance Imaging

Gestational Age (Weeks)	Subject Number	Pituitary Volume (mm^3) Mean ± SEM
Nonpregnant	20	300 ± 60
9	10	437 ± 90
21	11	534 ± 124
37	11	708 ± 123

Data from Gonzalez JG, Elizondo G, Saldivar D, et al. Pituitary gland growth during normal pregnancy: an in vivo study using magnetic resonance imaging. Am J Med 85:217, 1988.

Prolactin

Levels of prolactin in the maternal circulation increase throughout pregnancy, reaching concentrations of approximately 140 ng/mL at term (Fig. 28.8).[297] Although the maternal decidua is a major site of prolactin production during pregnancy, prolactin in the maternal circulation originates primarily from the maternal pituitary with small contributions from the maternal decidua and fetal pituitary. The observation that circulating levels of prolactin in pregnant women with pre-existing hypopituitarism remain low throughout pregnancy supports the hypothesis that very little decidual prolactin enters the maternal circulation.[298,299] Decidual production of prolactin leads to elevated levels in the amniotic fluid, which peaks at approximately 6000 ng/mL at the end of the second trimester.[296]

The hyperprolactinemia of pregnancy is likely due to increased circulating levels of estradiol-17β. Aside from this increase in basal levels, prolactin secretion by the maternal pituitary is stimulated by thyrotropin-releasing hormone (TRH),[300] arginine,[301] meals,[301] and sleep[302] in a manner similar to that seen in nonpregnant women. After delivery, maternal prolactin concentrations in nonlactating women decrease to prepregnancy levels within 3 months.[303] In

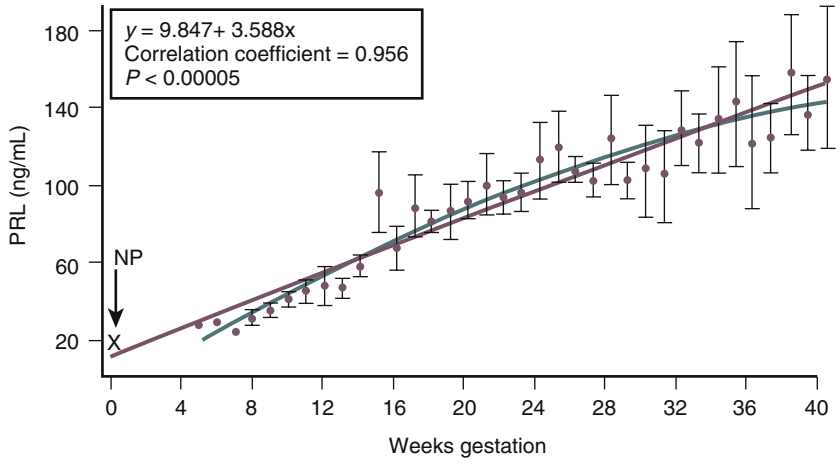


FIGURE 28.8 Prolactin concentration in maternal circulation throughout gestation. *(From Rigg LA, Lein A, Yen SSC. Pattern of increase in circulating prolactin levels during human gestation.* Am J Obstet Gynecol *129:454, 1977.)*

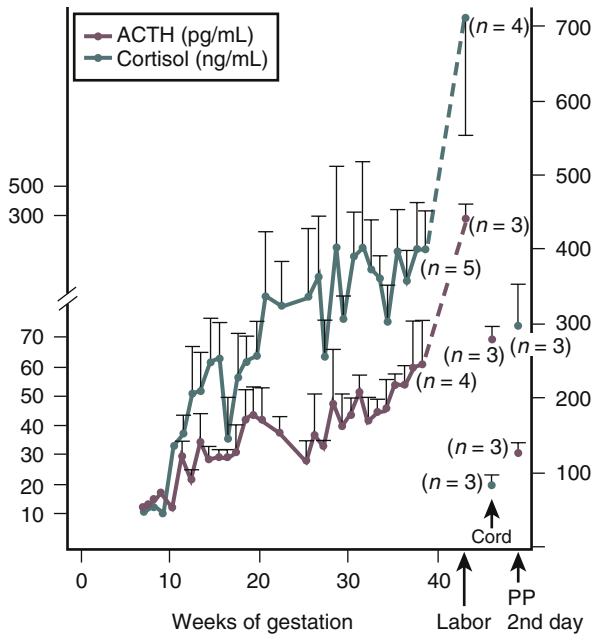


FIGURE 28.9 ACTH and total cortisol concentration in the maternal circulation throughout gestation. *(From Carr BR, Parker CR, Madden J D, et al. Maternal plasma adrenocorticotropin and cortisol relationships throughout human pregnancy.* Am J Obstet Gynecol *139:416, 1981.)*

lactating women, basal circulating prolactin levels decrease slowly to nonpregnant levels over a period of several months with intermittent episodes of hyperprolactinemia in conjunction with nursing.

Pregnancy is also associated with a shift in prolactin isoforms. In the nonpregnant state, the N-linked glycosylated isoform of prolactin (G-PRL) predominates in the circulation. As pregnancy progresses, increasing amounts of nonglycosylated prolactin appear in the circulation.[304] In the third trimester, the concentration of circulating nonglycosylated prolactin exceeds that of GPRL. Nonglycosylated prolactin appears to be more biologically active than G-PRL.[305] The precise function of the elevated circulating levels of prolactin in pregnancy is not clear, but it appears to be important in preparing breast tissue for lactation by stimulating glandular epithelial cell mitosis and increasing production of lactose, lipids, and certain proteins.[304-306] The role of prolactin in amniotic fluid is not known.

Pregnancy produces changes in prolactin secretion that persist long after delivery. Musey and colleagues[307] reported that the basal serum prolactin level and the prolactin response to perphenazine stimulation was lower after pregnancy than before pregnancy. They also found that the serum prolactin concentration was significantly lower in the parous women (mean, 4.8 ng/mL) than in the nulliparous women (8.9 ng/mL).[307] These and other studies[308,309] suggest that pregnancy permanently suppresses the secretion of prolactin by the maternal pituitary.

Adrenocorticotropic Hormone

ACTH levels in the maternal circulation increase from approximately 10 pg/mL in the non-pregnant state to 50 pg/mL at term,[72] and increase further to approximately 300 pg/mL in labor (Fig. 28.9).[72] Although the placenta can produce ACTH,[310] the majority of circulating ACTH appears to come from the maternal pituitary.[311] Placental production of corticotropin-releasing hormone (CRH) may be a major cause of the elevated levels of ACTH in the maternal circulation.[312-314] In nonpregnant women, serum CRH levels range from approximately 10 to 100 pg/mL. In the third trimester of pregnancy, these concentrations increase to 500 to 3000 pg/mL but then decrease precipitously after delivery.[314,315]

In addition to increasing pituitary ACTH secretion, chronically elevated levels of CRH in the maternal circulation reduce the ability of exogenous glucocorticoids to suppress the maternal ACTH–cortisol axis,[316-318] enhance the ability of vasopressin to induce an ACTH response, and diminish the effect of exogenous CRH.[312,313] CRH binding protein (CRH-BP) inactivates CRH, thereby preventing its action on the maternal or fetal pituitary. CRH-BP levels in the maternal circulation decrease during the last few weeks of pregnancy, resulting in an increase in free (biologically active) CRH.[319]

Although the maternal adrenal glands do not change in size, pregnancy is associated with significant changes in the circulating concentrations of adrenal hormones. For example, serum cortisol levels increase substantially in pregnancy. The majority of circulating cortisol is bound to cortisol-binding globulin (CBG), which is produced by the liver. Circulating levels of CBG increase during pregnancy in response to elevated levels of estrogen, and CBG may retard the clearance of these hormones. It is not surprising, therefore, that total cortisol levels increase in pregnancy. However, levels *of free* cortisol in the circulation[316,317]—as well as in saliva[320] and urine[73,316,317]—are also increased, which is likely due to the increased levels of ACTH in the maternal circulation. Maternal hypercortisolemia is also observed in complete molar pregnancy, suggesting that the increased cortisol is not derived from a fetal source. Other changes in adrenal hormone levels that occur in association with pregnancy include an increase in circulating levels of aldosterone[321] and adrenal androgens, primarily androstenedione and testosterone.

Growth Hormone

Maternal serum GH levels begin to increase at around 10 weeks' gestation, plateau at approximately 28 weeks, and can remain elevated for several months postpartum.[322] The majority of this GH is derived from the placenta (GHV), with a marked reduction in basal somatotropin (GH–N) production by the maternal pituitary.[45] Moreover, the release of GH-N in response to either insulin-induced hypoglycemia (Fig. 28.10)[43] or arginine stimulation[323] is markedly attenuated, suggesting that maternal pituitary GH secretory reserve is diminished in pregnancy.

Thyroid-Stimulating Hormone

In general, the concentration of TSH (thyrotropin) in the maternal circulation remains within the normal range during pregnancy.[324,325] Normative data from pregnant women suggest the upper reference range for TSH in pregnancy may be 2.5 to 3.0 mIU/L, rather than 4.0 mIU/L, the upper limit of normal in healthy, nonpregnant individuals.[326,327] At 9 to 13 weeks of gestation, there is a modest decline in circulating TSH levels (Fig. 28.11).[328,329] This coincides with the peak placental production of hCG, and some authorities

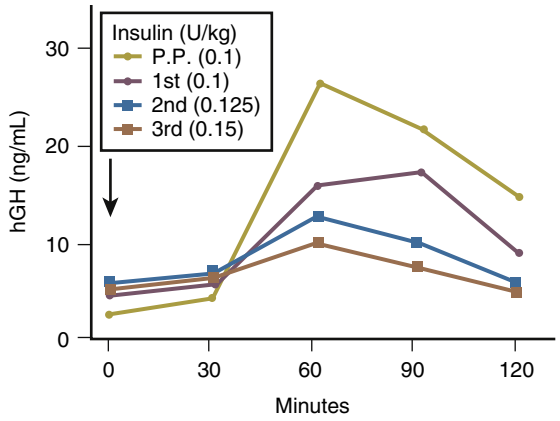


FIGURE 28.10 Human pituitary growth hormone response to insulin hypoglycemia in pregnancy. In pregnancy, pituitary growth hormone response to hypoglycemia is blunted. *(Adapted from Yen SSC, Vela P, Tsai CC. Impairment of growth hormone secretion in response to hypoglycemia during early and late pregnancy.* J Clin Endocrinol Metab *31:29, 1970.)*

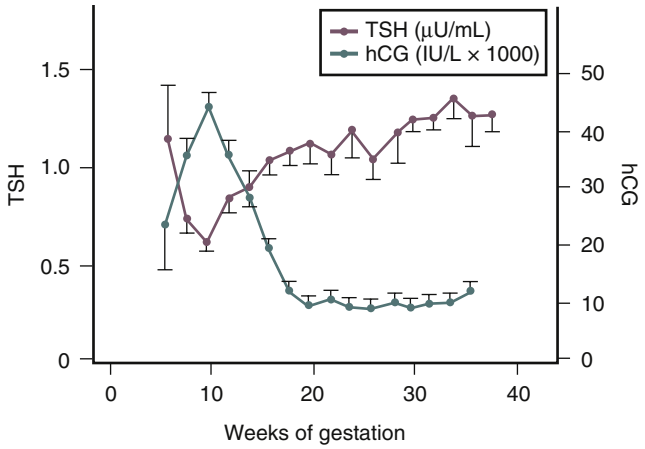


FIGURE 28.11 Maternal concentration of serum TSH and hCG as a function of gestational age. The decrease in serum TSH at approximately 10 weeks' gestation may be due to thyrotropic effects of hCG. *(Adapted from Glinoer D, de Nayer P, Bourdoux P, et al. Regulation of maternal thyroid during pregnancy.* J Clin Endocrinol Metab *71:276, 1990.)*

have suggested that the decrease in TSH may be due to the weak thyrotropic properties of hCG.[330-332] An alternative hypothesis is that the placenta may secrete a hormone with TRH or TSH-like properties, but this hypothesis is not supported by most data.[333]

Enlargement of the thyroid gland is a common finding during pregnancy. This growth is not due to any deficiency in the hypothalamic-pituitary-thyroid axis, but rather a result of relative iodide deficiency and increased demand for thyroid hormone secondary to elevated levels of thyroid-binding globulin (TBG).[328] Increased TBG concentrations result in the need for increased production of thyroxine (T_4) and triiodothyronine (T_3) to maintain adequate concentrations of free hormone. The thyroid responds to the need for increased production with increased vascularity, cellular hyperplasia, and ultimately glandular hypertrophy.[328] Indeed, the TSH response to exogenous TRH stimulation remains normal throughout pregnancy.[324,325] An appreciation of the physiological changes in thyroid hormone levels is important to accurately assess thyroid status in pregnancy. Total T_4 and T_3 levels are elevated due to TBG excess, and therefore have not typically been utilized to evaluate thyroid status during pregnancy. The most appropriate test to detect thyroid dysfunction during pregnancy is the TSH assay. If this is abnormal, classically free T_4 and free T_3 levels have been measured. However, more recent uncertainty about the accuracy of free T_4 immunoassays in pregnancy has led some to reconsider the use of adjusted total T_4 in pregnancy in select situations (see section on "Maternal Thyroid Function in Pregnancy" later in this chapter).[334,335]

Gonadotropins

Maternal serum LH and FSH levels are decreased by 6 to 7 weeks of pregnancy and are below the limits of detection of many radioimmunoassays by the second trimester.[336-338] The marked decrease in gonadotropin immunoreactivity in the pituitary glands of pregnant women[296,339] coupled with the blunted LH and FSH response to exogenous gonadotropin-releasing hormone (GnRH) stimulation (Fig. 28.12)[336-338] suggests that this effect is localized primarily to the pituitary. The suppression of pituitary LH and FSH synthesis and secretion likely results from elevated circulating levels of sex steroids (estradiol-17β, progesterone) and regulatory peptides (such as inhibin) during pregnancy.

Pituitary Tumors in Pregnancy

Mutations are the primary cause of pituitary tumors. Most pituitary tumors are monoclonal, indicating that a somatic mutation in a single progenitor cell is the cause of the tumor. In one study, 100% of GH-producing tumors and 75% of ACTH-producing tumors were found to be monoclonal.[340] In one series of GH-secreting tumors, mutations in the gene coding for the Gs protein were reported in 10 of 25 tumors, resulting in a constitutively active mutant Gs protein.[341] Other endocrine factors (such as the levels of estradiol-17β, progesterone, and dopamine) can influence tumor phenotype, and changes in these hormones during pregnancy may affect tumor growth. In general, pituitary tumors are benign and slow growing.

Pituitary tumors are commonly classified according to size as either microadenomas (less than 10 mm in diameter) or macroadenomas (larger than 10 mm). The clinical behavior of microadenomas and macroadenomas vary considerably during pregnancy. Macroadenomas may be associated with extrasellar extension, local invasion, or compression of the optic chiasm with resultant bitemporal hemianopia, and such conditions may become exacerbated in pregnancy. In one series of 60 pregnant women with macroadenomas, for example, 20% showed evidence of worsening visual field defects, significant enlargement on serial imaging studies, or neurological signs.[288] Urgent neurosurgical decompression may be required during pregnancy if the tumor enlarges markedly or causes neurological sequelae.

In contrast, microadenomas tend to behave in a relatively benign manner in pregnancy, with no evidence of functional

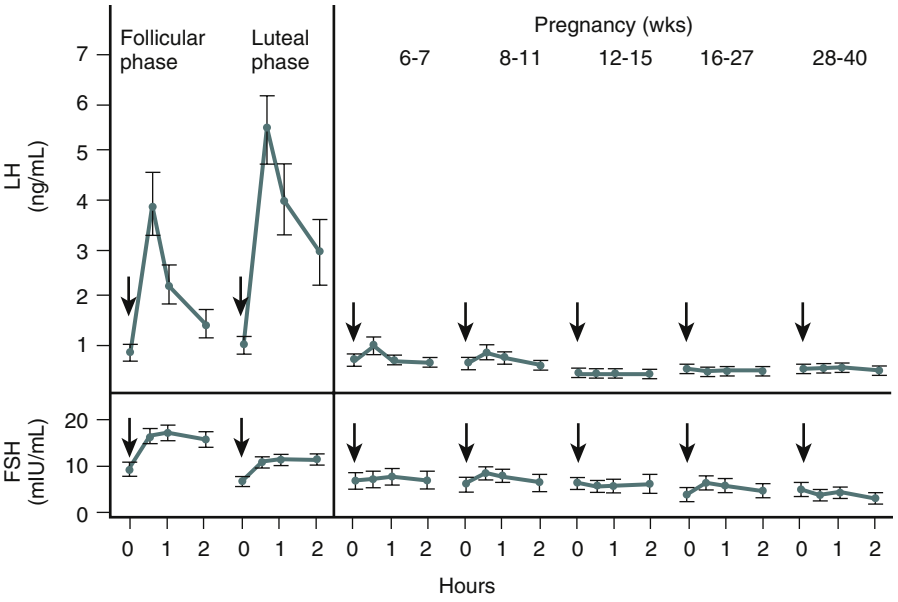


FIGURE 28.12 Effect of pregnancy on GnRH stimulation test. Serum levels of LH and FSH before and after a 100-μg bolus of GnRH *(arrow)* in pregnant women and normal menstruating women. During pregnancy, LH and FSH levels are markedly suppressed. *(From Miyake A, Tanizawa O, Aono T, Kurachi K. Pituitary responses in LH secretion to GnRH during pregnancy.* Obstet Gynecol *49:549–551, 1977, with permission from The American College of Obstetricians and Gynecologists.)*

pituitary deficiency and a low risk of neurological complications. In a longitudinal observational study of 215 pregnant women with microadenomas, for example, approximately 5% of women developed headaches, and less than 1% experienced worsening of visual field defects or demonstrated neurological signs.[288]

Prolactinoma

Prolactinoma refers to a tumor of prolactin-secreting lactotrope cells, and is typically associated with elevated levels of prolactin in the maternal circulation (see Chapter 3). In the initial evaluation of a suspected prolactinoma, measurement of circulating levels of thyroxine, TSH, and IGF-1 is important. This evaluation will exclude secondary causes of hyperprolactinemia, specifically hypothyroidism (thyroxine, TSH) and acromegaly (IGF-1). An imaging study of the hypothalamus and pituitary is also indicated, and computerized evaluation of the visual fields via automated perimetry is recommended if compression of the optic chiasm is suspected.[342]

Women with marked hyperprolactinemia are usually anovulatory and, as such, infertile. If such a patient does not desire pregnancy, treatment with combination estrogen–progestin therapy will reduce the risk of osteoporosis and regulate the menstrual cycle. This approach appears to be safe and is associated with few tumor-related complications, including minimal risk of tumor growth.[343] For infertile women with significant hyperprolactinemia who wish to conceive, treatment is usually required to induce ovulation. Controversy continues as to whether surgery or dopamine-agonist treatment represents the best first-line therapy for such women. Some authorities would recommend surgical treatment prior to conception to reduce both the need for dopamine-agonist treatment and the incidence of neurological complications during pregnancy.[344] However, microsurgical resection of a prolactinoma can result in death (in 0.3% of cases) or serious morbidity, such as a cerebrospinal fluid leak (0.4%).[345] Moreover, a long-term cure can be expected in only approximately 60% of women treated surgically. For these reasons, the weight of evidence in the literature suggests that medical treatment should be regarded as the best first-line therapy for infertile women with significant hyperprolactinemia.[346,347]

Having confirmed the diagnosis of a pituitary microprolactinoma, the goals of treatment are four-fold:

1. Suppress prolactin production and induce ovulation
2. Decrease tumor size
3. Preserve pituitary reserve
4. Prevent tumor recurrence

Treatment with a dopamine agonist can normalize circulating prolactin levels, establish regular ovulation, decrease tumor size, and preserve pituitary reserve.[348,349] A disadvantage of dopamine-agonist treatment is that it is not effective in preventing tumor recurrence once treatment is discontinued. Four dopamine agonists have been demonstrated to be effective in the treatment of hyperprolactinemia: bromocriptine, pergolide, quinagolide, and cabergoline. Cabergoline is administered once weekly and may be more effective than bromocriptine in the treatment of microadenomas.[350]

Little information is available concerning the effects of pergolide, quinagolide, and cabergoline on pregnancy, though increasing experience with cabergoline suggests that it is safe.[351,352] In contrast, there is substantial experience that bromocriptine is safe in pregnancy, with no significant increase in the overall rate of fetal congenital abnormalities.[353,354] The most common adverse effects associated with bromocriptine therapy are nausea, vomiting, and postural hypotension. Starting with low-dose therapy (0.625 mg daily) and increasing the dose slowly over a period of a few weeks can minimize these side effects. In some patients, doses as low as 2.5 mg daily may be effective. Prolactin levels should initially be checked every month for 3 months and thereafter every 3 months until the levels have returned to normal.

In women with microprolactinomas, bromocriptine can be discontinued once pregnancy is established. The majority of such women will have no further complications during

pregnancy. For those women who do experience neurological sequelae such as headache or cranial nerve dysfunction, bromocriptine therapy can be immediately reinstituted. Marked enlargement of a microprolactinoma or persistence of neurological sequelae despite medical treatment may be an indication for urgent neurosurgical intervention, but such complications are rare. In contrast, pituitary insufficiency and neurosurgical complications are far more common in women with macroadenomas. Such women should therefore be evaluated for panhypopituitarism before dopamine-agonist treatment is initiated. Women with macroprolactinomas are also more likely to develop complications in pregnancy.[288] One approach to the management of such women is to discontinue bromocriptine treatment once pregnancy is established, and to re-institute therapy if symptoms or signs of increasing tumor volume develop.[355] An alternative plan, which is equally appropriate, is to continue bromocriptine treatment throughout pregnancy.[356,357] Lactation does not appear to worsen the clinical course of women with prolactinomas, and such women should be encouraged to breastfeed.[358]

Cushing Disease

Cushing disease refers to the clinical syndrome resulting from excessive pituitary ACTH production. It is typically associated with depressed gonadotropin secretion, and spontaneous pregnancy is rare in women with untreated Cushing disease. Most cases of Cushing disease are due to pituitary microadenomas. As such, neurosurgical complications are rarely seen in pregnancy. However, the metabolical derangements associated with Cushing disease have been implicated as the cause of the observed increases in pregnancy-related complications, including premature labor, pregnancy-induced hypertension, and GDM.

Acromegaly

Acromegaly refers to the clinical syndrome associated with elevated circulating levels of GH. Acromegaly is often associated with anovulation, but spontaneous pregnancy can occur.[359,360] Except for complications associated with pituitary enlargement, acromegaly does not appear to adversely effect pregnancy outcome.[361] Since GH is an insulin antagonist, pregnancies complicated by excess circulating GH are at increased risk of hyperglycemia and diabetes.[362] In most women, definitive treatment for acromegaly can be deferred until after delivery. Bromocriptine, transsphenoidal surgery, and more recently octreotide, a somatostatin agonist, have been used successfully to treat acromegaly during pregnancy.[361]

Pituitary Insufficiency

Sheehan Syndrome

Sheehan syndrome (pituitary apoplexy) refers to the onset of acute hypothalamic–pituitary dysfunction that typically occurs after severe obstetric hemorrhage and resultant maternal hypotension at delivery. It is the most common cause of hypopituitarism worldwide, though not commonly seen in the United States.[363] During pregnancy, the pituitary volume increases by approximately 100%. This increase in pituitary size, coupled with the low-flow, low-pressure nature of the portal circulation, appears to make the pituitary and parts of the hypothalamus particularly susceptible to ischemia caused by obstetric hemorrhage and hypotension. The majority of cases of Sheehan syndrome occur in developing countries where deliveries are not performed in healthcare facilities by skilled attendants, increasing the risk of complications from obstetrical hemorrhage.

The hallmark of this syndrome is a loss of anterior pituitary hormone reserve, which may be complete or partial. Prolactin and GH deficiency are the most common abnormalities observed in Sheehan syndrome, but every imaginable pattern of pituitary hormone deficiency has been described. In a study of 10 African women with Sheehan syndrome, Jialal and co-workers[364] described the pituitary hormone response to a combined intravenous insulin (0.1 unit/kg), TRH (200 mg), and GnRH (100 mg) challenge test. The pattern of pituitary hormone response revealed the following loss of secretory reserve: 100% of these women had both prolactin and GH deficiency, 90% had cortisol deficiency, 80% had TSH deficiency, 70% had LH deficiency, and 40% had FSH deficiency.

The initial clinical manifestations of Sheehan syndrome include failure of lactation, failure of hair growth over areas shaved for delivery, poor wound healing after cesarean delivery, and generalized weakness. The best single test to confirm the diagnosis of Sheehan syndrome is to administer intravenous TRH (100 mg) and measure serum prolactin levels at 0 and 30 minutes. The ratio of prolactin measured at 30 minutes to that before TRH treatment (time 0) should be greater than 3.0.[365] If the ratio is abnormal, a complete evaluation for panhypopituitarism should be initiated.

In addition to loss of anterior pituitary hormone reserve, mild hypothalamic and posterior pituitary dysfunction is also frequently seen in women with Sheehan syndrome. Detailed neuropathological reports of autopsy specimens by Sheehan and Whitehead[366] have shown that 90% of women with postpartum hypopituitarism have evidence of atrophy and scarring of the neurohypophysis. Subsequent studies have also demonstrated atrophy of the supraoptic and paraventricular nuclei in such patients.[367] These observations have been confirmed in several clinical studies demonstrating that most women with Sheehan syndrome have mild functional defects in both vasopressin secretion and maximal urinary concentrating capability.[368,369]

Lymphocytic Hypophysitis

Lymphocytic hypophysitis is a rare disorder caused by infiltration of the adenohypophysis with lymphocytes and plasma cells. Most cases of lymphocytic hypophysitis occur in women in the third trimester of pregnancy or immediately postpartum.[370] In some cases, circulating antipituitary antinuclear or antimitochondrial antibodies have been detected. Pituitary enlargement can result in neurological complications (headache, visual field defects, cranial nerve palsy) requiring surgical intervention,[371] while pituitary cell damage may result in hyperprolactinemia, hypothyroidism, or adrenal insufficiency.[372] High-dose glucocorticoid therapy may be effective in treating some cases of lymphocytic hypophysitis when neurological sequelae are present.[373]

Diabetes Insipidus

Arginine vasopressin-antidiuretic hormone (AVP-ADH) is a cyclic nonapeptide secreted by the axonal terminals of the

neurohypophysis emanating from neurosecretory neurons located in the supraoptic and paraventricular nuclei of the hypothalamus. Blood osmolality is carefully monitored by sensitive osmoreceptors in the anterior hypothalamus. AVP-ADH is released in response to increasing osmotic pressures or decreasing hydrostatic pressures and act on the kidney to increase water retention. This system is designed to adjust blood osmolality over a relatively narrow range (±1.8%) with a mean of 285 mOsm/kg in nonpregnant women.[374] Pregnancy is associated with a decrease in plasma osmolality of approximately 9 to 10 mOsm/kg, which is evident early in the first trimester and persists throughout gestation[375] and appears to mirror changes in maternal hCG levels.[376] However, circulating AVP-ADH levels do not change in pregnancy.[377] These data suggest that pregnancy is associated with a modest resetting of the osmostat, leading to a 9 to 10 mOsm/kg decrease in the osmotic threshold for AVP-ADH release.

Diabetes insipidus (DI) involves the inappropriate loss of water resulting from failure of adequate tubular reabsorption by the kidney. The condition is characterized by polyuria (defined as more than 3 L of urine in 24 hours), polydipsia, and plasma hyperosmolarity. The causes of DI can be divided into two groups: central and peripheral.

Central (hypothalamic) DI refers to lesions of the hypothalamus or posterior pituitary that lead to inadequate production of AVP-ADH. The differential diagnosis of central DI includes pituitary surgery, trauma, infection, and infiltration of the neurohypophysis by tumors or inflammatory cells. Central DI is typically characterized by the acute onset of massive polyuria of 4 to 15 L per day. *Peripheral (nephrogenic) DI* refers to peripheral resistance to AVP-ADH action. Measurement of plasma AVP-ADH levels may be able to distinguish these two groups (levels are low in central DI and elevated in nephrogenic DI).

Transient nephrogenic DI can occur in pregnancy, usually in association with preeclampsia, HELLP (hemolysis, elevated liver enzymes, low platelet count) syndrome, or acute fatty liver of pregnancy.[378] High levels of placental vasopressinase may contribute to pregnancy-associated DI by degrading endogenous AVP-ADH. This increase in vasopressinase activity may also cause women with partial hypothalamic AVP-ADH deficiency to develop overt DI in pregnancy. D-arginine vasopressin (DDAVP) is resistant to degradation by placental vasopressinase. As such, DDAVP may be more effective than native AVP-ADH in the treatment of women with DI. In most cases, DI improves after delivery.[379]

DI is a rare disease in pregnancy. If suspected, the diagnosis of DI should be confirmed by performing a water-deprivation test. After an overnight fast, the patient is denied water until 3% of body weight is lost or urine osmolarity shows no increment in three successive hourly specimens. In women with DI, urine osmolarity remains low while plasma osmolarity increases significantly. This test is best performed by an endocrinologist because of the risks associated with dehydration and hypernatremia.[380] To help identify the cause, 10 μg of DDAVP can be administered immediately after the completion of the water-deprivation test. In women with central DI, there will be a decrease in urine output and an increase in urine osmolarity. In women with nephrogenic DI, on the other hand, there will be only a minimal change in urine output and osmolarity.[381]

Disorders of Thyroid Function

For reasons that are not fully understood, thyroid disease is 5 to 10 times more common in females than in males at all ages. Moreover, many of these conditions are autoimmune in nature with a peak incidence during the childbearing years. For these reasons, thyroid disease is one of the most common endocrine diseases affecting women of reproductive age and—despite the adverse effect of thyroid disease on fertility—such disorders are commonly encountered in pregnancy. Thyroid disease may also present for the first time during pregnancy. Pregnancy may complicate the management of functional thyroid disorders by affecting their clinical manifestations and limiting the approaches commonly used for diagnosis and treatment. The approach to other thyroid conditions like nodular disease must also be modified during pregnancy because of concern for the safety of the fetus.

Thyroid Physiology

Normal Thyroid Function

Thyroid hormone production is dependent in large part on the supply of iodine, which is derived solely from dietary sources and actively transported into the thyroid gland. The functional unit of the thyroid gland is the thyroid follicle, which is composed of a spherical alignment of cuboid epithelial cells surrounding a core of colloid. Colloid consists primarily of thyroglobulin, which provides tyrosine residues for serial iodination that results, through a complex series of biochemical and biophysical alterations, in the production of the thyroid hormones T_4 and T_3.[382] The thyroid gland is responsible for the production of all circulating T_4 and approximately 20% of T_3. Most of the body's supply of T_3 results from peripheral conversion of T_4 through a variety of tissue-specific deiodinase enzymes.

The thyroid hormones, T_4 and T_3—whose total serum concentrations in nonpregnant women are approximately 4 to 12 (μg/dL and 90 to 200 ng/mL, respectively) (Table 28.6)—circulate in a mostly bound form, such that less than 1% circulates as free hormone.[383] Thyroid hormone is bound primarily to a specific serum-binding protein known as thyroid-binding globulin (TBG), with lesser amounts bound to albumin and pre-albumin. As in most endocrine systems, it is the *free* fraction of these hormones, not the total concentration, that are physiologically important.

Regulation of Thyroid Hormone Secretion

Under normal circumstances, the circulating concentration of T_4, the most abundant and commonly measured thyroid hormone, is maintained within a narrow range that varies little from day to day. The follicular cell within the thyroid gland is responsible for the uptake of inorganic iodide from the circulation, its organification into iodinated thyronine compounds, storage of thyroid prohormone in the form of thyroglobulin, and re-uptake of formed thyroid hormone and its ultimate release into the systemic circulation.

Follicular cell activity is under the direct control of the hypothalamic-pituitary-thyroid axis (Fig. 28.13). The

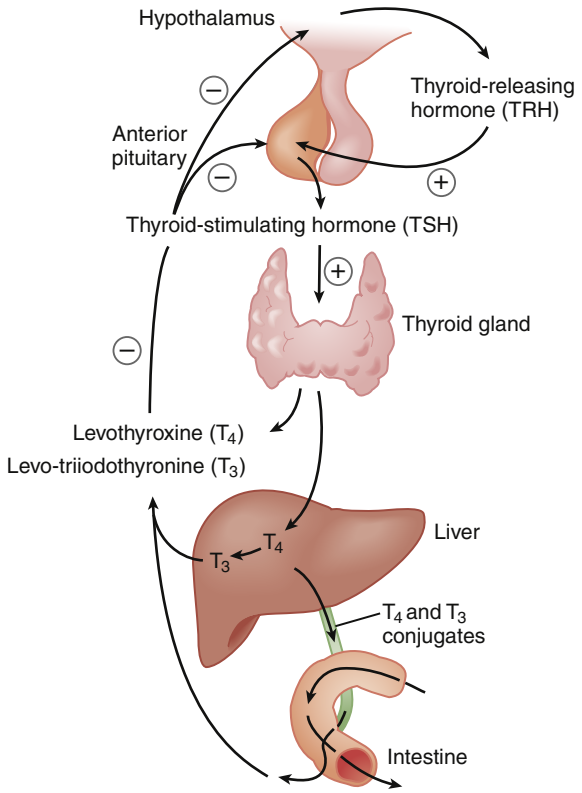


FIGURE 28.13 Diagrammatic representation of the hypothalamic-pituitary-thyroid axis. The hypothalamic-pituitary-thyroid axis and factors responsible for the regulation of thyroid hormone production and metabolism are shown.

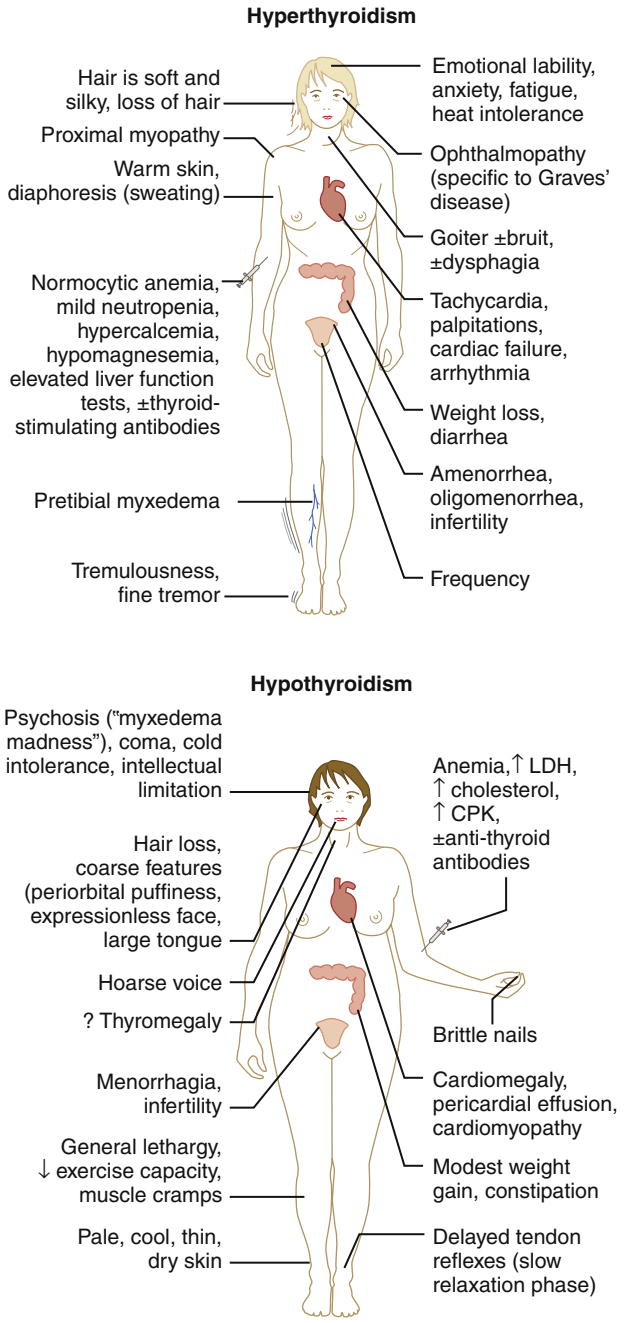


FIGURE 28.14 Diagnosis of maternal thyroid dysfunction in pregnancy. Common symptoms and signs associated with maternal hyperthyroidism and hypothyroidism. CPK, creatine phosphokinase; LDH, lactate dehydrogenase. *(From Norwitz ER, Schorge JO. Obstetrics and Gynecology at a Glance. Oxford: Blackwell Science Ltd, 2001, p 96, with permission.)*

hypothalamus produces the tripeptide TRH, which enters the portal circulation of the infundibular stalk and travels to the anterior lobe of the pituitary where it stimulates specific cells (thyrotropes) to produce TSH. TSH secretion varies diurnally with a peak secretion occurring between 11 PM and 4 AM.[384] TSH enters the systemic circulation and interacts with specific heptahelical, G-protein-coupled receptors on the surface of thyroid follicular cells, which triggers a series of signal transduction cascades culminating in the synthesis and release of thyroid hormones. Through a classical endocrine negative feedback loop, decreased circulating levels of thyroid hormones lead to an increase in TRH and TSH secretion (Fig. 28.13), which in turn leads to increased thyroid growth and activity.

Physiological Role of Thyroid Hormone

The precise role of thyroid hormone remains incompletely understood, although it clearly interacts with numerous biological systems. This fact is underscored by the complex series of symptoms and signs that are evident in patients with thyroid dysfunction (Fig. 28.14). At a cellular level, the active hormone (T_3) is transported into cells where it interacts with specific nuclear receptors. The T_3-receptor complex binds to specific thyroid hormone response elements within the promoter sequences of target genes and functions as a transcription factor, working along with other nuclear proteins to regulate gene expression.[385] In addition to these genomic effects, thyroid hormone also appears to have important extranuclear actions. These actions include regulation of deiodinase activity and, possibly, mitochondrial function.

Effect of Pregnancy on Thyroid Function

Maternal Thyroid Function in Pregnancy

In pregnancy, renal clearance of iodide increases (because of an increase in the glomerular filtration rate) and substantial amounts of iodide and iodothyronines are transferred to the fetus. As pregnancy progresses and fetal thyroid hormone

production increases, the fetus needs increasing amounts of iodide.[386,387] To meet this demand, the placenta is able to rapidly and efficiently transport available iodide from the maternal to the fetal circulation. The placenta is also capable of monodeiodination of iodothyronines, thereby making more iodide available for transport. The net result of these pregnancy-related physiological alterations is a decrease in the circulating concentration of inorganic iodide during pregnancy and a resultant increase in volume of the thyroid gland by 10% to 20% during pregnancy.[328] In light of this relative iodide deficiency during pregnancy, the recommended daily intake of iodine is increased from a baseline of 100 to 150 μg/day to approximately 250 μg/day. To achieve this daily dose, the American Thyroid Association (ATA) recommends that pregnant and lactating women supplement dietary iodine intake with a prenatal vitamin including 150 μg/day of iodine; this is the dose included in the majority of prenatal vitamins in the United States.[388,389]

Serum concentrations of TBG increase in pregnant women by 75% to 100% (the T_4 resin uptake decreases proportionally). Moreover, much of the increase in circulating TBG levels occurs during the first trimester and results from the effects of the hyperestrogenemic state on hepatocytes, with stimulation of TBG synthesis and reduced hepatic clearance due to estrogen-induced TBG sialylation.[390,391] The concentration of TBG plateaus at around 12 to 14 weeks' gestation and is associated with a concomitant increase in circulating total thyroid hormone concentrations (Fig. 28.15).

Indeed, mean concentrations of both total T_4 and T_3 in the maternal circulation increase by 10% to 30% in most longitudinal studies,[328,392,393] usually into a range that is considered elevated in the general population. In the first trimester, the increase in total T_4 exceeds the rise in TBG resulting in a slight increase in free T_4, although free hormone levels typically return to normal by the early second trimester (Fig. 28.15). However, these changes are so subtle that serum free T_4 concentrations in most pregnant women remain within the normal range for nonpregnant women.[328,391-394] While some studies report a substantial decrease in serum free T_4 with progression of gestation,[335,388,395-398] some report no change in T_4 concentrations as gestation progresses.[397] The negative-feedback control system of the hypothalamic—pituitary—thyroid axis functions normally in pregnant women.[391]

Recent data suggest that some free T_4 (and possibly T_3) immunoassays are unreliable in pregnancy, due to an increase in TBG concentration and lower albumin concentrations.[335] While the International Federation of Clinical Chemistry and Laboratory Medicine recommends using an isotope dilution-liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for measuring T_4 in the dialysate from equilibrium dialysis of serum to obtain a reference measurement for serum free T_4,[399] this assay technology is not currently widely available due to high cost. The ATA thus recommends the use of on-line extraction/LC/MS/MS to measure FT_4 in the dialysate or ultrafiltrate of serum samples. If this method is unavailable, they recommend method-specific and trimester-specific reference ranges for serum free T_4.[388] If these adjusted reference ranges are unavailable, or if free T_4 measurements are discordant with TSH measurements, consideration can be given to using adjusted serum total T_4 measurements to assess thyroid function.[335] Total T_4 and T_3 levels in pregnancy are typically 1.5-fold higher than in nonpregnant women, so an adjusted reference range should be used.

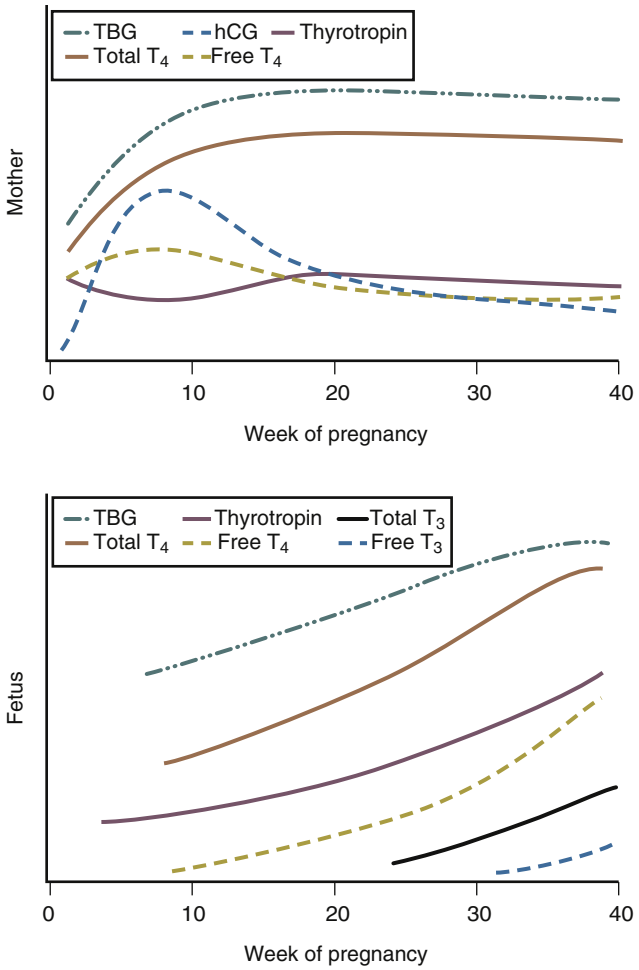


FIGURE 28.15 Relative changes in maternal and fetal thyroid function during pregnancy. The effects of pregnancy on the mother include a marked and early increase in hepatic production of thyroxine-binding globulin (TBG) and placental production of human chorionic gonadotropin (hCG). The increase in serum TBG, in turn, increases serum T_4 concentrations; hCG has thyrotropin-like activity and stimulates T_4 secretion. The transient hCG-induced increase in serum free T_4 inhibits maternal secretion of thyrotropin. *(Adapted from Burrow CN, Fisher DA, Larsen PR. Maternal and fetal thyroid function.* N Engl J Med *331:1072, 1994, with permission.)*

The pregnancy-specific glycoprotein hormone hCG is structurally similar to TSH and has some weak thyrotropic activity, which is estimated at approximately 0.025% that of TSH.[332,400] The production of hCG begins during the first week after fertilization and is highest near the end of the first trimester, after which it declines. This increase causes a transient increase in serum free T_4 concentrations, which in turn decreases serum TSH concentrations during the first trimester (Fig. 28.15).[327,332] Because hCG concentrations are higher in multiple gestations compared to singleton pregnancies, suppression of serum TSH concentrations is more marked in multiple gestations.[327] This cross-reactivity only becomes clinically significant if circulating levels of

hCG are markedly elevated, such as those seen in complete molar pregnancies.

Fetal Thyroid Function in Pregnancy

The fetal thyroid gland and pituitary-thyroid axis becomes functional late in the first trimester. Before that time, any thyroid hormone in the fetus must come from the maternal circulation. By 9 to 10 weeks of gestation, the fetal thyroid begins to concentrate iodine, thyroid follicles become visible, and T_4 synthesis can be demonstrated. TBG and T_4 are first detected in fetal serum at approximately 10 to 12 weeks of gestation, although the majority of fetal thyroid hormone synthesis occurs after the 18th to 20th week of gestation.[391] From this point forward, fetal thyroid secretion increases gradually, reaching a plateau at 35 to 37 weeks.[391,401] At term, fetal serum TSH concentrations are higher, free T_4 concentrations are slightly lower, and T_3 concentrations are one-half those in the maternal circulation.[391,401-405] The progressive increase in serum TBG concentrations with increasing gestational age presumably reflects maturation of the fetal liver and its responsiveness to estrogen stimulation. Increases in pituitary and serum concentrations of TSH during the second trimester coincide with the development of the hypothalamic-pituitary portal circulation, which facilitates the regulation of pituitary TSH secretion by hypothalamic TRH. The increased secretion of TRH despite higher serum free T_4 concentrations implies immaturity of the negative-feedback system that regulates the secretion of TSH and TRH *in utero*.[391,404,405]

Transplacental passage of thyroid hormones (T_4 and T_3) from the mother to the fetus does occur but is minimal, estimated at less than 0.1. This is likely due to the large amount of type III deiodinase enzyme in the placenta, which serves to maintain low serum T_3 concentrations in the fetus while protecting decidual cells from hypothyroidism.[391,404-407] As such, tests of fetal thyroid function—although rarely, if ever, indicated in clinical practice—accurately reflect functioning of the fetal thyroid and are largely unrelated to maternal thyroid status.[391] That said, however, in neonates with congenital hypothyroidism, enough maternal thyroid hormone is able to cross the placenta to prevent the overt stigmata of hypothyroidism at birth and maintain cord blood thyroid hormone levels at approximately 25% to 50% of normal.[408] Iodine, TRH, and TSH receptor immunoglobulins do cross the placenta[409] as does TSH, but to a far lesser extent.[410] Fetal thyroid function is completely dependent on the supply of iodine from the mother. Normal levels of thyroid hormone are critical for neuronal migration and myelination of the fetal brain. Thus, maternal and fetal iodine deficiency in pregnancy has adverse effects on neurocognitive function of offspring.[411-413] Children born to severely iodine-deficient mothers may exhibit profound mental retardation, deafmutism, and motor rigidity, a constellation of symptoms known as cretinism.[388]

Thyroid hormone is also present in measurable quantities in amniotic fluid. At term, total T_4 concentrations in the amniotic fluid are about 0.6 μg/dL, much lower than in maternal or fetal serum. Because protein and TBG concentrations are low in amniotic fluid, however, the free T_4 and T_3 concentrations are slightly higher than in maternal or fetal serum.[414] The source of this thyroid hormone is not known, but studies in fetuses with congenital hypothyroidism suggest that it may come from the maternal circulation.[415] Late in gestation, fetal swallowing appears to allow for the transfer of thyroid hormones from amniotic fluid to the fetal circulation. The physiological role of thyroid hormone in the amniotic fluid is not known.

Functional Thyroid Disorders in Pregnancy

Screening for Thyroid Disorders in Pregnancy

Data from the United States population suggest that 2% to 3% of pregnant women will have an elevated TSH level at the time of routine screening. Of screened women, 0.3% to 0.5% will have overt hypothyroidism (elevated TSH and low free T4, or a TSH level of 10 mIU/L or greater, regardless of free T4 levels), and 2% to 2.5% will have subclinical hypothyroidism (TSH levels above the 97.5th percentile for gestational age with normal free T4 concentration).[416,417] Hyperthyroidism is less common, and occurs in only 0.1% to 0.4% of pregnant women.[418] Overt thyroid dysfunction in pregnancy has consistently been associated with increased risk of adverse pregnancy outcomes and detrimental effects on fetal neurocognitive development (see sections on "Maternal Thyrotoxicosis" and "Maternal Hypothyroidism" later in this chapter). To date, there is no consistent evidence that screening and treatment for asymptomatic hypothyroidism will abrogate the posited association between subclinical hypothyroidism and neurocognitive impairment in offspring. Subclinical hyperthyroidism has not been associated with adverse maternal or fetal outcomes.[419]

While two decision analyses found that universal screening for thyroid dysfunction in pregnancy is cost-effective, both models assumed that treatment of subclinical hypothyroidism in pregnancy would increase offspring IQ, which has not been demonstrated in large randomized clinical trials.[420,421]Although large studies of a targeted case-finding strategy versus universal screening to identify women with hypothyroidism in pregnancy have demonstrated that a targeted case-finding approach may miss as many as 30% to 55% of women with thyroid abnormalities,[422,423] universal screening has not been shown to result in improved population outcomes.[424] Given these data, ACOG, the ATA, and the Endocrine Society recommend a targeted case-finding approach, rather than universal first-trimester screening of all pregnant women for subclinical hypothyroidism.[388,402,425,426] The ATA's suggested criteria for whom to screen for thyroid dysfunction in pregnancy are summarized in Box 28.4.[388] TSH measurement in the first trimester should be performed in these women, with free T_4 measurement if TSH is greater than 2.5 mIU/L. Screening is not indicated in asymptomatic pregnant women who have a mildly enlarged thyroid, in the absence of goiter.[402]

Maternal Thyrotoxicosis

Thyrotoxicosis is the clinical and biochemical state that results from an excess production of and exposure to thyroid hormone from any cause. In contrast, *hyperthyroidism* refers to thyrotoxicosis caused by hyperfunctioning of the thyroid gland.[402] Hyperthyroidism occurs in 0.05% to 0.2% of pregnancies. Graves disease is the most common cause of maternal hyperthyroidism in pregnancy, accounting for 95% of cases.[401,402,406-408,414,415,427] Other causes of thyrotoxicosis in pregnancy are summarized in Box 28.5.

Box 28.4 Women to Screen for Thyroid Dysfunction in Pregnancy

Family or personal history of thyroid disease
Symptoms suggestive of thyroid dysfunction (Fig. 28.14)
From an area with known moderate to severe iodine insufficiency
Type 1 diabetes or other autoimmune disorders frequently associated with autoimmune thyroid dysfunction (vitiligo, adrenal insufficiency, hypoparathyroidism, atrophic gastritis, pernicious anemia, systemic sclerosis, systemic lupus erythematosus, Sjogren's syndrome)
Known thyroid peroxidase antibodies
History of head or neck radiation
History of preterm delivery or recurrent miscarriage
Morbid obesity (BMI ≥ 40 kg/m^2)
Infertility
Age >30 years
Use of amiodarone or lithium
Recent administration of iodinated radiological contrast

Box 28.5 Etiology of Thyrotoxicosis in Pregnancy

- Graves disease: The most common cause of maternal thyrotoxicosis in pregnancy (95%). Results from the circulating thyroid-stimulating IgG autoantibodies, which can cross the placenta leading to fetal thyroid dysfunction.
- Thyroiditis (silent/postpartum [lymphocytic]; subacute [granulomatous]; suppurative [bacterial]): Characterized by hyperthyroidism and the presence of a large, palpable thyroid gland. An acute enlarged, painful, and tender thyroid is suggestive of subacute (De Quervain's) thyroiditis.
- Toxic multinodular goiter
- Solitary toxic nodule (also referred to as a hyperfunctioning thyroid adenoma)
- Gestational trophoblastic neoplasia: Including hydatidiform mole and choriocarcinoma. Probably secondary to elevated levels of hCG.
- Struma ovarii: Refers to thyroid tissue in a mature ovarian teratoma
- Exogenous thyroid hormone: Most commonly due to inadvertent ingestion of thyroid hormone
- Iodine-induced thyrotoxicosis
- TSH-secreting pituitary adenoma
- Gestational hyperthyroidism: Transient hyperthyroidism limited to the first half of pregnancy, frequently associated with elevated levels of hCG. Hyperemesis gravidarum is often coexistent. Typically self-limited, supportive care is indicated.
- Hyperemesis gravidarum: Characteristic symptoms and signs of hyperthyroidism are often absent.
- Familial gestational hyperthyroidism: TSH receptor mutation leading to functional hypersensitivity to hCG.

Of note, gestational hyperthyroidism, defined as "transient hyperthyroidism limited to the first half of pregnancy [and] characterized by elevated free T_4 or adjusted total T_4 and suppressed or undetectable serum TSH, in the absence of serum markers of thyroid autoimmunity"[428] is a frequent cause of thyrotoxicosis in pregnancy. Gestational hyperthyroidism occurs in 1% to 3% of pregnancies, and may be associated with hyperemesis gravidarum. Appropriate management includes supportive therapy and management of dehydration, if necessary. Anti-thyroid medications are not recommended as part of the standard management of gestational hyperthyroidism.[388] While gestational hyperthyroidism occurs in 1% to 3% of pregnancies, hyperemesis gravidarum, a syndrome of nausea and vomiting associated with weight loss of 5% or more in early pregnancy, occurs in 0.5 to 10 per 1000 pregnancies.[429,430] Hyperemesis gravidarum is characterized by higher serum hCG and estradiol concentrations than in nonaffected pregnant women,[428] and hCG has more thyroid-stimulating activity in women with hyperemesis gravidarum.[431,432]

Box 28.6 Effects of Drugs on Thyroid Hormone Synthesis and Metabolism

INHIBITION OF THYROID HORMONE SYNTHESIS BY THYROID GLAND
Iodine, sulfonylureas, lithium

INCREASE IN TSH
Iodine, cimetidine, dopamine agonists, lithium

DECREASE IN TSH
Glucocorticoids, dopamine agonists, somatostatin

INHIBITION OF THYROID HORMONE BINDING TO THYROID-BINDING GLOBULIN (TBG)
Phenytoin, diazepam, sulfonylureas, furosemide, salicylates

INHIBITION OF CONVERSION OF T4 TO T3 IN PERIPHERAL TISSUES (LIVER)
Glucocorticoids, propylthiouracil (PTU), ipodate, propranolol, amiodarone

INHIBITION OF GASTROINTESTINAL RESORPTION OF THYROID HORMONES
Cholestyramine, cholestipol, ferrous sulfate

Numerous drugs are known to interfere with thyroid hormone synthesis and metabolism (Box 28.6). Symptoms and signs may suggest the etiology of thyrotoxicosis in pregnancy (Fig. 28.14). For example, endocrine ophthalmopathy (lid lag, lid retraction) and dermopathy (localized or pretibial edema) are clinical signs that are specific to Graves disease. However, as in nonpregnant patients, confirmation of maternal hyperthyroidism in pregnancy requires thyroid function testing (Table 28.6). Measurement of TSH receptor antibodies and total T_3 may be helpful in trying to distinguish between Graves disease and gestational hyperthyroidism. Radioactive iodine scanning or radioiodine uptake determination should not be performed in pregnancy. There is not sufficient evidence to recommend for or against the use of thyroid ultrasound in distinguishing between possible etiologies of hyperthyroidism in pregnancy.[388]

As compared with well-controlled disease, inadequately treated maternal hyperthyroidism is associated with infertility and adverse perinatal outcome. Maternal complications in pregnancy include an increased risk of preeclampsia, cardiac failure, thyroid storm, and possibly spontaneous pregnancy loss.[401,402,406-408,414,415,427,433,434] Fetal and neonatal risks that are increased in the setting of poorly-controlled hyperthyroidism include preterm delivery, low birth weight, IUGR, stillbirth, central hypothyroidism, and increased perinatal mortality.[433,435-439] Because a large proportion of hyperthyroidism in pregnancy is mediated by IgG antibodies that cross the placenta (Graves disease and chronic

Table 28.6 Changes in Thyroid Function Test Results in Normal Pregnancy and Thyroid Disease

Thyroid Function Test	Units	Normal Non-pregnant Values	Normal Pregnant Values	Comparison to Nonpregnant Values	Hyperthyroidism	Hypothyroidism
Thyroid-stimulating hormone (TSH)	mIU/L	0.4-4.0	1st trimester: 0.1-2.5* 2nd trimester: 0.2-3.0* 3rd trimester: 0.3-3.0*	Decreased (largest decrease in 1st trimester)	Markedly decreased	Markedly increased
Thyroid-binding globulin (TBG)	mg/L	11-21	23-25	Increased	No change	No change
Total levothyroxine (T_4)	μg/dL	3.9-11.6	10.7-11.5	Increased	Increased	Decreased
Free levothyroxine (T_4)	ng/dL	0.8-2.0	Trimester-specific and method-specific ranges should be used	Decreased or no change	Increased	Decreased
Total L-triiodothyronine (T_3)	ng/dL	91-208	205-233	Increased	Normal to increased	Normal to decreased
Free L-triiodothyronine (T_3)	pg/dL	190-710	250-330	No change	Increased	Decreased

*For use if trimester-specific ranges for TSH are not available from the laboratory.

Data from Nissim M, Giorda G, Bailable M, et al. Maternal thyroid function in early and late pregnancy. *Horm Res* 36:196, 1991; O'Leary PC, Boyne P, Atkinson G, et al. Longitudinal study of serum thyroid hormone levels during normal pregnancy. *Int J Gynaecol Obstet* 38:171, 1992; Burrow GN, Lisher DA, Larsen PR. Maternal and fetal thyroid function. *N Engl J Med* 331:1072, 1994; American College of Obstetricians and Gynecologists. *Thyroid disease in pregnancy*. ACOG Practice Bulletin No. 37. Washington, DC; ACOG, 2002 (reaffirmed 2010); Stagnaro-Green A, Abalovich M, Alexander E, et al. Guidelines of the American Thyroid Association for the diagnosis and management of thyroid disease during pregnancy and postpartum. *Thyroid* 21:1081, 2011.

autoimmune thyroiditis), the fetus is at risk of immune-mediated thyroid dysfunction. This is true also of women with a history of Graves disease treated with thyroidectomy or radioactive iodine. Fetal sinus tachycardia (greater than 160 beats per minute persistent for over 10 minutes) is a sensitive index of fetal hyperthyroidism.[388] Only 1% to 5% of neonates born to women with poorly-controlled thyrotoxicosis will develop transient hyperthyroidism or neonatal Graves disease caused by the transplacental passage of maternal antithyroid antibodies.[436,440] In addition to fetal tachycardia, other signs of fetal hyperthyroidism include intrauterine growth restriction, presence of fetal goiter, and craniosynostosis or accelerated bone maturation.[441,442] Congestive heart failure and fetal hydrops may occur in severe cases. Fetuses of women with Graves disease are at risk not only for fetal and neonatal hyperthyroidism, but also for fetal and neonatal hypothyroidism due to overtreatment with antithyroid drugs,[443] and central hypothyroidism.[437-439] High titers of serum thyroid receptor antibodies between 22 and 26 weeks' gestation are a risk factor for fetal or neonatal hyperthyroidism, and thus determination of maternal serum thyroid receptor antibodies is recommended at 20 to 24 weeks' gestation in women with a history of Graves disease.[388,441,444-446] Fetal surveillance with serial ultrasounds is recommended in pregnant women with Graves disease, uncontrolled hyperthyroidism, or those with thyroid receptor antibody levels greater than two to three times the upper limit of normal.[388,402,441,447]

In order to minimize complications, hyperthyroidism is best diagnosed and treated prior to conception, and the use of contraception is advisable until a euthyroid state is achieved. If a patient has high TSH receptor antibody titers and is planning pregnancy in the next 2 years, surgical therapy may be considered. TSH receptor antibody titers tend to increase and remain elevated for many months after radioactive iodine (^{131}I) therapy.[448] If ^{131}I ablative therapy is performed, pregnancy should be delayed for 6 months after thyroid ablation.

The goal of therapy during pregnancy is to control thyrotoxicosis while avoiding fetal and transient neonatal hypothyroidism. The mainstay of treatment for hyperthyroidism in pregnancy is thioamide drugs, specifically propylthiouracil (PTU) and methimazole (the active metabolite of carbimazole), which decrease thyroid hormone synthesis by blocking the organification of iodide.[449,450] PTU also reduces the peripheral conversion of T_4 to T_3, and may therefore have a quicker suppressive effect than methimazole.

Traditionally, PTU has been preferred in pregnant patients because methimazole was believed to pass the placenta more easily and was associated with methimazole embryopathy (including choanal or esophageal atresia, tracheo-esophageal fistula, patent vitellointestinal duct and dysmorphic facies) and fetal aplasia cutis congenita, a rare congenital skin defect of the scalp.[401,402,406-408,414,415,427,433,440,451] Recent data, however, called these associations into question. No difference in free T_4 and TSH concentrations has been demonstrated in the umbilical cord blood of fetuses delivered to women treated with PTU or methimazole,[452] and no definitive increase in the incidence of aplasia cutis or structural anomalies over healthy controls has been shown in women treated with methimazole in pregnancy.[451-454] A recent study including all birth defects related to maternal treatment with carbimazole or PTU over nearly 50 years in the United Kingdom did suggest an elevated risk of structural anomalies in women taking carbimazole in pregnancy compared to those taking PTU,[455] but these data should be interpreted with caution given that carbimazole was historically prescribed far more frequently in the UK than was PTU.

Despite concern about theoretical risk of methimazole embryopathy and fetal aplasia cutis, PTU treatment is associated with an increased risk for fulminant hepatotoxicity and agranulocytosis.[456-459] Hepatotoxicity may occur at any time during PTU treatment and there are no data that monitoring of liver function tests is effective in preventing fulminant hepatotoxicity. Thus, an advisory committee to

the United States Food and Drug Administration recommended limiting the use of PTU to the first trimester of pregnancy.[460] PTU treatment is usually initiated at 50 to 300 mg daily in divided doses (the lowest dose is preferable to minimize the risk of fetal hypothyroidism). Following the first trimester, consideration should be given to transitioning to methimazole, usually initiated at 5 to 15 mg daily. Methimazole is approximately 10 to 15 times as potent as PTU; thus 100 milligrams of PTU is equivalent to 7.5 to 10 mg of methimazole.[388] The goal is to utilize the least amount of drug to maintain maternal free T_4 at the upper limits of normal.[402] TSH and T_4 levels should be checked monthly and treatment adjusted accordingly. Clinicians should remember that stored hormone may not be depleted for 3 to 4 weeks, therefore delaying a clinical response. Complete blood counts should be monitored monthly because of the risk of drug-induced agranulocytosis.[402]

Radioactive iodine administration to ablate the thyroid gland is absolutely contraindicated in pregnancy. Moreover, breastfeeding should be avoided for at least 120 days after ^{131}I treatment.[461] Surgery is best avoided, but may be performed in the second trimester if indicated for failed medical therapy.

Subclinical hyperthyroidism, defined as low TSH with normal free T_4 and T_3 in an asymptomatic patient, is not associated with adverse perinatal outcome.[419] Long-term subclinical hyperthyroidism is associated with osteoporosis, atrial fibrillation, and increased mortality and, as such, should be treated. However, there is little data on the appropriate course of action during pregnancy. Many cases resolve spontaneously. The presence of suppressed serum TSH in the first trimester (TSH less than 0.1 mIU/L) should be investigated with a history and physical examination, as well as free T_4 measurements in all patients.[388]

Thyroid Storm

Thyroid storm (thyrotoxic crisis) is a medical emergency characterized by a severe acute exacerbation of the signs and symptoms of hyperthyroidism. It is a rare complication, occurring in approximately 1% of pregnant patients with hyperthyroidism, but is associated with a high rate of maternal mortality and morbidity.[99] Thyroid storm is diagnosed by a combination of the following symptoms and signs in patients with thyrotoxicosis: fever, tachycardia out of proportion to the fever, altered mental status (restlessness, nervousness, confusion, seizures), diarrhea, vomiting, and cardiac arrythmia.[99] An inciting event (infection, surgery, labor and delivery) can be identified in many instances. The diagnosis can be difficult to make, however, and requires expeditious treatment to avoid severe consequences like shock, stupor, coma, and death.

If thyroid storm is suspected, serum TSH and free T_4 and T_3 levels should be evaluated to help confirm the diagnosis. If the clinical index of suspicion is high, however, *treatment should not be withheld pending the results of the biochemical tests*. The treatment of thyroid storm is summarized in Box 28.7. The goals of treatment are to:

- Reduce synthesis and release of hormone from the thyroid gland (using thioamides like PTU or methimazole, supplemental iodide, and glucocorticoids)
- Block the peripheral actions of thyroid hormones (using glucocorticoids, PTU, and high-dose beta blockers)
- Treat complications and support physiological functions (supplemental oxygen, fluid and caloric replacement)
- Identify and treat precipitating events (such as hypoglycemia, thromboembolic events, and diabetic ketoacidosis)

As with other acute maternal illnesses, fetal well-being should be evaluated and consideration given to delivery, if appropriate.

Box 28.7 Treatment of Thyroid Storm in Pregnant Women

- **Propylthiouracil (PTU),** 600-800 mg orally stat, then 150-200 mg orally every 4-6 hours. If oral administration is not possible, use methimazole rectal suppositories.
- Starting 1-2 hours after PTU, administer **saturated solution of potassium iodide (SSKI),** 2-5 drops orally every 8 hours, or **sodium iodide**, 0.5-1.0 g intravenously every 8 hours, or **Lugol solution**, 8 drops every 6 hours, or **lithium carbonate**, 300 mg orally every 6 hours.
- **Dexamethazone**, 2 mg intravenously or intramuscularly every 6 hours for four doses.
- **Propranolol**, 20-80 mg orally every 4-6 hours or 1-2 mg intravenously every 5 minutes for a total of 6 mg, then 1-10 mg intravenously every 4 hours. If the patient has a history of severe bronchospasm, **reserpine** (1-5 mg intramuscularly every 4-6 hours), **guanethidine** (1 mg/kg orally every 12 hours), or **diltiazem** (60 mg orally every 6-8 hours) should be given.
- **Phenobarbital**, 30-60 mg orally every 6-8 hours as needed for extreme restlessness.

Data from Ecker JL, Musci TJ. Thyroid function and disease in pregnancy. *Curr Probl Obstet Cynecol Fertil* 23:109, 2000.

Maternal Hypothyroidism

Hypothyroidism is caused by inadequate thyroid hormone production. It complicates 0.3% to 0.5% of all pregnancies but is more common in women with other autoimmune diseases, such as type 1 diabetes.[462] The classic signs and symptoms of hypothyroidism (Fig. 28.14) may suggest the diagnosis. Again, however, thyroid function testing is required for a definitive diagnosis (Table 28.6). The causes of hypothyroidism in pregnancy are summarized in Box 28.8. In developed countries, chronic autoimmune thyroiditis (Hashimoto disease) is the most common cause.[401,402,406-408,414,415,427,433,440,451-453,461-463] Worldwide, however, the most common cause of hypothyroidism is iodine deficiency.[401,402,406-408,414,415,427,463] Women previously treated for Graves disease (by radioactive iodine or surgery) may manifest with posttherapy hypothyroidism. Although such women may themselves be asymptomatic, their fetuses remain at risk for thyroid dysfunction as circulating antithyroid antibodies are still present. In women with preexisting Hashimoto disease, pregnancy may actually result in a transient improvement of symptoms.

Untreated or inadequately treated maternal hypothyroidism in pregnancy is associated with an increased risk of adverse pregnancy outcome, including preeclampsia, low birth weight, placental abruption, preterm birth, and stillbirth.[401,402,406-408,414,415,427,463] It is not clear whether untreated hypothyroidism is a risk factor for IUGR

Box 28.8 Etiology of Hypothyroidism in Pregnancy

PRIMARY HYPOTHYROIDISM

- Iodine deficiency (the most common cause of hypothyroidism worldwide)
- Chronic autoimmune thyroiditis (Hashimoto): Characterized by hypothyroidism, a firm goiter, and the presence of circulating antithyroglobulin and/or antimicrosomal autoantibodies
- Silent/postpartum thyroiditis (hypothyroid phase)
- Prior treatment for hyperthyroidism (includes women previously treated with radioactive iodine or surgery [thyroidectomy] leading to post-therapy hypothyroidism)
- Prior high-dose external beam neck irradiation
- Infectious (suppurative) thyroiditis: Characterized by fever and a painful, swollen thyroid gland. Common infections include *Staphylococcus aureus, S. hemolyticus,* and fungi.
- Subacute thyroiditis (hypothyroid phase): Similar to suppurative thyroiditis, but it is usually the result of a viral infection and is self-limiting
- Thyroid agenesis/dysgenesis
- Drug-induced hypothyroidism
- Dietary goitrogens (includes such drugs as thioamides and lithium)
- Organification enzyme defects

SECONDARY HYPOTHYROIDISM

- Pituitary adenoma
- Pituitary necrosis/hemorrhage
- Lymphocytic hypophysitis
- Central nervous system sarcoidosis
- Prior hypophysectomy
- Cranial irradiation
- Suprasellar/parasellar
- Traumatic injury to pituitary/hypothalamus

independent of other complications. Thyroid hormones also have important roles in embryogenesis and fetal maturation. Maternal hypothyroxinemia is associated with neonatal hypothyroidism and with defects in IQ and long-term neurological function in the offspring.[402,464,465] Women with iodine-deficient hypothyroidism are at particularly high risk of having a child with congenital cretinism (growth failure, mental retardation, and other neuropsychological deficits).

Early diagnosis and treatment of maternal hypothyroidism is essential to avoid antepartum pregnancy complications and impaired neonatal and childhood development. Indeed, in an iodine-deficient population, treatment with iodine in the first and second trimesters of pregnancy has been shown to significantly reduce the incidence of congenital cretinism.[466] With the advent of routine newborn screening for congenital hypothyroidism, it has become clear that size, weight, appearance, behavior, extrauterine adaptation, and immediate postnatal development are usually normal in infants with hypothyroidism, even those with thyroid agenesis.[467]

Although untreated hypothyroidism is associated with adverse perinatal outcome, the maternal and fetal consequences of isolated maternal hypothyroxinemia (normal TSH in conjunction with free T4 concentration in the lower 5th or 10th percentile of the reference range), and subclinical hypothyroidism (SCH) during pregnancy are not as clear. One study utilizing stored serum samples of nearly 17,300 pregnant women reported that isolated maternal hypothyroxinemia was not associated with adverse pregnancy outcomes.[468] However, another study utilizing nearly 11,000 stored serum samples from the multicenter, prospective First- and Second-Trimester Evaluation of Risk (FASTER) trial found that women with hypothyroxinemia and normal TSH had 1.6 times the odds of preterm labor, nearly twice the odds of macrosomia, and 1.7 times the odds of gestational diabetes.[469]

The majority of studies suggest that SCH is associated with increased perinatal risk.[402,464,465,470,471] A retrospective cohort study of over 17,000 women demonstrated that untreated subclinical hypothyroidism was associated with a threefold risk of placental abruption and a 1.8-fold risk of preterm birth before 34 weeks, compared to euthyroid controls.[471] Subclinical hypothyroidism in pregnancy has also been associated with a fourfold risk of fetal death,[416] an increased risk for severe preeclampsia,[472] and an increased miscarriage rate even in thyroid peroxidase antibody (TPOAb)-negative women.[473-475] However, other large studies have failed to demonstrate an association between SCH and adverse obstetrical outcomes.[469,476,477] The only trial to directly address the impact of treatment for SCH randomized over 4500 pregnant women to either a case-finding group or universal thyroid screening strategy.[424] Negro and colleagues found that the universal screening approach did not result in an overall decrease in adverse perinatal outcomes, but TPOAb-positive women with SCH (TSH greater than 2.5 mIU/L) who were treated with levothyroxine had a lower risk of adverse obstetrical outcomes, including miscarriage, hypertension, preeclampsia, gestational diabetes, placental abruption, cesarean delivery, congestive heart failure, preterm labor, respiratory distress, neonatal intensive care unit admission, low and high birth weight, preterm or very preterm delivery, low Apgar score, and perinatal death.[424] This finding failed to reach statistical significance, however, due to the broad range of outcomes identified as adverse events and the high proportion of adverse outcomes in euthyroid women.

Most controversial is the association between subclinical hypothyroidism, isolated maternal hypothyroxinemia, and neurological impairment in the offspring.[463,478,479] A large case-control study reported a 7-point reduction in IQ scores in children born to mothers with untreated TSH elevations in pregnancy, compared to children born to euthyroid mothers.[463] Pop and colleagues reported impaired psychomotor development in offspring born to women with isolated hypothyroxinemia.[478] Similarly, a retrospective cohort study of Chinese women found that SCH, hypothyroxinemia, and elevated TPOAb titers were associated with lower intelligence scores and impaired motor development of offspring at 25 to 30 months.[480] The Generation R study, a prospective, nonrandomized population-based cohort from the Netherlands, found that while SCH was not significantly associated with cognitive outcomes in offspring, both mild (OR 1.44) and severe (OR 1.8) hypothyroxinemia were associated with expressive language delay, and severe maternal hypothyroxinemia predicted a higher risk of nonverbal cognitive delays (OR 2.03).[481]

Other studies, however, have failed to find an association between maternal subclinical hypothyroidism and cognitive performance in offspring.[481-483] To date, only one randomized controlled trial has investigated whether levothyroxine

therapy for women with either overt or subclinical hypothyroidism is associated with an improvement in childhood intellectual development.[483] The Controlled Antenatal Thyroid Screening (CATS) study enrolled nearly 22,000 women with singleton pregnancies, and randomized them before 16 weeks' gestation to screening and treatment for TSH greater than the 97.5th percentile and/or free T_4 less than the 2.5th percentile, or storage of serum samples until after completion of pregnancy. The CATS study found no significant difference in mean IQ or in the proportion of children with IQ less than 85 at 3 years of age, in children born to 390 treated mothers compared to 404 untreated mothers.[483] The Maternal Fetal Medicine Unit of the National Institutes of Health is currently conducting a multicenter randomized controlled trial to evaluate the effects of levothyroxine therapy for pregnant women with SCH. The primary outcome of this study is child IQ at age 5; the results are anticipated in 2015.[388] Thus, while an association between maternal SCH-isolated hypothyroxinemia and adverse fetal neurocognitive outcomes is biologically plausible, such an association has not been clearly demonstrated in prospective, randomized controlled trials. The most recent guidelines of the ATA therefore recommend levothyroxine therapy for pregnant women with SCH and anti-TPO antibodies, but conclude there is insufficient evidence to recommend for or against treating women with SCH in the absence of TPO antibodies, and do not recommend therapy for isolated hypothyroxinemia in pregnancy.[388]

Levothyroxine is the treatment of choice for pregnant and nonpregnant women with overt hypothyroidism. Treatment should be initiated at an oral dose of 100 to 150 μg daily. TSH levels should be measured serially every 4 weeks, and the dose of levothyroxine adjusted to maintain TSH levels within trimester-specific ranges. As thyroid hormone production is increased during pregnancy, most women will need an increase in their daily dose by approximately 30% to 50% during pregnancy.[484,485]

Postpartum Thyroiditis

Postpartum thyroiditis is an autoimmune inflammation of the thyroid gland that presents as new-onset, painless hypothyroidism, transient thyrotoxicosis, or thyrotoxicosis followed by hypothyroidism within 1 year postpartum. The condition occurs in approximately 5% (range 4% to 10%) of women without preexisting thyroid disease and may also occur after early pregnancy loss.[486-488] Thirty-three percent to 50% of women with antithyroid antibodies in the first trimester will develop postpartum thyroiditis, with higher titers conferring a greater risk.[488] The classic form begins with transient thyrotoxicosis, followed by transient hypothyroidism, with a return to euthyroid state by the end of the first postpartum year.[488] Studies have found that the classic presentation of postpartum thyroiditis is seen in approximately 20% of cases, while the majority of women (44% to 48%) with postpartum thyroiditis have isolated hypothyroidism (with fatigue, weight gain, and depression), and approximately 30% experience isolated thyrotoxicosis (characterized by dizziness, fatigue, weight loss, and palpitations).[486,488,489]

The diagnosis of postpartum thyroiditis requires a high clinical index of suspicion. The diagnosis is confirmed by documenting abnormal serum levels of TSH and T_4 in a previously euthyroid patient. Differentiating postpartum thyroiditis from Graves disease can be challenging. Thyroid receptor antibodies are often positive in Graves disease but usually are negative in postpartum thyroiditis, and radioiodine uptake is elevated or normal in Graves disease and low in postpartum thyroiditis. ^{123}I or technetium scans are preferable to ^{131}I scans in breastfeeding women, due to the shorter half-life.[488] The need for treatment in women with postpartum thyroiditis is not clear, although it may be warranted to control symptoms.[402,489] In one prospective study of 605 asymptomatic pregnant and postpartum women, none of the women with thyrotoxicosis and only 40% of women with hypothyroidism required treatment.[489] If treatment is required, it can usually be tapered within 1 year. The majority of studies examining the impact of postpartum thyroiditis on long-term thyroid function have found that between 10% and 20% of women with postpartum thyroiditis will require long-term treatment.[489-495] A recent prospective study of 169 women with postpartum thyroiditis, however, reported a much higher rate of 54% of women with persistent hypothyroidism after 1 year.[496] Women with the highest levels of TSH, high TPO antibody titers, multiparous women, older women, and those with a history of miscarriage have the highest risk for developing permanent hypothyroidism.[402,488,489] Both levothyroxine and iodine treatment during pregnancy have been investigated as a potential strategy to prevent postpartum thyroiditis in women with antithyroid antibodies, but neither intervention was effective.[497,498] A more recent prospective placebo-controlled study evaluating the efficacy of selenium in preventing postpartum thyroiditis in TPOAb-positive women found that selenium decreased both the incidence of postpartum thyroiditis and permanent hypothyroidism.[499] Selenium administration was also associated with a significant decrease in postpartum TPOAb titers. Given that only one trial has demonstrated this benefit, there is insufficient evidence at this time to recommend selenium supplementation during pregnancy in TPOAb-positive women. Even in women whose thyroid function returns to normal, the risk of recurrent postpartum thyroiditis in a subsequent pregnancy is approximately 70%.[500,501] Women with a prior history of postpartum thyroiditis should have annual TSH screening to evaluate for permanent hypothyroidism.[388]

Structural Thyroid Disorders in Pregnancy

Goiter

Goiter refers to enlargement of the entire thyroid gland. Goiters can be classified into several categories according to the functional status of the gland (hypothyroid, hyperthyroid, or euthyroid) or to its clinical or scintigraphic appearance (diffuse or multinodular). The most common causes of goiter are summarized in Box 28.9.

Treatment is rarely necessary for diffuse goiter if the patient is asymptomatic and thyroid function testing is normal. With time, however, diffuse enlargement of the thyroid gland typically evolves into multinodular goiter, with progressive autonomous functioning of one or more follicles and occasional progression to thyrotoxicosis. This progression is seen most often in the largest goiters, those with nodules greater than 2.5 cm in size, and in older patients.[502] Large, dominant nodules should undergo fine-needle aspiration because malignant neoplasms can coexist with this typically benign condition. A trial of thyroid hormone

Box 28.9 Most Common Causes of Goiter

- Endemic goiter (iodine deficiency)
- Sporadic goiter (diffuse non-toxic goiter; multinodular goiter)
- Diffuse toxic goiter (Graves disease)
- Thyroiditis (chronic autoimmune [Hashimoto disease]; subacute; silent/postpartum; suppurative)
- Drugs (thioamides, iodides, lithium)
- Dietary/environmental goitrogens
- Organification enzyme defects
- Diffuse malignant disease (lymphoma, anaplastic carcinoma)
- Infiltrative diseases (Riedel thyroiditis, sarcoidosis, amyloidosis)

suppression is reasonable in most patients, although less than 50% of nodules will respond to medical therapy by decreasing in size.[503] The approach to therapy in the patient with a toxic multinodular goiter includes thyroid ablation with radioactive iodine, thioamides, or thyroidectomy.

Thyroid Nodules

Nodules of the thyroid are common; they are palpable in 5% of the general population and may be even more common in areas of relative iodine deficiency. Careful attention must be taken to examination of the thyroid nodule and surrounding tissues because of the small but real potential of malignancy. Malignant transformation is more common in the largest nodules, in those with progressive growth, in older women, and in women with other risk factors for malignancy (such as prior neck irradiation).

The majority of thyroid nodules are found on pathological examination to be either hyperplastic or adenomatous in origin. Benign and malignant neoplasms of the thyroid are listed in Box 28.10. Papillary and follicular carcinomas represent the majority of the cancers. The incidence of thyroid cancer in pregnancy is 1/1000.[504] Pregnancy itself does not appear to increase the risk of malignant transformation[461-465] or alter the course of thyroid cancer.[504,505] Moreover, treatment for thyroid cancer does not appear to increase the risk of congenital anomalies, low birth weight, or stillbirths.[506]

Any thyroid nodule discovered during pregnancy should be further evaluated, because malignancy may be found in up to 40% of these nodules.[502,504-507] Fine-needle aspiration coupled with careful cytopathological examination of the aspirate is the technique of choice for evaluation of a thyroid nodule. Ultrasound examination may be helpful in distinguishing simple cysts from solitary nodules, in the evaluation of a multinodular goiter, or in the follow-up of known thyroid lesions. However, ultrasonography is a purely anatomical study and does not provide any functional or histological information. Similarly, scintigraphy (with either technetium or radioiodine) can provide functional information that may be important, because functional ("hot") nodules are rarely malignant and almost all carcinomas are non-functional ("cold"). However, such testing cannot definitively exclude malignancy. As such, neither of these diagnostic tests can replace fine-needle aspiration for the initial evaluation of a thyroid nodule.

If a diagnosis of thyroid cancer is made, a multidisciplinary treatment plan should be established. Management options include pregnancy termination, treatment during pregnancy, and preterm or term delivery with definitive treatment after pregnancy. The decision will be affected by the gestational age at diagnosis and by the tumor characteristics.[402] Definitive treatment for thyroid cancer is thyroidectomy and radiation. If necessary, thyroidectomy can be performed during pregnancy, preferably in the second trimester. However, given the slow progression of most thyroid cancers, surgery can often be delayed until after delivery.[504] Radiation is best deferred until after pregnancy.

Box 28.10 Most Common Causes of Thyroid Nodules

BENIGN

- Follicular adenoma
- Colloid nodule
- Hiirthle cell adenoma
- Multinodular goiter
- Simple cyst
- Nodular autoimmune thyroiditis
- Marine-Lenhart nodule (in Graves disease)

MALIGNANT

- Papillary carcinoma
- Follicular carcinoma
- Hürthle cell carcinoma
- Medullary carcinoma
- Anaplastic carcinoma
- Lymphoma
- Metastases to the thyroid

Disorders of Calcium Metabolism

Total calcium stores in the mother are distributed between a large skeletal pool of "inert" calcium (1 kg) and a small extracellular pool of bioavailable calcium. These two pools of calcium are maintained in a state of dynamic equilibrium, controlled on the one hand by parathyroid hormone (PTH) and by calcitonin on the other. PTH stimulates release of calcium from bone and promotes calcium uptake from the gastrointestinal tract, while calcitonin suppresses calcium release from bone. Calcium uptake from the gastrointestinal tract is also regulated by vitamin D metabolites. Calcium is excreted by the kidneys and is sequestered by the fetoplacental unit to build the fetal skeleton.

Pregnancy is associated with a net accumulation of calcium. At term, the total accumulation of calcium in the mother is approximately 25 to 30 g, most of which is sequestered in the fetal skeleton. This is due primarily to elevated circulating levels of biologically active 1,25-hydroxyvitamin D (cholecalciferol) leading to an increase in calcium absorption from the gastrointestinal tract.[508-510] The decidua may be a major source of 1,25-hydroxyvitamin D in pregnancy.[511] Calcitonin levels do not change in pregnancy. Although initial studies suggested that serum PTH levels increase during pregnancy[512] subsequent studies using more sensitive dual-antibody assays have shown that PTH levels are lower throughout gestation.[513,514] Urinary excretion of calcium increases during pregnancy,[515] but the ratio of urinary calcium to creatinine decreases,[516] suggesting an attempt by the kidneys to reabsorb and conserve calcium even in the face of an increased glomerular filtration rate. In general, bone density

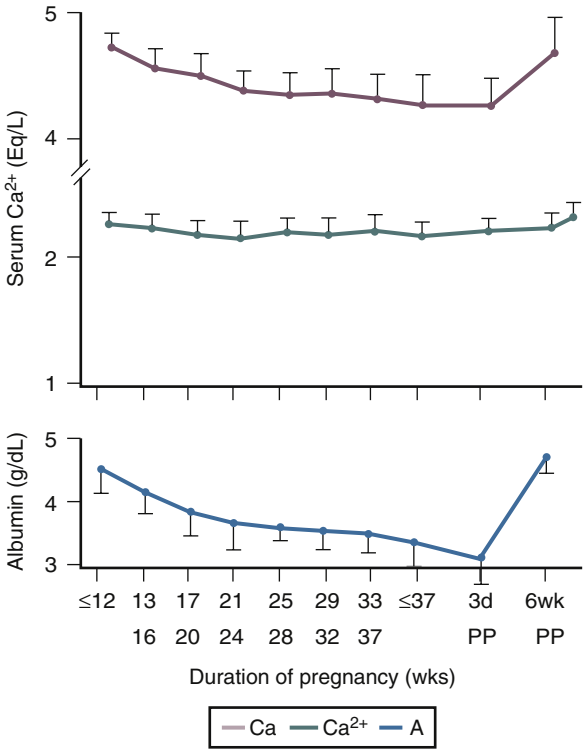


FIGURE 28.16 Serum calcium, ionized calcium, and albumin concentrations during pregnancy. During pregnancy, there is a marked decrease in circulating albumin concentration; this results in a decrease in total calcium. There is no change in ionized calcium. A, albumin. *(From Pitkin RM, Reynolds WA, Williams CA, Hargis CK. Calcium metabolism in normal pregnancy: A longitudinal study.* Am J Obstet Gynecol *133:781, 1979.)*

remains relatively constant during pregnancy, although some investigators have reported a slight decrease in bone density in the third trimester.[517] Pregnancy is also associated with a decrease in serum albumin and a concomitant decrease in total calcium concentrations (Fig. 28.16).[512,513] The upper limit of normal for total serum calcium in pregnancy is approximately 9.5 mg/dL. However, ionized calcium concentrations do not change significantly during pregnancy.[513]

Calcium from the maternal compartment is actively transported across the placenta against a concentration gradient to the fetal compartment. This process is regulated, at least in part, by the production of PTH-related protein (PTHrP), a PTH homolog, by the fetal parathyroid glands.[518] PTHrP levels rise in maternal serum throughout gestation.[514,519] In mice lacking the gene coding for PTH-related protein, calcium transport from the maternal to the fetal compartment is markedly impaired but can be rescued completely by administration of exogenous PTH-related protein.[520] Compared with the mother, the human fetus is relatively hypercalcemic, hypercalcitonemic, and hypoparathyroid. Separation of the fetus from the mother is associated with a fall in serum calcium and a compensatory rise in serum PTH and a fall in calcitonin levels.

Hyperparathyroidism

Primary hyperparathyroidism in pregnancy is rare; only a few hundred cases have been reported. Causes of hyperparathyroidism include a solitary parathyroid adenoma (80 of cases), generalized hyperplasia (15), multiple adenomas (3%), and carcinoma (less than 2%). Maternal complications of untreated or poorly controlled hyperparathyroidism include hyperemesis gravidarum, generalized weakness, headache, confusion, emotional lability, nephrolithiasis, pancreatitis, and hypertension.[521] Spontaneous abortion and perinatal mortality rates are also increased in pregnancies complicated by hyperparathyroidism[522]; however, improved diagnosis and treatment have led to a substantial decrease in perinatal mortality.[523] Most authorities recommend surgical excision of the parathyroid adenoma in symptomatic women,[521,524,525] but controversy persists as to the optimal management for asymptomatic women and women with mild hyperparathyroidism. At birth, neonatal hypocalcemic tetany is common and typically occurs in the first 2 weeks of life.[526,527]

Unusual causes of hypercalcemia in pregnancy include familial hypocalciuric hypercalcemia (FHH) and sporadic cases of inappropriate secretion of PTH-related protein.[528] Women with FHH typically present with mild hypercalcemia, mild elevations in circulating PTH concentrations, and low urinary calcium. Because of the autosomal-dominant nature of the disease and high penetrance, infants can present with either hypercalcemia (if the neonate has FHH) or hypocalcemia (if the neonate does not have FHH but is responding to maternal hypercalcemia).[528]

Hypoparathyroidism

The most common cause of maternal hypoparathyroidism is incidental resection of the parathyroid glands at the time of thyroidectomy. This complication occurs in approximately 1% of thyroidectomy cases. Symptoms of hypocalcemia include numbness and tingling of the fingers and orofacial area. Chvostek sign (twitching of the facial muscles when the facial nerve is tapped) and Trousseau sign (induction of carpopedal spasm by applying pressure to the upper arm with a blood pressure cuff) are often present.

If untreated, maternal hypocalcemia can lead to compensatory hyperparathyroidism in the fetus leading to bone demineralization. The treatment of maternal hypoparathyroidism is calcium (1.2 g daily) and either vitamin D (50,000 to 150,000 IU daily) or the active metabolite, calcitriol (0.25 to 3 μg daily).[529] If circulating calcium levels can be maintained at or near the normal range, pregnancy outcome will not be adversely affected.[530] In women with hypocalcemia, labor may be complicated by generalized tetany that requires intravenous calcium administration. Vitamin D is secreted into breast milk and may lead to hypercalcemia in the newborn.[531] As such, breastfeeding may not be advisable in women receiving high-dose vitamin D therapy.

Adrenal Diseases

Adrenal Insufficiency

Adrenal insufficiency may be either primary or secondary. *Primary adrenal insufficiency* (Addison's disease) results from destruction of both adrenal cortices. The most common cause of primary adrenal insufficiency is autoimmune destruction of the adrenal glands, which can occur

Box 28.11 Autoimmune Polyglandular Syndromes

COMMON TYPE 1*
- Addison disease
- Hypoparathyroidism
- Mucocutaneous candidiasis

LESS COMMON TYPE 1
- Hypogonadism
- Malabsorption
- Vitiligo
- Pernicious anemia
- Alopecia
- Hypothyroidism

COMMON TYPE 2
- Addison disease
- Thyroid dysfunction
- Type 1 diabetes mellitus

LESS COMMON TYPE 2
- Hypogonadism
- Myasthenia gravis
- Vitiligo
- Pernicious anemia
- Alopecia

*Type 1 is also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome.

Data from Neufeld M, MacLaren NK, Blizzard RM. Two types of autoimmune Addison's disease associated with different polyglandular autoimmune syndromes. *Medicine (Baltimore)* 60:355, 1981.

in isolation or in association with other autoimmune endocrinopathies like autoimmune polyglandular diseases, type I and type II (Box 28.11).[532] The presence of circulating antibodies against cytochrome P450 monooxygenases involved in steroidogenesis may suggest the diagnosis of autoimmune adrenal insufficiency.[533] Other causes of primary adrenal insufficiency include human immunodeficiency virus (HIV) disease, tuberculosis, sarcoidosis, and adrenal leukodystrophy.

Secondary adrenal insufficiency, on the other hand, results from an abnormality in the hypothalamic-pituitary-adrenal axis that leads to ACTH deficiency and resultant adrenocortical atrophy. In secondary adrenal insufficiency, the zona glomerulosa of the adrenal gland (and thus mineralocorticoid production) are preserved, because they are under the control of the renin-angiotensin system.[534,535]

Regardless of the cause, the most common symptoms of adrenal insufficiency are generalized weakness, fatigue, nausea, anorexia, diarrhea, and weight loss. Pigmentation in the creases of the palms of the hands, knuckles, and knees can be seen in some patients with primary adrenal insufficiency (Addison disease) due to an increase in secretion of melanocyte-stimulating hormone from an overactive adenohypophysis. Laboratory features of Addison disease include hyponatremia, hyperkalemia, and an increase in plasma blood urea nitrogen. The diagnosis of Addison disease can be confirmed using the ACTH stimulation test, in which a serum cortisol level is measured 60 minutes after an intravenous bolus of 0.25 mg synthetic[1,24] ACTH (cosyntropin). A normal ACTH stimulation test result is associated with a serum cortisol measurement of greater than 18 μg/dL.[535] The absence of an adequate cortisol response is highly suggestive of primary adrenal insufficiency. Secondary adrenal insufficiency should be suspected if there is a suboptimal cortisol response to the ACTH stimulation test, but a normal serum aldosterone concentration.[536]

Adrenal insufficiency in pregnancy has been described, and initial reports suggested a high perinatal mortality rate. More recent experience suggests good outcomes with the use of glucocorticoid therapy,[537,538] although fetal growth restriction has been described.[539]

Treatment of Addison disease should include physiological replacement of cortisol as well as mineralocorticoid, if necessary. Endogenous cortisol production rates are typically in the range of 20 to 30 mg daily, but may be as high as 300 mg daily. Hydrocortisone (cortisol) at a dose of 20 to 30 mg daily (two thirds in the morning and one third in the late afternoon or early evening) is typically prescribed as replacement for both pregnant and nonpregnant women. An alternative is oral prednisone at 2.5 to 7.5 mg daily. Fluorohydrocortisone (Florinef) at a dose of 0.1 mg daily should adequately treat mineralocorticoid deficiency. Mineralocorticoid therapy is not necessary for secondary adrenal insufficiency.

Addisonian crisis (adrenal crisis) refers to a state of acute adrenal insufficiency. This condition is rarely seen during pregnancy but frequently develops in the immediate postpartum period.[540] This is likely because, in pregnancy, the kidney synthesizes large amounts of deoxycorticosterone (a weak glucocorticoid and mineralocorticoid) from progesterone.[541] The immediate treatment of adrenal crisis should include intravenous hydration with normal saline, glucose replacement, and high doses of cortisol given either intramuscularly or intravenously (100 mg bolus every 6 to 8 hours for the first 24 hours).

Women with Addison disease undergoing surgery should be given stress doses of cortisol. On the day of surgery, 100 mg of cortisol can be administered either intramuscularly or intravenously and repeated every 6 to 8 hours during surgery and in the immediate postoperative period. This dose can be reduced by 50 mg daily until oral glucocorticoid replacement can be reinstated. Consideration should also be given to administering stress doses of cortisol in labor. By the second trimester, very little cortisol crosses the placenta intact due to high placental type 2 11β-hydroxysteroid dehydrogenase activity that converts cortisol to cortisone. As such, the fetus is highly resistant to adrenal suppression caused by maternal ingestion of glucocorticoids.[542]

Cushing Syndrome

Cushing syndrome results from exposure to excessive circulating levels of cortisol. The condition may be ACTH-dependent, as in ACTH-secreting pituitary adenoma (Cushing disease) and ACTH- or CRH-secreting tumors like bronchial carcinoids. Cushing syndrome can also be ACTH-independent, resulting from exogenous glucocorticoids, adrenal adenoma, or carcinoma. In nonpregnant women, Cushing disease is threefold more common than adrenal adenomas. In pregnancy, however, adrenal adenomas are the most common cause of Cushing syndrome (Table 28.7).[543]

The most common clinical features of Cushing disease include proximal muscle weakness, centripetal obesity

Table 28.7 Etiology of Cushing Syndrome in Nonpregnant and Pregnant Populations

Etiology	Nonpregnant (n = 108)	Pregnant (n = 58)
ACTH-secreting pituitary tumor leading to bilateral adrenal hyperplasia	64 (59%)	19 (33%)
Adrenal adenoma	17 (16%)	29 (50%)
Adrenal carcinoma	10 (9%)	6 (10%)
Ectopic ACTH	17 (16%)	1 (2%)
Unknown	0 (0%)	3 (5%)

Data from Buescher MA, McClamrock HD, Adashi EY. Cushing's syndrome in pregnancy. *Obstet Gynecol* 79:130, 1992. Reprinted with permission from The American College of Obstetricians and Gynecologists.

("potato stick" person with thick trunk and thin limbs), facial plethora, supraclavicular and dorsal ("buffalo hump") fat pads, violaceous striae, hirsutism, personality changes, and hypokalemia. In nonpregnant women, significant and persistent elevation in urinary free cortisol excretion (greater than 200 μg/day) confirms the diagnosis.

Cushing syndrome can be difficult to recognize and diagnose during pregnancy, however, because normal pregnancy is associated with physiological hypercortisolism and increased urinary free cortisol excretion. Initial studies suggested that urinary free cortisol excretion was significantly increased in pregnancy, with a mean of 130 (range, 60 to 250) μg/day.[544] However, more recent studies using high-performance liquid chromatography have shown that normal pregnant subjects have a 24-hour urinary free cortisol excretion of approximately 23 μg, which is increased to 165 to 3360 μg daily in the setting of Cushing syndrome.[545] As such, urinary free cortisone and cortisol as determined by high-performance chromatography may have greater sensitivity and specificity in the diagnosis of Cushing syndrome than does the measurement of urinary free cortisol by standard assay techniques.[546]

Once diagnosis has been confirmed, every effort should be made to identify the cause. Given the high incidence of adrenal adenomas and carcinomas in pregnant women with Cushing syndrome (Table 28.7), it may be appropriate to first perform a high-resolution imaging study of the adrenal glands, either with computed tomography or magnetic resonance imaging. If the imaging study is negative, then tests should be performed to identify an ACTH-secreting pituitary tumor (CRH stimulation followed by petrosal sinus sampling for ACTH) or an extrapituitary source of ACTH or CRH (computed tomography of the chest).[547]

Cushing syndrome is associated with an increased risk of pregnancy-related complications, including hypertension (65%), diabetes (325), preeclampsia (10%), congestive heart failure, and maternal death.[548] Perinatal morbidity and mortality are also increased in such pregnancies. Adverse perinatal outcomes include an increased risk of prematurity (65% t), IUGR (26%), and perinatal death (16%).[548]

Cushing syndrome is a rare condition in pregnancy due to the high frequency of ovulation dysfunction. As such, it has not been possible to systematically examine and compare treatment strategies for this condition. Given the high maternal and perinatal morbidity associated with this condition, aggressive antepartum and intrapartum management is indicated. Surgery (unilateral adrenalectomy) should be considered if a functional adrenal adenoma is identified. In the case of an ACTH-secreting pituitary tumor, transsphenoidal resection can be performed during pregnancy.[549] Medical treatment options include antiglucocorticoids and inhibitors of adrenal steroidogenesis. Metyrapone,[550,551] aminoglutethimide,[552] and ketoconazole[548,553] have all been used to treat Cushing syndrome in pregnancy. However, the efficacy or safety of these agents during pregnancy has yet to be established.

Congenital Adrenal Hyperplasia

Congenital adrenal hyperplasia (CAH) refers to a group of genetic disorders of steroidogenesis. It occurs in approximately 1 in 14,000 live births but is more common in certain populations. In the Yupik Inuit, for example, CAH occurs at a frequency of 1 in 300 live births.[554] The most common cause of CAH is a deficiency of 21-hydroxylase enzyme activity, which leads, in turn, to a decrease in circulating cortisol levels, a compensatory increase in ACTH production, and an increase in adrenal androgen production.

The exposure of a female fetus to high levels of androgens during early embryonic development can lead to clitorimegaly, labioscrotal fusion, ambiguous genitalia, abnormal course of the urethra, and virilization. In severe forms of 21-hydroxylase deficiency, the conversion of progesterone to mineralocorticoids (deoxycorticosterone, corticosterone, and aldosterone) is reduced, resulting in "salt wasting." If undetected and untreated, the saltwasting form of the disease may be associated with neonatal hyponatremia, hyperkalemia, and death. In CAH due to 21-hydroxylase deficiency, 75% of cases are associated with salt wasting and 25% present with virilization but no salt wasting. For the obstetrician evaluating a newborn with ambiguous genitalia, the most important disease to exclude is 21-hydroxylase deficiency associated with salt wasting. Failure to recognize this condition can lead to discharge of the newborn resulting in neonatal dehydration and death.

If a mother has previously delivered a child with CAH, or if either she or the father has CAH, then appropriate recommendations include antenatal genetic counseling, prenatal glucocorticoid treatment, and genetic testing of the neonate after delivery. Women with non-classical 21-hydroxylase deficiency who have not previously delivered a child with CAH do not require prenatal intervention because the probability that the fetus is severely affected is low (less than 1%) in most cases.

For parents wishing to pursue glucocorticoid therapy to reduce the risk of virilisation of a female fetus, the suggested management of these pregnancies is summarized in Figure 28.17.[555] In such cases, the mother should be treated with dexamethasone (20 μg/kg daily in three divided doses) as soon as the pregnancy is confirmed. This dose has been shown to normalize 17-hydroxyprogesterone levels in the amniotic fluid of affected fetuses.[556] Chorionic villus sampling should be offered at 9 to 11 weeks' gestation. If a male fetus is identified, steroid therapy can be discontinued. If the fetus is a female, prenatal diagnosis can be achieved

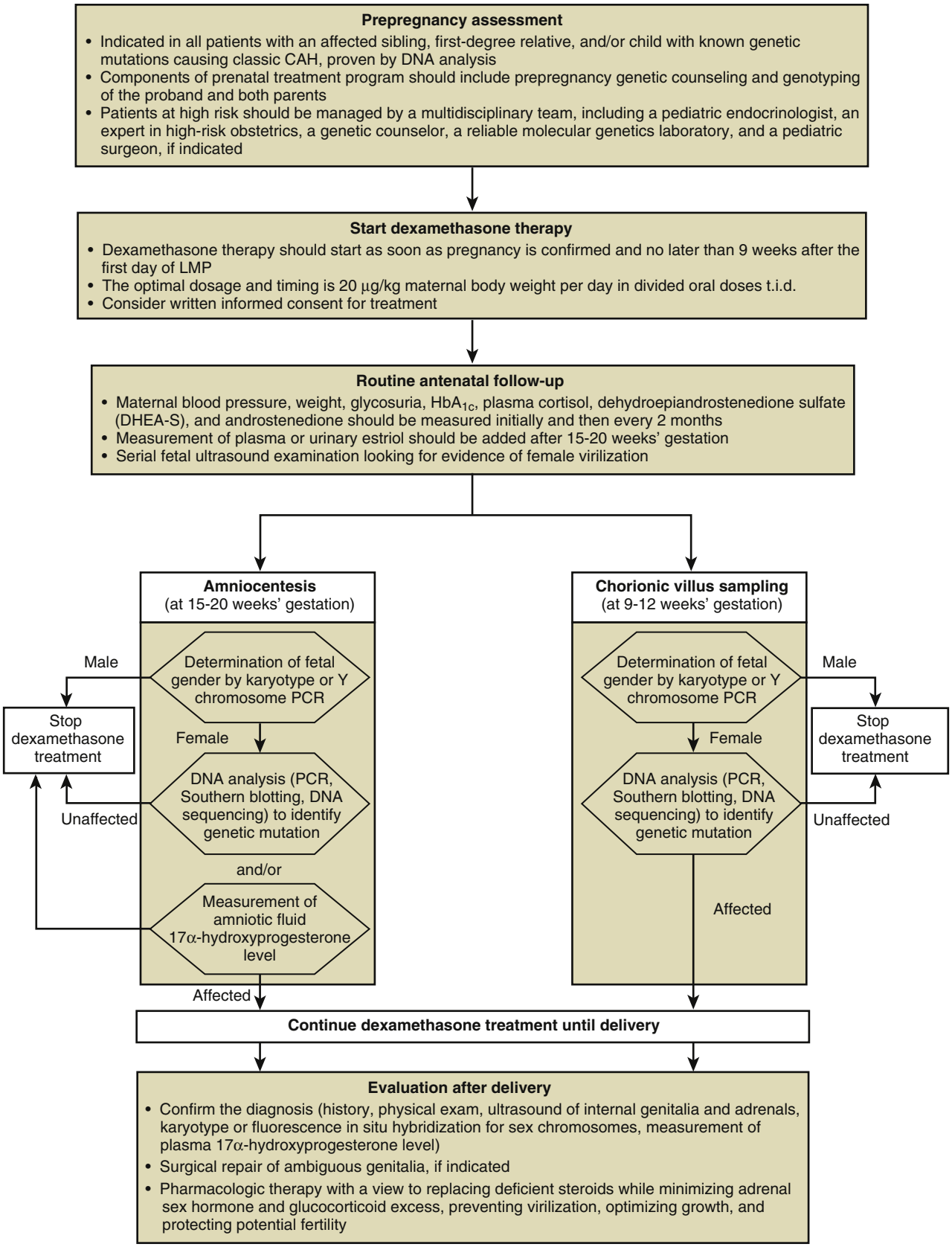


FIGURE 28.17 Proposed algorithm for the management of pregnant women at risk for congenital adrenal hyperplasia. CVS, chorionic villus sampling. *(From LWPES/ESPE CAH Working Croup. Consensus statement on 21-hydroxylase deficiency from The Lawson Wilkins Pediatric Endocrine Society and The European Society for Pediatric Endocrinology.* J Clin Endocrinol Metab *87:4048, 2002.)*

by CYP2 genotyping. If an affected female fetus is identified, glucocorticoid treatment should be continued for the entire pregnancy. The goal of this protocol is to suppress endogenous androgen production by the fetal adrenal and thereby prevent virilization of an affected female fetus. If dexamethasone therapy is initiated too late in gestation (typically regarded as later than 9 weeks' gestation), clitoromegaly and labioscrotal fusion may already have occurred.

Because estriol in the maternal circulation is derived almost exclusively from placental metabolism of fetal androgens (primarily 16-hydroxy dehydroepiandrosterone sulfate [16-OH DHEAS]), estriol levels can be used to monitor the efficacy of maternal glucocorticoid therapy to suppress fetal adrenal steroidogenesis. An estriol level of less than 0.2 nM in the maternal circulation is associated with marked suppression of fetal adrenal steroidogenesis, whereas a level of greater than 10 nM is associated with inadequate suppression of the fetal adrenal.[557]

Exactly how effective this protocol is in preventing virilization is not clear. In one study, implication of this protocol in 14 pregnancies at risk resulted in the treatment of two affected female fetuses, one of whom was virilized.[558] A subsequent report described the effects of antenatal glucocorticoid treatment on 15 female fetuses found to have CAH on the basis of antepartum genetic testing. Of the 15 female infants, 5 responded completely and 10 responded partially.[558] In the largest experience to date of 61 affected female fetuses, New and colleagues demonstrated that, for affected females, the average Prader score (an objective scoring system of virilisation graded on a scale from 1 [mild] to 5 [severe]) for those treated prenatally at or before 9 weeks' gestation was 0.96, whereas those with no prenatal treatment had an average Prader score of 3.75.[559] Taken together, these data suggest that approximately 85% of affected female fetuses who receive glucocorticoid therapy will have either no virilization or mild virilization.[555]

Potential complications of long-term antepartum dexamethasone treatment include the development of iatrogenic Cushing syndrome in the mother[557] and subtle cognitive and motor deficits in the child,[560,561] although not all studies have confirmed this association.[562]

Pheochromocytoma

Pheochromocytomas are catecholamine-secreting tumors that arise from chromaffin cells. Although the majority (90%) occur in the adrenal gland, these tumors can also be found outside of the adrenal, primarily at the base of the bladder and at the aortic bifurcation (organ of Zuckerkandl). Of all cases, 10% are bilateral and 10% are malignant. In a minority of cases (10%), pheochromocytomas may occur in association with other systemic disorders like neurofibromatosis (von Recklinghausen disease), multiple endocrine neoplasia type IIA (Sipple syndrome), and von Hippel-Lindau syndrome.

Pheochromocytomas account for 0.1% of hypertension in adults. These tumors are rare in pregnancy, and only a few hundred cases have been reported in the literature. However, they pose a very high risk for both mother and fetus. Anesthesia, vaginal delivery, uterine contractions, or even vigorous fetal movements can precipitate fatal maternal hypertension. Fetal growth restriction is a common finding due primarily to uteroplacental insufficiency. If untreated, the overall maternal mortality rate ranges from 4% to 17%, with a fetal loss rate of 11% to 26%.[563-565] Antenatal diagnosis reduces the maternal mortality rate to 0% to 2% and the fetal loss rate to 1% to 15% [563-565]; however, only half of such cases are diagnosed prior to conception.[563] Delay in diagnosis is, therefore, associated with considerable morbidity and mortality. Any pregnant woman with paroxysms ("spells") of labile hypertension, headaches, palpitations, sweating, flushing, blurred vision, anxiety, emesis, dyspnea, or convulsions should prompt an investigation to exclude the diagnosis. It should be remembered, however, that 50% of women with pheochromocytoma will present with sustained hypertension.

Maternal and fetal survival depends on early diagnosis, aggressive medical therapy, and correct timing of delivery and surgery. Confirmation of the diagnosis requires documentation of elevated circulating levels of metanephrines and catecholamines in either urine or blood. Although testing for fractionated plasma free metanephrines appears to be the most sensitive, it is not as specific as the standard 24-hour urine catecholamines and metanephrines measurements.[566] Given the ease of a random plasma test over a 24-hour urine collection, some authorities have advocated the former as a first line test, especially in women with a high clinical suspicion for pheochromocytoma.[567] Plasma catecholamines and urinary vanillylmandelic acid (VMA) are used less commonly because of their poor accuracy.[567] Catecholamine levels are not affected by pregnancy. Localization of the tumor can be achieved by radiological imaging (magnetic resonance imaging is the modality of choice) or, rarely, by selective sampling of the adrenal veins. Scintigraphy (^{131}I) is contraindicated in pregnancy.

Initial treatment should include pharmacological control of hypertension and tachycardia. Alpha-adrenergic blockage should be initiated immediately, preferably with phenoxybenzamine (starting at 10 mg every 8 hours and increasing gradually until orthostatic hypotension is achieved). Alternative agents include prazosin or labetalol. Beta-blockade should be reserved for women with persistent tachycardia or arrhythmias and selective and short-acting agents are preferred (such as metoprolol or atenolol). Optimal management of a pregnant patient with pheochromocytoma involves collaboration between obstetricians, endocrinologists, anesthesiologists, and general surgeons. The decision of whether or not to proceed with surgical resection depends on the success of medical treatment, the size of the tumor, estimated risk of malignancy, and gestational age.[563-565] In the first and second trimesters, surgical resection of the tumor is associated with good fetal outcome. More recently, laparoscopic tumor resection has been described in pregnancy.[568] In later pregnancy, delivery by elective cesarean followed by tumor resection is commonly recommended

Ovarian Endocrine Tumors

The precise incidence of adnexal masses in pregnancy is not known. Increasing use of ultrasonography during pregnancy suggests that sizable adnexal masses complicate as many as 1 in 200 pregnancies.[569-571] These data are consistent with a large retrospective study that showed significant adnexal

neoplasms were identified in 1 of 197 cesarean deliveries.[572] The majority of adnexal masses occurring in pregnant women are simple cysts less than 5 cm in diameter, which carry a small risk of complications such as malignancy (less than 5%), torsion, rupture, or hemorrhage.[570,571,573] Some of these adnexal masses will be functional endocrine tumors of the ovary.

The major risk of an ovarian endocrine tumor during pregnancy is virilization of a female fetus. Levels of testosterone[574,575] and androstenedione[575] increase in the maternal circulation throughout gestation, peaking in the third trimester. However, circulating levels of free testosterone remain relatively unchanged prior to 28 weeks' gestation, suggesting that the increase in total testosterone is due in large part to increases in sex hormone–binding globulin.[576] These data are consistent with the observation that the clearance of testosterone decreases during pregnancy.[577] After 28 weeks, however, levels of both total and free testosterone appear to increase in the maternal circulation. In contrast, DHEA and DHEAS concentrations decrease precipitously in pregnancy.[578] This decrease occurs despite an increase in maternal DHEAS production[579] and is likely due to increased metabolical clearance by the placenta.[578,579]

These endocrine changes serve to protect a female fetus from virilization. Other protective mechanisms include a high capacity of the placenta to aromatize androgens such as testosterone and androstenedione to estrogens (estrone, estradiol-17β, and estriol).[580] In this way, the placenta is able, at least in part, to protect a female fetus from exposure to excessive concentrations of testosterone and androstenedione. Dihydrotestosterone, on the other hand, is not a substrate for aromatization. As such, the placenta may be less effective in preventing this steroid from crossing into the fetal compartment and causing virilization. Some female fetuses appear to be uniquely resistant to the virilizing effects of androgens, especially in the second and third trimesters of pregnancy. Although the mechanism is not clear, there are several cases in which markedly elevated levels of androgens have been demonstrated in the umbilical cord blood of female infants without evidence of virilization.[581]

Three ovarian endocrine tumors can cause virilization: luteomas, gestational theca-lutein cysts (hyperreactio luteinalis), and Sertoli-Leydig cell tumors (arrhenoblastomas),[582] all of which are associated with markedly elevated levels of testosterone, dihydrotestosterone, and androstenedione. Luteomas are derived from luteinization and hyperplasia of theca interna cells,[583,584] and are bilateral in approximately 45% of cases.[582] Maternal virilization or hirsutism occurs in approximately 35% of such women, and the risk of virilization of a female fetus is high.

In contrast, theca-lutein cysts are associated with a lower risk of virilization of a female fetus. This disorder occurs typically in conditions with elevated circulating levels of hCG (such as gestational trophoblastic tumors, diabetes, and Rh isoimmunization), which directly stimulates ovarian steroid production. In the majority of cases, the cysts are bilateral.

The risk of both maternal and fetal virilization is highest with Sertoli-Leydig cell tumors. These tumors are usually unilateral. Fortunately, such tumors are usually associated with chronic anovulation and infertility, and are therefore rarely seen in pregnancy.

Preeclampsia

Preeclampsia (gestational proteinuric hypertension) complicates 6% to 8% of all pregnancies.[585,586] The second most common cause of maternal mortality in the United States (after thromboembolic disease), preeclampsia accounts for 12% to 18% of all pregnancy-related maternal deaths (around 70 maternal deaths per year in the United States and an estimated 50,000 maternal deaths per year worldwide).[586-589] It is also associated with a high perinatal mortality and morbidity, due primarily to iatrogenic prematurity.[590]

Preeclampsia is an idiopathic multisystem disorder specific to human pregnancy and the puerperium.[585] More precisely, it is a disease of the placenta because it has also been described in pregnancies where there is trophoblast but no fetal tissue (complete molar pregnancies).[591] Similarly, in the rare situation of an advanced extrauterine intraabdominal ectopic pregnancy complicated by preeclampsia, removal of the placenta is not possible at the time of delivery of the fetus and, as such, preeclampsia persists postpartum instead of resolving.[592]

Despite aggressive research efforts, the pathogenesis of preeclampsia remains poorly understood. Pathological and physiological observations as well as examination of epidemiological studies and biochemical aberrations (Table 28.8) have led to a number of theories to explain preeclampsia. At present, four major hypotheses are the subject of intense investigation:

1. Genetic imprinting
2. Immune maladaptation
3. Placental ischemia
4. Generalized endothelial dysfunction[593,594]

The data in support of each theory are summarized in Box 28.12.[595,596] In addition to endocrine changes, preeclampsia is also associated with abnormalities in the nervous system. For example, Schobel and colleagues[597] have shown that postganglionic sympathetic nerve activity is increased in women with preeclampsia compared with pregnant women without hypertension. This finding suggests that the increase in peripheral vascular resistance and blood pressure that characterize preeclampsia may be due, at least in part, to increased firing of sympathetic neurons. Interestingly, heart rate was not increased in the women with preeclampsia, implying either that increased vagal tone suppressed sympathetic activity in the heart or that the increase in peripheral sympathetic activity is a secondary compensation to plasma volume contraction.

Despite a plethora of hypotheses, there is as yet no single unifying theory that can account for all of the findings in preeclampsia. It is clear, however, that the blueprint for its development is laid down early in pregnancy. Researchers have suggested that the pathological hallmark of preeclampsia is a complete or partial failure of the second wave of trophoblast invasion from 16 to 20 weeks' gestation, which is responsible in normal pregnancies for destruction of the muscularis layer of the spiral arterioles.[598-602] As pregnancy progresses, the metabolical demands of the fetoplacental unit increase. Because of the abnormally shallow invasion of the placenta, however, the spiral arterioles are unable to

Table 28.8 Biochemical Aberrations Associated with Preeclampsia*

Elevated†	Diminished†	Unchanged†
Vasoactive Agents		
Endothelin-1	Prostacyclin (PGI_2)	
Thromboxane A_2 (TXA_2)	Prostaglandin E_2 (PGE_2)	
Nitric oxide synthase activity	Antithrombin III activity	
Nitric oxide production	Placental endothelin-1 production	
	Endothelin A and B receptors	
	Urinary excretion of nitric oxide metabolites (including nitrate and nitrite)	
	? Plasma levels of nitric oxide metabolites	
Markers of Endothelial Cell Dysfunction and/or Injury		
Fibronectin and fibronectin degradation products	PGI_2	Total cholesterol
Endothelin-1	LDL-I, LDL-II, and high-density lipoprotein (HDL)	Intermediate-density lipoprotein
Elastase		Total LDL
Triglycerides, very-low density lipoprotein (VLDL), low-density lipoprotein-III (LDL-III)	VLDL and LDL receptors	
Lipid peroxidases	Nitric oxide	
Malonydialdehyde (metabolite of lipid peroxidation)		
Urinary protein excretion		
Markers of Neutrophil Activation		
Neutrophil elastase		
Neutrophil defensins		
Soluble L-selectin		
Leukocyte adhesion molecules, including E-selectin, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1)		
Neutrophil reactive oxygen species (ionized oxygen, hydrogen peroxide, hydroxyl radical)		
Markers of Platelet Activation		
Platelet endothelial cell adhesion molecule-1		
Cytokines/Growth Factors		
Interleukin-6 (IL-6)	Insulin-like growth factor-1 (IGF)	Interleukin-8 (IL-8)
Tumor necrosis factor- α (TNF α)	IGF-binding proteins (IGFBP-1 and placental protein 12)	Interleukin-4 (IL-4)
TNF α soluble receptors	? Granulocyte-macrophage colony-stimulating factor (GM-CSF)	Interleukin-10 (IL-10)
Platelet-derived growth factor		
Vascular endothelial growth factor (VEGF)		
VEGF receptor (flt1)	Placental growth factor	
Soluble endoglin		
Interferon-γ (IFN- γ)		
IL-6 and IL-1 receptor antagonists		
Activin A		
Inhibin A		
Hormones		
Testosterone		Estradiol-17β
β-human chorionic gonadotropin (β-hCG)		Dehydroepiandrosterone sulfate (DHEAS)
Corticotropin-releasing factor		Sex hormone-binding globulin (SHBG)
Leptin		
Coagulation Factors		
Von Willebrand factor	Platelets	Soluble fibrin
Thrombomodulin	Thrombopoietin	Thrombin–antithrombin III complexes
Tissue plasminogen activator		Fibrin degradation products
Plasminogen activator inhibitor I		
Miscellaneous		
Uric acid	Albumin	Sodium
Free fatty acids (oleic, linoleic, and palmitic acids)	Antioxidant vitamins (vitamins C and E)	Glucose
Urinary albumin excretion	Urinary calcium excretion	Folk acid
Free radical superoxide formation	Magnesium	Lactoferrin
Homocysteine	Zinc	C-type natriuretic peptide
Ceruloplasmin	Calcium	α-Fetoprotein

Continued

Table 28.8 Biochemical Aberrations Associated with Preeclampsia*—cont'd

Elevated†	Diminished†	Unchanged†
Haptoglobin	β-Carotene	
α_1-Antitrypsin	Transferrin	
Serotonin (5-hydroxytryptamine)	Vitamin B12	
Atrial and brain natriuretic peptide		
Circulating fetal erythroblasts		
Circulating syncytiotrophoblast microvilli fragments		

*Data reflects an overall summary of published literature by the author. Unless otherwise indicated, measurements refer to maternal serum concentrations.
†Relative to measurements in pregnancies not complicated by preeclampsia.

Box 28.12 Theories of Etiology of Preeclampsia and Their Supportive Evidence

GENETIC
- Familial inheritance pattern (increased incidence in women with a positive family history of preeclampsia[595] and in pregnancies conceived by men born themselves of preeclamptic pregnancies[792])
- Increased incidence in African-American race[793]
- Increased incidence in women with a prior history of preeclampsia[612]
- Association with Factor V Leiden mutation[613]
- Association with angiotensinogen gene variant[794]
- Association with protein C and/or protein S deficiency[795]
- Association between fetal 3-hydroxyacyl-coenzyme A dehydrogenase deficiency and HELLP syndrome[796]

IMMUNOLOGICAL MALADAPTATION
- Increased incidence in first pregnancies (nulliparity)[797]
- Increased incidence with changed paternity (new partner)[798,799]
- Advanced maternal age >40 years)[611]
- Association with maternal-fetal HLA-DR discordance[614]
- Association with reduced *in vitro* lymphocyte activity[800]
- Lower plasma levels of T lymphocytes[801]
- Higher plasma levels of immune complexes and complement[802,803]
- Lower level of HLA-G mRNA expression in chorionic tissue[804]
- Elevated end-organ deposition of immune complexes and immunoglobulins[805,806]
- Lower incidence of preeclampsia in women who have received a blood transfusion[807]
- Lower incidence of preeclampsia with an extended duration of sexual cohabitation prior to conception[799]
- Higher incidence of preeclampsia in women using contraception that prevent exposure to sperm[808]
- Higher incidence of preeclampsia in women impregnated by men from a different racial group[809]
- Association with autoimmune disease (such as systemic lupus erythematosis)[598]

PLACENTAL ISCHEMIA
- Association with abnormal placentation[599-601]
- Association with excessive placental mass (increased incidence with increasing gestational age, in molar and multifetal pregnancies,[609] in nonimmune hydrops fetalis,[810] and in women with malaria[811]
- Association with intrauterine fetal growth restriction[812]
- Pathological evidence of placental thrombosis and infarction

GENERALIZED ENDOTHELIAL INJURY
- Increased incidence in women with chronic hypertension[615,616]
- Increased incidence in women with chronic renal disease[617]
- Increased incidence in women with antiphospholipid antibody syndrome[618]
- Increased incidence in women with diabetes mellitus[619]
- Association with imbalance in prostaglandin synthesis (elevated thromboxane A2/prostacyclin ratio)[813]
- Association with coagulopathy
- Association with abnormalities in free fatty acid, lipoprotein, and lipid peroxidase metabolism[596]

dilate to accommodate the required increase in blood flow resulting in "placental dysfunction" that manifests clinically as preeclampsia.

A number of recent studies have provided an improved understanding of the pathophysiology of preeclampsia, suggesting that aberrant expression of placentally-derived circulating anti-angiogenic factors may lead to widespread maternal endothelial injury.[603] Vascular endothelial growth factor (VEGF) is an important mitogen involved in angiogenesis and the maintenance of vascular integrity. VEGF acts by binding to two cell surface receptors, VEGF receptors type 1 (flt1) and type 2 (KDR). The flt1 receptor exists in two major isoforms: a functional transmembrane isoform and a soluble truncated isoform known as soluble fms-like tyrosine kinase-1 (sFlt-l). sFlt-1 lacks a membrane binding domain and thus exists free in the maternal circulation where it binds and functionally inactivates VEGF and placental growth factor (PlGF) leading to increased vascular permeability.[604] Levels of sFlt-1 in the maternal circulation increase during pregnancy, but increase earlier and to higher levels in pregnancies destined to develop preeclampsia as compared with normotensive controls. The source and molecular mechanisms responsible for the increase in circulating sFlt-1 levels in preeclampsia remain unclear. Interestingly, anti-VEGF antibodies have been used as pharmacological therapy in nonpregnant individuals for conditions resulting from inappropriate or excessive angiogenesis, such as neoplastic disorders and macular degenerative disease. Common side effects associated with this therapy include hypertension and proteinuria.[605]

More recently, another anti-angiogenic factor—known as soluble endoglin (sEng)—has been implicated in the

Table 28.9 Epidemiological Risk Factors for Preeclampsia

Factor	Risk Ratio
Nulliparity[797]	2.9:1
African-American race[793]	1.2:1
Advanced maternal age >40 years[797]	2:1
Interval between pregnancies (<1 year)[620]	1.2:1
Body mass index[236]	
→ 26–35 kg/m^2	1.6:1
→ ±35 kg/m^2	3.3:1
Blood pressure[234]	
→ Initial systolic blood pressure 120-136 mm Hg	4:1
→ Initial diastolic blood pressure 60-84 mm Hg	2:1
Multiple gestation[797]	2.9:1
Family history of preeclampsia (first-degree relative)[797]	2.9:1
History of preeclampsia[613]	
→ History of mild preeclampsia	10:1
→ History of severe preeclampsia	7:1
→ History of eclampsia	3:1
→ History of preeclampsia ±30 weeks' gestation	5:1
Chronic hypertension[615]	10:1
Chronic renal disease[617]	20:1
Antiphospholipid antibody syndrome[618]	10:1
Diabetes mellitus[619]	2:1
Factor V Leiden mutation[815]	
→ Homozygous	?
→ Heterozygous	1.8:1
Maternal-fetal HLA-DR discordance[614]	3:1
Angiotensinogen gene T235[794]	
→ Homozygous	20:1
→ Heterozygous	4:1

Box 28.13 Features of Severe Preeclampsia

SYMPTOMS
- Symptoms of central nervous system dysfunction (headache, blurred vision, scotomata, altered mental status)
- Symptoms of liver capsule distention or rupture (right upper quadrant and/or epigastric pain)

SIGNS
- Severe elevation in blood pressure (defined as >160/110 mm Hg on two separate occasions at least 6 hours apart)
- Pulmonary edema
- Eclampsia (generalized seizures and/or unexplained coma in the setting of preeclampsia and in the absence of other neurological conditions)
- Cerebrovascular accident
- Cortical blindness
- Fetal intrauterine growth restriction (IUGR)

LABORATORY FINDINGS
- Proteinuria (>5 g/24 hours)
- Renal failure (rise in serum creatinine concentration by 1 mg/dL over baseline) or oliguria (<500 mL per 24 hours)
- Hepatocellular injury (serum transaminase levels ≥2 × normal)
- Thrombocytopenia (<100,000 platelets/mm^3)
- Coagulopathy
- HELLP syndrome (Hemolysis, Elevated Liver enzymes, Low Platelets)

pathogenesis of preeclampsia. This factor serves as a coreceptor for transforming growth factor-β (TGF-β), and its use in rats leads to a syndrome similar to severe preeclampsia.[606] Like sFlt-1, levels of sEng are elevated in the circulation of women with preeclampsia several weeks before the disease becomes evident clinically.[607]

Although an attractive hypothesis, the role of the antiangiogenic factors (sFlt-1 and sEng) in the pathogenesis of preeclampsia and the ability to use these factors to predict, diagnose, and potentially treat this disorder require further study. It is likely that preeclampsia is not a single disease entity, but rather a clinical syndrome encompassing three distinct elements:

1. New-onset hypertension (defined as a sustained sitting blood pressure of at least 140/90 mm Hg in a previously normotensive woman)
2. New-onset proteinuria (defined as greater than 300 mg/24 hours or at least 1+ on a clean-catch urinalysis in the absence of urinary infection)
3. New-onset significant nondependent edema,[585] although more recent consensus reports have eliminated edema as a criterion for the diagnosis[608]

The diagnosis of preeclampsia can only reliably be made after 20 weeks' gestation. Evidence of proteinuric hypertension prior to 20 weeks' gestation should raise the possibility of an underlying molar pregnancy,[591] multiple pregnancy,[609] drug withdrawal, antiphospholipid antibody syndrome, uniparental disomy in the placenta, or, rarely, a chromosomal abnormality (trisomy) in the fetus.[610]

Although numerous risk factors for the development of preeclampsia have now been defined (Table 28.9),[585,609,611-620] it is not possible to predict with any certainty which pregnancies are going to be complicated by this disease.[621,622] Moreover, despite intensive research efforts, there is no effective way at this juncture in time to prevent the development of preeclampsia in women at high risk.[622,623] As such, the current focus of obstetric care providers is regular prenatal visits with routine blood pressure and urinary protein screening with a view to early identification of preeclampsia followed by aggressive and gestational age-appropriate management.

Preeclampsia is classified as either *mild* or *severe*. A diagnosis of severe preeclampsia should be entertained in women with new-onset proteinuric hypertension accompanied by one or more of a series of complications (Box 28.13). It should be emphasized that only *one* such criterion is required for the diagnosis of severe preeclampsia. Fetal IUGR was excluded from the criteria in 2000 by the National High Blood Pressure in Pregnancy Working Group[608] because of inconsistencies in its definition, but was still included as a criterion for the diagnosis of severe preeclampsia by ACOG in 2002.[585] Mild preeclampsia includes all women with preeclampsia who do not have any features of severe disease.

The only definitive treatment of preeclampsia is delivery of the fetus and placenta to prevent potential maternal complications. Delivery is recommended for women with mild preeclampsia at or near term. In contrast, delivery should be recommended for all women with severe preeclampsia, regardless of gestational age.[585] That said, there are three situations in which expectant management of women with severe preeclampsia may be appropriate:

1. Severe proteinuria (greater than 5 g in 24 hours), because this finding alone is not associated with serious maternal or fetal sequelae[624]
2. Mild IUGR (fifth to tenth percentile) remote from term, as long as antepartum fetal testing remains

reassuring, oligohydramnios is not severe, umbilical artery diastolic flow is not reversed on Doppler velocimetry, and there is progressive fetal growth

3. Severe preeclampsia by blood pressure criteria alone before 32 weeks' gestation that is managed with antihypertensive therapy[625,626]

The rationale for delaying delivery in these pregnancies is to reduce perinatal morbidity and mortality by delivery of a more mature fetus and, to a lesser degree, to achieve a more favorable cervix for vaginal birth. The risk of prolonging pregnancy is continued poor perfusion of major organs in both the mother and fetus, with the potential for severe end organ damage to the brain, liver, kidneys, placenta and fetus, and hematological and vascular systems. Delivery should be initiated—after a course of antenatal corticosteroid therapy, if possible—when there is poorly controlled severe hypertension, thrombocytopenia, eclampsia, elevated liver function tests with epigastric or right upper quadrant pain, pulmonary edema, renal insufficiency, placental abruption, or persistent symptoms (severe headache or visual changes) (Box 28.13). Fetal indications for immediate delivery include nonreassuring fetal testing *(fetal distress)*, severe oligohydramnios, or severe fetal growth restriction (less than the fifth percentile).[585]

Delivery is usually by the vaginal route, with cesarean delivery reserved for appropriate obstetrical indications. Severe preeclampsia does not mandate immediate cesarean birth.[627] The decision to proceed with cesarean or induction of labor and attempted vaginal delivery should be individualized based on such factors as parity, gestational age, cervical examination (Bishop score), maternal desire for vaginal delivery, and fetal status and presentation. Cervical ripening agents may be used if the cervix is not favorable prior to induction, but prolonged inductions should be avoided.[585] The rate of vaginal delivery after labor induction decreases to about 33% at less than 34 weeks, primarily because of nonreassuring fetal testing and failure to progress in labor.[628,629]

The use of antihypertensive agents to control mildly elevated blood pressure in the setting of preeclampsia has not been shown to alter the course of the disease or diminish perinatal morbidity or mortality.[630] Indeed, such therapy may adversely affect uteroplacental perfusion, leading to reduced birth weight.[631] Moreover, the use of antihypertensive agents in preeclampsia may provide a false sense of security by masking an increase in blood pressure as a sensitive measure of worsening disease, and is therefore not generally recommended. These studies serve to confirm that hypertension is a clinical feature, and not the underlying cause, of preeclampsia.

The cause of the blood pressure elevation in preeclampsia is not clear. It has been suggested that it may represent an attempt of the body to maintain perfusion through an underperfused (ischemic) placenta, and may be triggered by a distress signal from the fetoplacental unit.[632] Although treatment of mild to moderate hypertension has not been shown to improve maternal or perinatal outcome, antihypertensive agents should be administered to prevent a maternal cerebrovascular accident from severe hypertension, which accounts for 15% to 20% of deaths from preeclampsia. The risk of hemorrhagic stroke correlates directly with the degree of elevation in systolic blood pressure (and is less related to the diastolic pressure), but there is no clear threshold systolic pressure above which emergent therapy should be instituted.[633] The National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy has recommended initiating therapy when systolic blood pressure exceeds 150 to 160 mm Hg and diastolic pressure exceeds 100 to 110 mm Hg.[608] However, this threshold has not been tested prospectively and the cerebral vasculature of women with underlying chronic hypertension can probably tolerate higher systolic pressures without injury.

The traditional drug of choice for the antepartum management of chronic hypertension is the central acting agent, methyldopa, primarily because of the extensive experience with its use during pregnancy. However, other agents have been increasing in popularity including β-blockers (primarily labetalol) and calcium channel blockers (nifedipine). Hydralazine, labetalol, and nifedipine have all been used for the management of acute hypertensive episodes in pregnancy.[585,608,630]

In term nulliparous women, the duration of labor is not affected by either preeclampsia or magnesium sulphate seizure prophylaxis.[585,634] Close and continuous monitoring of both the mother and fetus is indicated during labor to identify worsening hypertension, deteriorating maternal hepatic, renal, cardiopulmonary, and/or hematological function, as well as uteroplacental insufficiency with evidence of nonreassuring fetal testing. Anticonvulsant therapy is generally initiated during labor or while administering corticosteroids or prostaglandins prior to a planned delivery and continued until 24 to 48 hours postpartum, when the risk of seizures is decreased.

Magnesium sulfate is the drug of choice for seizure prevention.[585,632,634-637] The safety and efficacy of magnesium sulfate seizure prophylaxis was illustrated in the largest study performed on preeclamptic women, the Magpie (Magnesium Sulfate for Prevention of Eclampsia) Trial.[637] The trial followed over 10,000 pregnant women with preeclampsia (defined as blood pressures of at least 140/90 mm Hg on two occasions and proteinuria of 1+ or more) in whom the obstetric care provider was uncertain about the benefit of starting magnesium sulfate therapy. The women were randomly assigned to receive magnesium sulfate (4 g intravenous loading dose then 1 g/hour, or 5 g intramuscularly into each buttock followed by 5 g intramuscularly every 4 hours) or placebo for 24 hours. The maintenance dose was given only if a patellar reflex was present (loss of reflexes being the first manifestation of symptomatic hypermagnesemia), respirations exceeded 12 per minute, and the urine output exceeded 100 mL per 4 hours. Approximately 25% of patients met criteria for severe preeclampsia.

The major finding from this trial was that magnesium sulfate significantly reduced the risk of eclampsia as compared with normotensive controls (0.8% vs 1.9%, respectively). This reduction in eclamptic seizures was observed regardless of the severity of preeclampsia, gestational age, or parity. The data also demonstrated a reduction in maternal mortality (0.2% vs 0.4%, respectively), but no difference in maternal morbidity, perinatal mortality, and neonatal morbidity (except for a lower rate of abruption in treated women [2% vs 3.2%, respectively]). The study concludes that, to prevent one convulsion, 63 women with severe

preeclampsia or 109 women with mild preeclampsia would need to be treated. Magnesium sulfate therapy should therefore be considered for prevention of eclampsia in all women with preeclampsia, including those with mild disease.[637-639] However, some authors have questioned the value of treating all preeclamptic women to prevent seizures in only 0.6% to 3.2% of patients.[640] The incidence of seizures is much lower (approximately 0.1%) in women with nonproteinuric hypertension.[636] For this reason, it may be safe to withhold seizure prophylaxis in such women.

Hypertension due to preeclampsia resolves postpartum, often within a few days, but sometimes takes a few weeks. Elevated blood pressures that persist beyond 12 weeks postpartum are unlikely to be related to preeclampsia. Population-based retrospective cohort studies and large case-control studies have demonstrated that a history of preeclampsia confers an increased risk for future premature cardiovascular disease, and a lifetime increased risk of maternal mortality from cardiovascular causes.[641-651] The relative risk of cardiovascular disease is comparable for women with a history of preeclampsia, and women with known conventional cardiovascular risk factors such as hypertension, obesity, diabetes mellitus, and dyslipidemia, leading some to conclude that preeclampsia or gestational hypertension should be considered a known risk factor for cardiovascular disease in women.[641] Irgens and colleagues reported that women with severe preterm preeclampsia were eight times more likely to die from cardiovascular causes than women without the disease or those with mild preeclampsia, suggesting that preterm preeclampsia may be the most significant mediator of long-term morbidity and mortality.[642]

The underlying mechanisms of future cardiovascular risk in women with preeclampsia are not well understood. Similar biological mechanisms for preeclampsia and cardiovascular disease have been suggested, including inflammation, hypercoagulability, and insulin dysregulation.[652-658] Whether pregnancy acts as a "stress test," unmasking a predisposition to hypertension and cardiovascular disease that would otherwise not have been apparent until a later age,[659,660] or whether a cause-effect relationship exists between preeclampsia and the subsequent development of cardiovascular disease, remains unclear. The finding that women with preeclampsia have a higher prevalence of chronic hypertension as early as 6 months postpartum suggests that early-onset hypertension may be one of the mechanisms by which preeclampsia increases the risk for long-term cardiovascular sequelae.[661] Studies have demonstrated that levels of sFLT-1 remain elevated an average of 18 months postpartum in women with a history of preeclampsia, and markers of endothelial activation may remain elevated for over 15 years after preeclampsia.[658,662] The persistence of an anti-angiogenic milieu long after pregnancy may contribute to these women's predisposition to develop cardiovascular disease.[663]

Parturition

Labor is the physiological process by which the products of conception are passed from the uterus to the outside world (see also Chapter 12). The definition of labor includes an increase in myometrial activity or, more precisely, a switch in the myometrial contractility pattern from irregular *contractures* (long-lasting, low-frequency activity) to regular *contractions* (high-intensity, high-frequency activity)[664] leading to effacement and dilatation of the uterine cervix. An initial cervical examination of at least 2 cm dilatation or at least 80% effacement in the setting of regular phasic uterine contractions is also accepted as being sufficient for the diagnosis of labor in nulliparous women. A bloody discharge ("show") is often included in the description of labor, but is not a prerequisite for the diagnosis.

Considerable evidence suggests that, in most viviparous animals, the fetus is in control of the timing of labor.[665-672] Horse-donkey crossbreeding experiments performed in the 1950s, for example, resulted in a gestational length intermediate between that of horses (340 days) and that of donkeys (365 days), suggesting a role for the fetal genotype in the initiation of labor.[667,670] However, the factors responsible for the initiation and maintenance of labor in humans remain poorly defined. The slow progress in our understanding of the biochemical mechanisms involved in the process of labor in humans reflects, in large part, the difficulty in extrapolating from the endocrine control mechanisms in various animals to the paracrine and autocrine mechanisms of parturition in humans—processes which, in humans, preclude direct investigation.

Regardless of whether the trigger for labor begins within the fetus or outside the fetus, the final common pathway for labor ends in the maternal tissues of the uterus and is characterized by the development of regular phasic uterine contractions. As in other smooth muscles, myometrial contractions are mediated through the ATP-dependent binding of myosin to actin. This process is dependent in large part on the phosphorylation of myosin light chain by a calcium-dependent enzyme, myosin light chain kinase. In contrast to vascular smooth muscle, however, myometrial cells have a sparse innervation that is further reduced during pregnancy.[673] The regulation of the contractile mechanism of the uterus is therefore largely humoral and dependent on intrinsic factors within myometrial cells.

A "parturition cascade" likely exists in the human (Fig. 28.18), responsible for the removal of mechanisms maintaining uterine quiescence and for the recruitment of factors acting to promote uterine activity.[672] Given its teleological importance, such a cascade would likely have multiple redundant loops to ensure a fail-safe system of securing pregnancy success and ultimately the preservation of the species. In such a model, each element is connected to the next in a sequential fashion, and many of the elements demonstrate positive feed-forward characteristics typical of a cascade mechanism. A comprehensive analysis of each of the individual paracrine and autocrine pathways implicated in the process of labor has been reviewed in detail elsewhere.[665,667-669,672,674]

In brief, human labor is a multifactorial physiological event involving an integrated set of changes within the maternal tissues of the uterus (myometrium, decidua, and uterine cervix) that occur gradually over a period of days to weeks. Such changes include, but are not limited to, an increase in prostaglandin synthesis and release within the uterus, an increase in myometrial gap junction formation, and upregulation of myometrial oxytocin receptors (uterine activation). Once the myometrium and cervix are prepared, endocrine or paracrine and autocrine factors from

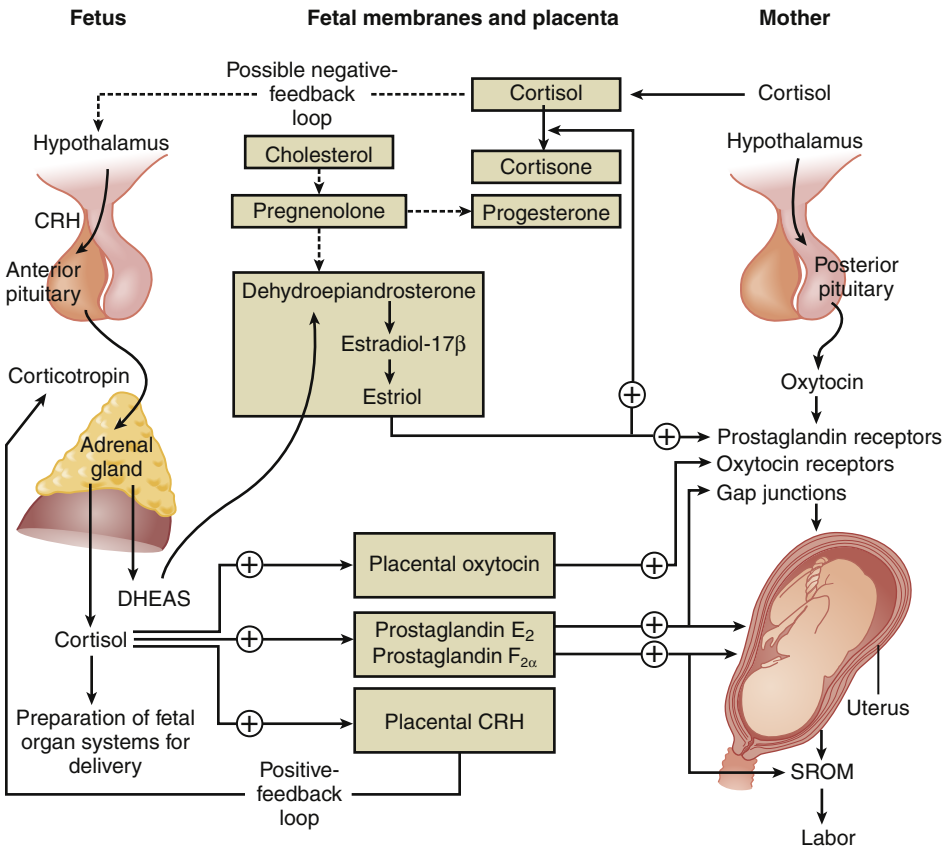


FIGURE 28.18 Proposed mechanism of labor induction at term. The major hormones and paracrine/autocrine factors responsible for promoting uterine contractions at term in an integrated parturition cascade are shown. Plus signs indicate activation or up-regulation. CRH, corticotropin-releasing hormone; DHEAS, dehydroepiandrosterone sulfate; SROM, spontaneous rupture of fetal membranes. *(Adapted from Norwitz ER, Robinson JN, Challis JRC. The control of labor.* N Engl J Med *341:660, 1999.)*

the fetoplacental unit bring about a switch in the pattern of myometrial activity from irregular contractures to regular contractions (uterine stimulation). The fetus may coordinate this switch in myometrial activity through its influence on placental steroid hormone production, through mechanical distention of the uterus, and through secretion of neurohypophyseal hormones and other stimulators of prostaglandin synthesis.

The final common pathway for the initiation of labor appears to be activation of the fetal hypothalamic-pituitary-adrenal axis, resulting in an increase in C-19 steroid (DHEA) production from the intermediate (fetal) zone of the fetal adrenal, the primary substrate for estrogen synthesis in the placenta. This activity is necessary because the human placenta is an incomplete steroidogenic organ, and estrogen synthesis by the placenta has an obligate need for C-19 steroid precursor from the fetus.[669] In the rhesus monkey, infusion of C19 precursor (androstenedione) leads to preterm delivery.[675] This effect is blocked by concurrent infusion of an aromatase inhibitor,[676] demonstrating that conversion to estrogen is important. However, systemic infusion of estrogen failed to induce delivery, suggesting that the action of estrogen is likely paracrine-autocrine.[675,677,678]

In humans, activation of the fetal HPA axis results in increased estrogen production, which is responsible for upregulating myometrial gap junctions and preparing the myometrium for contractions and labor.[679] The major estrogen produced in the human placenta is estriol, which is found in concentrations 10 to 20 times higher than other estrogens. Unlike other estrogens that are made primarily by maternal tissues, estriol is produced almost exclusively by fetal and placental tissues. Because estriol passes easily from the placenta to the maternal circulation, its relative concentrations in serum and saliva have been used as a direct marker of the activity of the fetal HPA axis.[680] Estriol is detectable as early as 9 weeks' gestation, and increases gradually after 30 weeks with a rapid rise prior to the onset of labor.[681,682] This surge in circulating estriol levels occurs approximately 3 to 5 weeks prior to the onset of labor, both at term and preterm,[682,683] but does not occur in patients requiring induction of labor or whose pregnancies are complicated by premature rupture of membranes in the absence of labor.[684] Although critical for the maintenance of early pregnancy, the role of progesterone in the latter half of pregnancy and in labor is less clear. Unlike most other mammalian species, systemic progesterone withdrawal is not a prerequisite for labor in humans[685] and the administration of a progesterone antagonist at term does not result in labor.[686,687]

The exact mechanism responsible for activation of the fetal HPA axis at term remains unclear although changes in corticotropin releasing hormone (CRH) seem to be important. McLean and colleagues suggested that CRH may serve as a "placental clock" that controls the length of gestation, and that elevated levels of CRH in the maternal circulation may predict the timing of labor and delivery.[688] Unlike total CRH levels, CRH bioactivity does not increase throughout gestation due to increased production of CRH binding protein (CRH-BP) by the liver. Approximately 3 to 5 weeks before the onset of labor, levels of CRH-BP in the maternal circulation fall precipitously leading to an increase in free (biologically active) circulating CRH.[688]

The CRH gene is expressed in the human placenta. CRH is found in low concentrations in nonpregnant women, rises in the 2nd and 3rd trimesters of pregnancy, and rises exponentially in the last 3 to 5 weeks of pregnancy for both term and preterm births.[314,689,690] Fetal and maternal glucocorticoids, such as cortisol, stimulate placental CRH production.[691,692] In response, CRH stimulates the production of corticotropin (ACTH) in the mother and fetus resulting in further cortisol release. Thus, a positive feed forward system is initiated that results ultimately in the onset of labor.[693]

The mean duration of human singleton pregnancy is 280 days (40 weeks), dated from the first day of the last normal menstrual period. A term pregnancy is defined as the period from 37 weeks (259 days) to 41 weeks, 6 days (293 days). Preterm (premature) labor is defined as labor occurring prior to 37 weeks of gestation.

Preterm Labor

Preterm birth occurs in 7% to 12% of all deliveries, but accounts for over 85% of all perinatal morbidity and mortality.[694,695] Preterm labor likely represents a syndrome rather than a diagnosis because the etiologies are varied. Approximately 20% of preterm deliveries are iatrogenic and are performed for maternal or fetal indications, including IUGR, preeclampsia, placenta previa, and nonreassuring fetal testing.[696] Of the remaining cases, approximately 30% occur in the setting of preterm premature rupture of the membranes, 20% to 25% result from intraamniotic inflammation and/or infection, and the remaining 25% to 30% are due to spontaneous (unexplained) preterm labor.

Spontaneous preterm labor may reflect a breakdown in the normal mechanisms responsible for maintaining uterine quiescence[665] or a short-circuiting or overwhelming of the normal parturition cascade.[672] The demonstration of endocrine differences, sequence divergence in pregnancy-related genes, and distinctive physiological changes of pregnancy in different mammalian species suggest that human parturition is unique.[697] Evolutionary adaptations associated with human pregnancy may at times provide protection against, and at times promote, preterm birth.[697] Human gestation is relatively long compared to other species, and in the setting of an infected or compromised gestation, preterm birth may provide an evolutionary advantage. An important feature of the proposed parturition cascade would be the ability of the fetoplacental unit to trigger labor prematurely if the intrauterine environment became hostile and threatened the well-being of the fetus. For example, up to 25% of preterm births are thought to result from intraamniotic infection.[696,698,699] In many patients with infection, elevated levels of lipoxygenase and cyclooxygenase pathway products can be demonstrated.[698,700,701] There are also increased concentrations of cytokines in the amniotic fluid of such women. Cytokines and eicosanoids appear to interact and to accelerate each other's production in a cascade-like fashion, which may act to overwhelm the normal parturition cascade and result in preterm labor. Recently, thrombin has been shown to be a powerful uterotonic agent,[702,703] providing a physiological mechanism for preterm labor secondary to placental abruption.

Numerous risk factors for preterm birth have been identified (Box 28.14), and several tests have been developed in an attempt to predict women at risk of preterm delivery (Box 28.15).[247,688,704-718] Prevention of preterm labor, however, has been largely unsuccessful (Box 28.16).[719-722] Improvements in perinatal outcome during this same time period have resulted primarily from antepartum corticosteroid administration and from advances in neonatal care.

Box 28.14 Risk Factors for Preterm Delivery

NONMODIFIABLE RISK FACTORS

- Prior preterm birth
- African-American race
- Social stress
- Age <18 years or >40 years
- Poor nutrition
- Low prepregnancy weight
- Low socioeconomic status
- Absent prenatal care
- Cervical injury or anomaly
- Uterine anomaly or fibroid
- Excessive uterine activity
- Premature cervical dilatation (>2 cm) or effacement (>80%)
- Overdistended uterus (twins, polyhydramnios)
- Vaginal bleeding

MODIFIABLE RISK FACTORS

- Cigarette smoking
- Illicit drug use
- Anemia
- Bacteriuria/urinary tract infection
- Lower genital tract infections (including bacterial vaginosis, *Neisseria gonorrhoea, Chlamydia trachomatis*, Group B streptococcus, *Ureaplasma urealyticum*, and *Trichomonas vaginalis*)
- Gingival disease
- Strenuous work
- High personal stress

Guidelines for the management of preterm labor are summarized in Box 28.17. In many instances, premature labor represents a necessary escape of the fetus from a hostile intrauterine environment and, as such, aggressive intervention to stop labor may be counterproductive. Every effort should be made to exclude contraindications to expectant management and tocolysis, including, among others, intrauterine infection, unexplained vaginal bleeding, non-reassuring fetal testing, and intrauterine fetal demise. Bed-rest and hydration are commonly recommended for the treatment of preterm labor, but without confirmed efficacy.[719,723] Although there is substantial data that broad-spectrum antibiotic therapy can prolong latency in the setting of preterm premature rupture of the membranes remote from term, there is no consistent evidence that such an approach can delay delivery in women with preterm labor and intact membranes.[724]

Pharmacological tocolytic therapy remains the cornerstone of management for acute preterm labor. Although a number of alternative agents are now available (Table 28.10),[672,725-728] there are no reliable data to suggest that any of these agents are able to delay delivery in women presenting with preterm labor for longer than 48 hours. Because no single agent has a clear therapeutic advantage, the adverse effect profile of each of the drugs will often determine which to use in a given clinical setting.

Box 28.15 Efficacy of Screening Tests Used to Identify Women at High Risk for Preterm Delivery

RISK FACTOR SCORING

Risk factor scoring systems based on historical factors, epidemiological factors, and daily habits have been developed in an attempt to predict women at risk of preterm birth. However, reliance on risk factor-based screening protocols alone will fail to identify over 50% of pregnancies that deliver preterm (low sensitivity) and the majority of women who screen positive will ultimately deliver at term (low positive predictive value).[247,704]

HOME UTERINE ACTIVITY MONITORING (HUAM)

HUAM of women at high risk of preterm delivery has not been shown to reduce the incidence of pre-term birth.[710] Such an approach, however, does increase antepartum visits, obstetric intervention, and the cost of antepartum care.[710] There is no role for HUAM to prevent preterm birth.

CERVICAL ASSESSMENT (DIGITAL AND SONOGRAPHIC EXAMS)

Serial digital evaluation of the cervix in women at risk for preterm delivery is useful if the exam remains normal. However, an abnormal cervical exam (shortening and/or dilatation) is associated with preterm delivery in only 4% of low-risk women and 12% to 20% of high-risk women.[714]

Ultrasound has demonstrated a strong inverse correlation between cervical length and pre-term birth.[706,709] If the cervical length is below the 10th percentile for gestational age, the pregnancy is at a sixfold increased risk of delivery prior to 35 weeks.[709] A cervical length of <15 mm at 23 weeks occurs in under 2% of low-risk women, but is predictive of delivery prior to 28 weeks and 32 weeks in 60% and 90% of cases, respectively.[706]

BIOCHEMICAL MARKERS (FETAL FIBRONECTIN)

Elevated level of fetal fibronectin (fFN) in cervicovaginal secretions is associated with preterm delivery.[712]

However, in a low-risk population, the positive predictive value of a positive fFN test at 22 to 24 weeks' gestation for spontaneous preterm delivery prior to 28 weeks and 37 weeks is only 13% and 36%, respectively.[705] The value of this test lies in its negative predictive value (99% of patients with a negative fFN test will not deliver within 7 days[708]), which may prevent unnecessary hospitalization.

ENDOCRINE MARKERS (SALIVARY ESTRIOL, CORTICOTROPIN-RELEASING HORMONE)

Salivary estriol accurately mirrors the level of biologically active (unconjugated) estriol in the maternal circulation.[680] Elevated levels of estriol in maternal saliva (>2.1 ng/mL) is predictive of delivery prior to 37 weeks in a high-risk population with a sensitivity of 68% to 87%, a specificity of 77%, and a false-positive rate of 23%.[707,713]

Corticotropin-releasing hormone (CRH) can stimulate prostaglandin production from the decidua and fetal membranes,[711] and can potentiate the contractile effects of oxytocin and prostaglandins on the myometrium.[715] Maternal plasma CRH levels increase and CRH-BP levels decrease prior to the onset of labor, both at term and preterm, resulting in a marked increase in bioactive CRH. Some authorities have proposed that CRH may serve as a "placental clock" that controls the duration of pregnancy, and that measurement of plasma CRH levels in the late second trimester may predict the onset of labor.[688] However, recent studies have shown that such tests are not clinically useful.[433,435,716-718] In light of these data, the use of plasma CRH as a predictor of preterm labor—as well as other serum markers such as activin A—remains investigational.[717]

Box 28.16 Guidelines for the Prevention of Preterm Delivery

STRATEGIES THAT HAVE NO PROVEN EFFICACY

- Bed rest[719]
- Regular prenatal care[720]
- Treatment of asymptomatic lower genital tract infection[721]
- Treatment of gingival disease[814]

STRATEGIES THAT MAY HAVE SOME EFFICACY

- Prevention and early diagnosis of sexually transmitted diseases and genitourinary infections
- Treatment of symptomatic lower genital tract infection[722]
- Cessation of smoking and illicit substance use
- Prevention of multifetal pregnancies
- Cervical cerclage, if indicated
- Intramuscular progesterone, if indicated
- Vaginal progesterone, if indicated

Box 28.17 Guidelines for the Management of Preterm Delivery

- Confirm the diagnosis of preterm labor
- Exclude contraindications to expectant management and/or tocolysis
- Administer antenatal corticosteroids, if indicated
- Group B β-hemolytic streptococcus (GBS) chemoprophylaxis, if indicated
- Pharmacological tocolysis
- Consider transfer to tertiary care center

Maintenance tocolytic therapy beyond 48 hours has not been shown to confer any therapeutic benefit, but does pose a substantial risk of adverse effects.[518,729,730] As such, maintenance tocolytic therapy is not generally recommended. Similarly, the concurrent use of two or more tocolytic agents has not been shown to be more effective than a single agent alone, and the cumulative risk of adverse effects generally precludes this course of management.[731] In the setting of preterm premature rupture of the fetal membranes, tocolysis has not been shown to be effective and is best avoided.[732]

There is increasing evidence that prophylactic supplemental progesterone may reduce the rate of preterm birth in women at high risk. While the exact mechanism by which progestogens help prevent preterm birth is unknown, the best evidence appears to favor the following mechanisms: (1) Progesterone helps maintain uterine quiescence in the latter half of pregnancy through inhibition of the expression of contraction-associated protein genes within the myometrium, stimulation of transcription of genes that inhibit uterine contractile activity, and by decreasing synthesis of stimulatory prostaglandins and cytokines.[672,733-737] (2) Although the drop in circulating progesterone levels that

Table 28.10 Management of Acute Preterm Labor

Tocolytic Agent	Route of Administration (Dosage)	Efficacy*	Maternal Adverse Effects	Fetal Adverse Effects
Magnesium sulfate	IV (4-6 g bolus, then 2-3 g/hr infusion)	Effective	Nausea, ileus, headache, weakness	Decreased beat-to-beat variability
			Hypotension	Neonatal drowsiness, hypotonia
			Pulmonary edema	
			Cardiorespiratory arrest	? Ileus
			? Hypocalcemia	? Congenital ricketic syndrome (with treatment >3 weeks)
β-Adrenergic agonists				
-Terbutaline sulfate	IV (2 μg/min infusion to a maximum of 80 μg/min)	Effective	Jitteriness, anxiety, restlessness, nausea, vomiting, rash	Fetal tachycardia Hypotension Ileus
	SC (0.25 mg q20min)	Effective	Cardiac dysrhythmias, myocardial ischemia, palpitations, chest pain	Hyperinsulinemia, hypoglycemia (more common with isoxsuprine)
-Ritodrine hydrochloride†	IV (50 μg/min infusion to a maximum of 350 μg/min)	Effective	Hypotension, tachycardia (more common with isoxsuprine)	Hyperbilirubinemia hypocalcemia
	IM (5-10 mg q2-4hr)	Effective	Pulmonary edema Paralytic ileus Hypokalemia Hyperglycemia, acidosis	? Hydrops fetalis
Prostaglandin inhibitors				
-Indomethacin	Oral (25-50 mg q4-6hr)	Effective	Gastrointestinal effects (nausea, heartburn), headache, rash	Transient oliguria, oligohydramnios
	Rectal (100 mg q12hr)		Interstitial nephritis	Premature closure of the neonatal ductus arteriosus and persistent pulmonary hypertension
			Increased bleeding time	? Necrotizing enterocolitis, intraventricular hemorrhage
Calcium channel blockers				
-Nifedipine	Oral (20-30 mg q4-8hr)	Effective	Hypotension, reflex tachycardia (especially with verapamil) Headache, nausea, flushing Potentiates the cardiac depressive effect of magnesium sulfate Hepatotoxicity	
Oxytocin antagonists				
-Atos iban	IV (1 μM/min infusion to a maximum of 32 μM/min)	Effective	Nausea, vomiting, headache, chest pain, arthralgias	? Inhibit lactation
Phosphodiesterase inhibitor				
-Aminophylline	Oral (200 mg q6-8hr) IV (0.5-0.7 mg/kg/hr)	? Effective ? Effective	Tachycardia	Fetal tachycardia
Nitric oxide donor				
-Nitroglycerine	TD (10-50 mg q day) IV (100 μg bolus, then 1-10 μg/kg/min infusion)	Unproven Unproven	Hypotension, headache	Fetal tachycardia

*Efficacy is defined as proven benefit in delaying delivery by 24-48 hr compared with placebo or standard control.
†The only tocolytic agent approved by the Food and Drug Administration.
IM, intramuscular; IV, intravenous; SC, subcutaneous; TD, transdermal.

occurs at the onset of labor in rodents and ruminants does not occur in humans,[733] recent molecular data suggest that alterations in levels of human progesterone isoforms may result in a functional withdrawal of progesterone activity at the level of the uterus.[697,738] (3) Progesterone may help prevent preterm premature rupture of membranes (PPROM), a common cause of preterm birth, via prevention of apoptosis in fetal membrane explants.[739]

The efficacy of progesterone supplementation for prevention of preterm birth appears to depend primarily on appropriate patient selection. *In vitro* and animal research suggests that the type of progestin, formulation, dose, and delivery route may also have a significant impact on efficacy.[740,741] There are two settings in which progesterone supplementation has been shown to reduce the rate of spontaneous preterm birth: (1) Women with a history of spontaneous preterm birth, (2) women with a short cervix on ultrasound examination in the current pregnancy.

The seminal study demonstrating the benefit of intramuscular progesterone supplementation to prevent recurrent preterm birth was the Maternal Fetal Medicine Units Network trial, in which Meis and co-investigators randomized 459 women with a history of spontaneous preterm delivery to weekly intramuscular injections of

17 alpha-hydroxyprogesterone caproate (17-OHP) or placebo beginning at 16 to 20 weeks of gestation and continuing until 36 weeks.[742] Weekly progesterone supplementation was associated with a significant reduction in the rate of recurrent preterm delivery, with greatest benefit seen in women with a prior spontaneous preterm birth before 34 completed weeks of gestation. Subsequent randomized trials using vaginal progesterone preparations to prevent recurrent spontaneous preterm birth have had mixed results.[743-745] Meta-analyses of randomized trials have demonstrated that progesterone supplementation is protective against recurrent preterm birth.[746-750] The most recent meta-analysis of six randomized controlled trials demonstrated a significant reduction in preterm delivery less than 37 weeks (RR 0.77, 95% CI 0.67-0.87), less than 35 weeks (RR 0.77, 95% CI 0.63-0.96), and less than 32 weeks (RR 0.61, 95% CI 0.45-0.82), as well as reduced rates of perinatal and neonatal death, respiratory distress syndrome, and necrotizing enterocolitis in the newborn.[749] The balance of data suggests that intramuscular 17-OHP is more efficacious than vaginal progesterone for prevention of recurrent spontaneous preterm birth.[735] A randomized controlled trial including 657 nulliparous women with singleton gestations found that intramuscular 17-OHP was not efficacious in preventing preterm birth in women with a short cervix (cervical length less than 30 mm at 16 to 22 weeks).[751]

Fonseca and colleagues performed the seminal study demonstrating the benefit of vaginal progesterone supplementation to prevent preterm birth in women with sonographically short cervix.[752] These investigators randomized 413 women with cervical length of 15 mm or less to receive nightly vaginal progesterone suppositories or placebo from 24 to 34 weeks' gestation, finding that vaginal progesterone was associated with a significant decrease in spontaneous preterm delivery before 34 weeks' gestation (from 34.4% to 19.2%, RR 0.56, 95% CI 0.36-0.86). A recent meta-analysis of five randomized placebo-controlled trials, including data from 775 pregnancies, demonstrated the benefits of progesterone supplementation in women with asymptomatic short cervix on midtrimester ultrasound.[753] Treatment with vaginal progesterone in women with a singleton gestation and sonographic short cervix was associated with a significant reduction in the rate of preterm birth, and decreased neonatal morbidity and mortality. These benefits were similar at progesterone doses of 90, 100, and 200 mg daily.[753] The same meta-analysis demonstrated that in twin pregnancies with a short cervix, treatment with vaginal progesterone was associated with a nonsignificant reduction in preterm birth at less than 33 weeks' gestation, and a significant reduction in composite neonatal morbidity and mortality.

In the absence of short cervix, progesterone supplementation in twin pregnancies does not appear to be beneficial. A randomized, double-blind, placebo-controlled study found that routine administration of vaginal progesterone does not prevent early preterm birth (<34 weeks' gestation) in twin gestations.[754] This finding was confirmed in another double-blind, placebo-controlled randomized trial including 677 women.[755] Routine administration of intramuscular 17-OHP also failed to reduce the rate of preterm birth in women with twin gestations in randomized controlled trials.[756-758] Randomized controlled trials in triplet pregnancies have also failed to demonstrate efficacy of intramuscular 17-OHP in preventing preterm birth.[759,760] The mechanism leading to preterm birth in multiples may be different than that in singleton pregnancies. It has been proposed that excessive uterine stretch may precipitate preterm birth multiple gestations. If this is indeed the mechanism, a recent *in vitro* study demonstrating lack of progesterone inhibition of stretch-induced mitogen activated protein kinase (MAPK) activation or gene expression in myometrial cells may shed light on the failure of progesterone supplementation to prevent preterm birth in these cases.[761,762]

Enthusiasm for widespread use of progesterone supplementation to prevent preterm birth should be tempered by recognition that the long-term effects on both women and their offspring have not yet been fully elucidated.[763] While concerns about an increased risk of miscarriage and stillbirth in women exposed to progestins in pregnancy[742,754,756,764] have largely been laid to rest,[759,760,765] concern persists regarding a potential increased incidence of hypospadias in male fetuses exposed to exogenous progestins before 11 weeks' gestation.[766,767] Recent work has demonstrated that a negative-feedback loop involving inhibitory transcription factors zinc finger E-box binding homeobox proteins 1 and 2 (ZEB1 and ZEB2) and members of the microRNA (miRNA)-200 family may mediate the effect of progesterone on contraction-associated proteins in the uterus during pregnancy.[736] Caution should be used, however, when targeting the ZEB-miRNA-200 loop for therapeutic intervention, as it has also been implicated in cancer progression.[737,768]

Postterm Pregnancy

Postterm (prolonged) pregnancy refers to a pregnancy that has extended to or beyond 42 weeks (294 days) of gestation. Approximately 10% (range 3% to 14%) of all singleton pregnancies continue beyond 42 weeks of gestation and 4% (2% to 7%) continue beyond 43 completed weeks in the absence of obstetric intervention.[769] Accurate pregnancy dating is critical to the diagnosis. The lowest incidence of postterm pregnancy is reported in studies using routine sonography for confirmation of gestational age.[770]

Although the majority of postterm pregnancies have no known cause, an explanation may be found in a minority of cases. Primiparity and prior postterm pregnancy are the most common identifiable risk factors for prolongation of pregnancy.[769,771,772] Genetic predisposition may also play a role,[771,772] as concordance for postterm pregnancy is higher in monozygotic twins than dizygotic twins.[773] Women who themselves are a product of a prolonged pregnancy are at 1.3-fold increased risk of having a prolonged pregnancy, and recurrence for prolonged pregnancy is increased twofold to threefold in women who previously delivered after 42 weeks.[774,775] Rarely, postterm pregnancy may be associated with placental sulfatase deficiency or fetal anencephaly (in the absence of polyhydramnios) or CAH.[776]

Perinatal mortality after 42 weeks of gestation is twice that at term (4 to 7 versus 2 to 3 deaths per 1,000 deliveries), and is increased fourfold at 43 weeks and fivefold to sevenfold at 44 weeks compared with 40 weeks.[776-778] Uteroplacental insufficiency, asphyxia (with and without meconium), intrauterine infection, and "fetal dysmaturity (postmaturity) syndrome" (which refers to chronic IUGR

due to uteroplacental insufficiency) all contribute to the excess perinatal deaths. Postterm infants are larger than term infants, with a higher incidence of macrosomia (2.5% to 10% vs 0.8% to 1%).[779,780] Complications associated with fetal macrosomia include prolonged labor, cephalopelvic disproportion, and shoulder dystocia with resultant risks of orthopedic or neurological injury. Prolonged pregnancy does not appear to be associated with any long-term neurological or behavioral sequelae.[781]

Postterm pregnancy is also associated with risks to the mother, including an increase in labor dystocia (9% to 12% vs 2% to 7% at term), an increase in severe perineal injury related to macrosomia (3.3% vs 2.6% at term), and a doubling in the rate of cesarean delivery.[744,782,783] The latter is associated with higher risks of complications such as endometritis, hemorrhage, and thromboembolic disease.

The management of postterm pregnancy should include confirmation of gestational age, antepartum fetal surveillance, and induction of labor if spontaneous labor does not occur. Postterm pregnancy is a universally accepted indication for antenatal fetal monitoring.[769,770,784] However, the efficacy of this approach has not been validated by prospective randomized trials.[770] No single method of antepartum fetal testing has been shown to be superior.[770,785] ACOG has recommended that antepartum fetal surveillance be initiated between 41 and 42 weeks of gestation, without a specific recommendation regarding type of test or frequency.[769,784] Many investigators would advise twice-weekly testing with some evaluation of amniotic fluid volume.

Delivery is typically recommended when the risks to the fetus by continuing the pregnancy are greater than those faced by the neonate after birth. In high-risk pregnancies, the balance appears to shift in favor of delivery at around 38 to 39 weeks of gestation. Management of low risk pregnancies is more controversial. Factors that need to be considered include results of antepartum fetal assessment, favorability of the cervix, gestational age, and maternal preference after discussion of the risks, benefits, and alternatives to expectant management with antepartum monitoring versus labor induction. Delivery should be affected immediately if there is evidence of fetal compromise or oligohydramnios.[786,787]

In low-risk postterm gravida, both expectant management and labor induction are associated with low complication rates. However, the risk of unexplained intrauterine fetal demise—which, in one large series, was 1 in 926 at 40 weeks, 1 in 826 at 41 weeks, 1 in 769 at 42 weeks, and 1 in 633 at 43 weeks[788]—disappears after a fetus is delivered. Hannah and colleagues[789] randomly assigned 3407 low-risk women with uncomplicated singleton pregnancies at 41 weeks of gestation to induction of labor (with or without cervical ripening) within 4 days of randomization or expectant management until 44 weeks. Elective induction resulted in a lower cesarean delivery rate (21.2% vs 24.5%, respectively), primarily related to fewer surgeries performed for nonreassuring fetal testing. These findings have been confirmed by a subsequent large randomized clinical trial.[790] In addition, a meta-analysis of 26 trials of routine versus selective induction of labor in postterm patients found that routine induction after 41 weeks was associated with a lower rate of perinatal mortality (OR 0.20; 95% CI, 0.06- 0.70) and no increase in the cesarean delivery rate.[785,791] Taken together, these data suggest that there does appear to be an advantage to routine induction of labor at 41 weeks of gestation, using cervical ripening agents when indicated, regardless of parity or method of induction.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 29

Hormone Responsive Cancers

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Breast Cancer

Etiology

The development of *de novo* cancer is a complex process involving several mutational and epigenetic events. Hanahan and Weinberg delineated six key requirements for cancer to develop: "sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis."[1] These attributes allow "cancer cells to generate their own mitogenic signals, resist exogenous growth-inhibitory signals, evade apoptosis, proliferate without limits (i.e., to undergo immortalization), undergo angiogenesis and, in more advanced cancers, invade and metastasize."[1]

Genome wide analyses suggest that multiple mutations are present in breast cancer, some of which regulate the six key pathways and some that are merely bystanders.[2] Vogelstein and his colleagues have coined the term: *driver mutations* for those that regulate the six key processes and *passenger mutations* for the bystanders.[2,3] The Vogelstein group identified 167 mutated genes in the breast cancers examined, with 14 considered to be candidate "driver" genes and the other 153 "passenger" genes. These mutations probably alter no more than 20 key signaling pathways.[3] This dynamic of cancer development is consistent with the idea that a large number of mutations, each associated with a small growth or cell death advantage, drive tumor progression.[4] Based upon these concepts, the specific signaling pathways altered by mutations are considered more important for emphasis than identification of individual mutations.

A number of specific mutations have been associated with an increased incidence of breast cancer.[5] Whole genome and GWAS (gene wide association studies) have identified more than 20 mutations, each of which impart only a small increase in frequency of breast cancer in the populations studied.[6-11] The most common include *BrCa1* and *BrCa2*, *FGFR2*, *IKBKE*, *ATM*, *BRIP-1*, *BR1P1*, and *TP53* genes.[12-18] Mutations of the BrCa1 and BrCa2 genes are associated with approximately 5% of breast cancer cases.[19] Rarer genetic syndromes include mutations of the p53 gene in the Li-Fraumeni syndrome; impaired cell cycle check point surveillance in the ataxia-telangiectasia syndrome (ATM); mutations in the *PTEN* gene in the Cowden syndrome; the *MLH1/MSH2* genes in the Muir-Torre syndrome; a *STK 11* mutation the Peutz-Jeaghers syndrome and *Chek 2* mutations.[5] Studies in identical twins suggest that approximately 27% of breast cancers are associated with genetic factors but the specific genes are unknown in 22% of these.[20]

Risk Factors

Dietary, Environmental, and Lifestyle Factors

As key roles in breast cancer etiology, these factors contribute to the fourfold differences in incidence between Japan with a rate of 23 women per 100,000 per year and the United States at 90/100,000 per year.[21-25] Epidemiological observations suggest a role for high fat diet, alcohol, exercise, and obesity in the genesis of breast cancer.[13,26,27] In Japan, the rate of breast cancer peaks at the age of menopause but in the United States, incidence continues to increase until age 90. The difference in postmenopausal patterns may result from the increase in obesity and associated aromatase increments in women in the United States compared to Japan. This differential breast cancer rate does not appear genetic since Japanese women who move to the United States experience an increased rate of breast cancer that later approaches that of North American women.

Hormonal Factors

Hormonal factors and particularly estrogens contribute to the development of breast cancer. Administration of exogenous estrogens to various animal species results in breast neoplasms.[28] Spontaneous development of breast cancer in aging rats can be prevented by oophorectomy or administration of aromatase inhibitors to block estrogen production. As outlined in Figure 29.1, epidemiological studies implicate several hormonal factors which are associated with an increased incidence of breast cancer.

Intensity of Estrogen Exposure

The majority of risk factors for breast cancer relate to the duration or intensity of a woman's exposure to endogenous or exogenous estrogens (Fig. 29.1). Early menarche and/or late menopause increase breast cancer risk.[29] Elevations

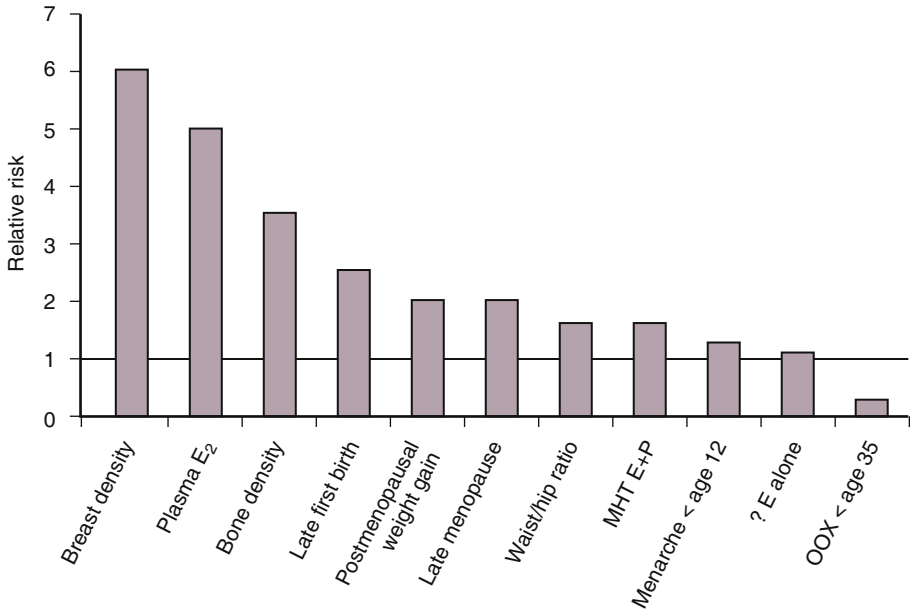


FIGURE 29.1 Relative risk of breast cancer as a function of several factors which relate to the long-term exposure to estradiol (E_2). The question mark indicates data are conflicting, but the bar represents the majority opinion. E, estrogen; MHT, menopausal hormone therapy; OOX, oophorectomy; P, progestine. *(From Santen R. Endocrine–responsive cancer. In Kronenberg HM, Melmed S. Polonski KS, Larsen PR [eds].* Williams Textbook of Endocrinology, *11th ed, Philadelphia, WB Saunders, 2008, Figure 42.2.)*

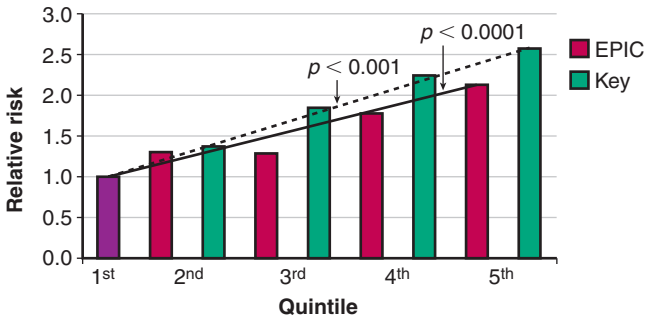


FIGURE 29.2 Risk of breast cancer related to levels of free plasma estradiol (i.e., non-sex-hormone bound estradiol) in postmenopausal women. The relative risk of breast cancer is related to the levels of free plasma estradiol divided into quintiles for the first to the fifth quintile. The solid and dotted lines indicate the trend analyses of increased relative risk versus the quintile of estradiol. *(Adapted from [30; 31.] Reprinted with the permission of the Endocrine Society from Yager JD and Santen RJ. Mechanisms relating estrogens to breast cancer,* Translational Endocrinology and Metabolism *3[1]:75–93, 2012.)*

in circulating estradiol levels predict the risk of developing breast cancer over the ensuing years in postmenopausal women (Fig. 29.2).[30-31] Estrogen levels provide information independent of other known risk factors.[32] An estradiol in the top quintile may increase the relative risk of breast cancer by threefold to fivefold (Fig. 29.2).[30,31,33] An index weighing several circulating estrogens (E1, E2, E3, E1-S, E2-S) provides a higher estimate of risk than estradiol alone.[34] Estrogen metabolites, which do not act through the estrogen receptor, are also associated with an increased risk of breast cancer. For example, weighted averages of several estradiol metabolites involving the 4-hydroxylation pathway predict a greater risk of breast cancer than levels of E2 alone.[35]

Putative markers of long-term estrogen exposure such as bone density are also predictive. Women in the top quartile of bone density have a threefold increased risk of breast cancer (Fig. 29.1); a history of fracture or height loss lowers the risk.[36-37] Late first birth increases the risk 2.8-fold and is believed to relate to the lack of the differentiating effect of pregnancy on type of breast lobule present.[38] Gain of at least 20 kg as an adult increases breast cancer risk twofold and weight loss reduces the risk.[21,39] Increased waist-hip ratio exerts a similar increase in risk.[22] Combined analyses of multiple studies indicate that alcohol intake can increase the risk of breast cancer, perhaps by decreasing the clearance of estradiol.[26] Increased exposure to estradiol *in utero*, as shown by twin studies, may increase risk of breast cancer by as much as twofold. Early pregnancy and prolonged duration of breastfeeding diminish the risk.[27] More dramatic is the 75% reduction in risk caused by bilateral oophorectomy before age 35 or use of anti-estrogens by premenopausal and postmenopausal women.[40-43]

Sources of Estrogen

The estradiol present in breast tissue is synthesized in three sites: the ovary, extra-glandular tissues, and the breast itself. Direct glandular secretion by the ovary results in delivery of estradiol to the breast through an endocrine mechanism in premenopausal women. After the menopause, extra-glandular production of estrogen from ovarian and adrenal androgens in fat and muscle provides the second source of estradiol. Thirdly, the breast itself can synthesize estradiol via aromatization of androgens to estrogens, cleavage of estrone-sulfate to free estrone via the enzyme sulfatase, and conversion of estrone to estradiol via 17βOH-HSD. Classic studies involving infusion of radiolabeled androgens and estrogens into women provide direct evidence of both *in situ* production in breast and uptake from plasma.[44] Recent studies relating plasma estrogen levels to expression of estrogen responsive genes in breast tumor tissue suggest that ER mediated uptake might predominate over *in situ* synthesis.[45] Several factors regulate *in situ* estradiol synthesis but the most important is the degree of obesity which increases the amount of aromatase in breast and consequently, estradiol production. The mechanism for obesity related induction of aromatase involves leptin, adiponectin, and AMP-kinase which allow CRTC2 to become detached

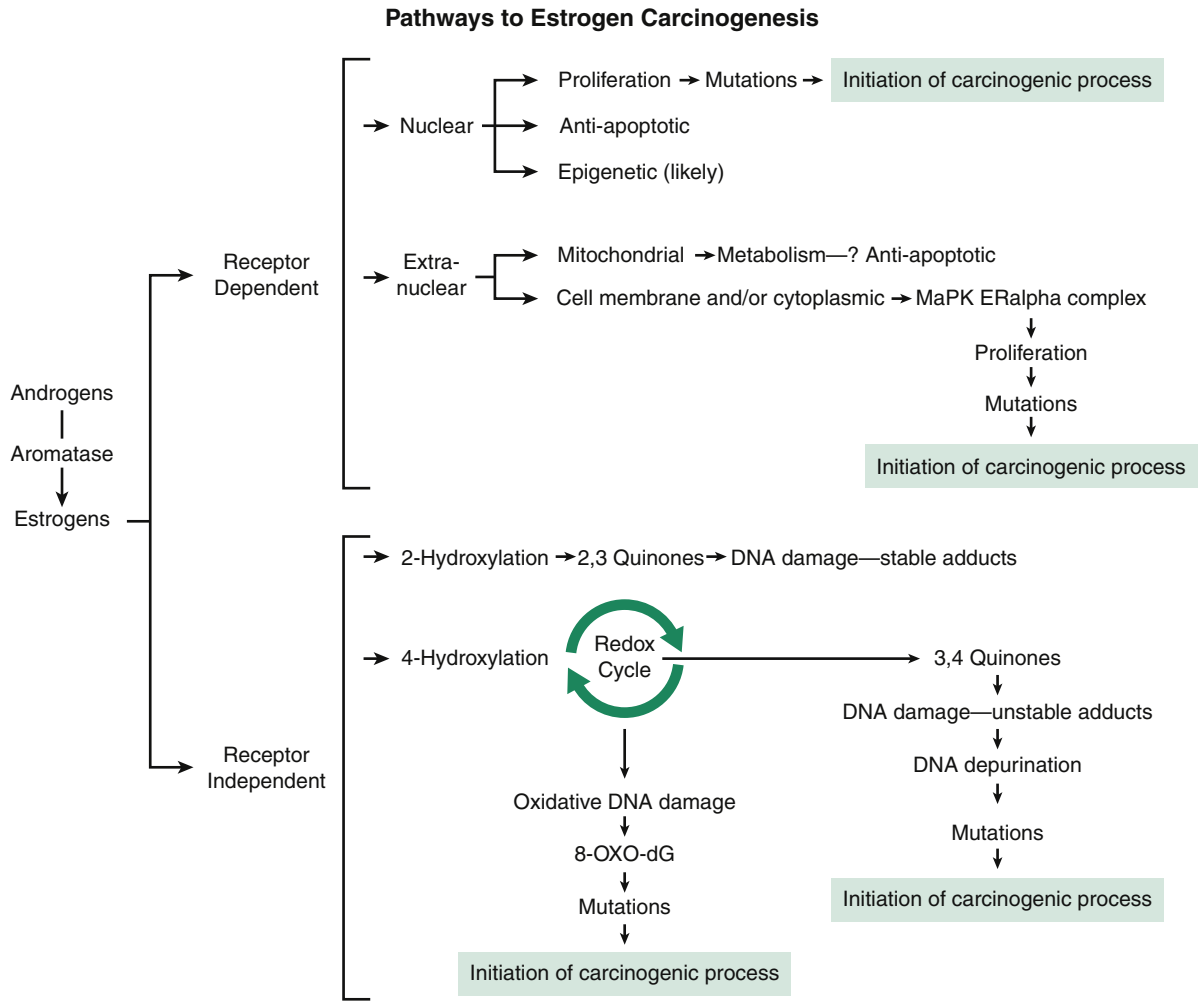


FIGURE 29.3 Diagrammatic pathway to illustrate the ER dependent and ER independent mechanisms whereby estrogens contribute to the development of breast cancer. ER, estrogen receptor. *(Reprinted with the permission of the Endocrine Society from Yager JD and Santen RJ. Mechanisms relating estrogens to breast cancer,* Translational Endocrinology and Metabolism *3[1]:75–93, 2012.)*

from a 14-3-3 protein, and enter the nucleus to increase aromatase transcription.[46]

Estrogen Induced Carcinogenesis

The precise mechanisms for estrogen induced carcinogenesis are becoming better understood. Current data suggest that both ERα dependent and independent mechanisms are involved in the initiation of *de novo* breast cancer (Fig. 29.3).[28,47] Via ERα, estradiol increases the rate of proliferation of breast cells through nuclear transcriptional mechanisms and through extranuclear effects resulting from activation of MAP kinase. After entering the nucleus, activated MAP kinase and ERα bind to estrogen response elements (ERE) on the DNA cistrome.*[48] ERα also binds to separate ERE sites both in the absence of MAP kinase or through tethering, to adaptor protein-1 (AP-1) and specificity protein-1 (SP-1) proteins to induce additional proliferation stimulatory genes.

As the number of cell divisions increase through accelerated proliferation, the frequency of mutations is enhanced pari passu.[47] As a contributory factor, less time is available for DNA repair when cells are dividing rapidly. Estrogens also enhance tumor promotion by increasing the rate of propagation of mutated cells. Enhanced cell proliferation is considered to be a key feature of the carcinogenic process, not just in the breast, but in several organs. These steps are generally considered the primary processes by which estrogens initiate mutations which ultimately lead to neoplastic transformation.[47]

Accumulating evidence implicates estrogen metabolism as an additional mutagenic process in breast but this mechanism is not fully proven and remains controversial. ERα independent mutational effects of estrogen involve metabolites of estradiol which may directly damage DNA through a pathway involving the CYP 1B1 cytochrome p450 enzyme (Fig. 29.3).[28] This p450 enzyme catalyzes conversion of estradiol to 4-0H-estradiol, a catechol-estrogen which is then further metabolized to 3, 4 estradiol-quinone. This highly reactive species binds covalently to guanine or adenine

*Cistrome is defined as the set of cistrons within the genome where a cistron is a type of gene which contains a cis-acting target (DNA binding site) of a trans-acting factor (e.g., transcription factor, pioneer factor, restriction enzyme, etc.) and promotes transcription of a unique series of genes involved in proliferation.

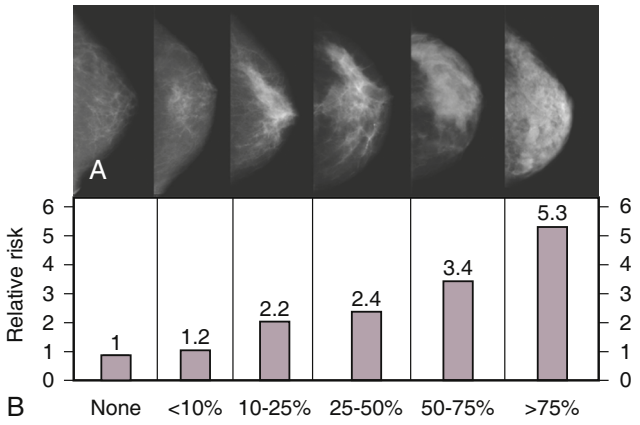


FIGURE 29.4 Relative risk of breast cancer as a function of the degree of mammographic density. **A,** Six categories of breast density as illustrated by mammograms. **B,** Relative risk of breast cancer for each category of density. *(From Boyd NF, Bying JW, Jong RA, et al. Quantitative classification of mammographic densities and breast cancer risk: results from the Canadian National Breast Screening Study,* J Natl Cancer Inst *87[9]:670–675, 1995.)*

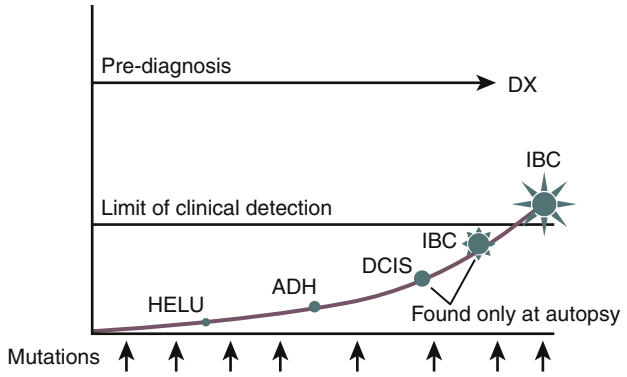


FIGURE 29.5 Diagram representing the sequential development of breast cancer. The first predisposing lesion is called a hyperplastic elongated lobule unfolded (HELU) which progresses to atypical ductal hyperplasia (ADH) and then ductal carcinoma *in situ* (DCIS) and finally to invasive breast cancer (IBC). It should be noted that the role of the HELU is not universally accepted. It has been recently estimated that 14 "driver" mutations on average are found in established breast cancers (see text). A lesion must reach 1 cm before it is detected clinically by mammogram. Accordingly, there is a large reservoir of undiagnosed tumors too small to be detected by palpation or imaging techniques (see text). DX, diagnosis.

molecules in the DNA helix and forms unstable complexes which result in depurination. Error prone or replicative repair of the depurinated sites leads to point mutations.[28] The 3, 4 estradrol-quinones can also be back converted to 4-OH–estradiol by quinone reductase (a process enhanced by tamoxifen) resulting in a redox cycle which generates reactive oxygen species.[49] Oxygen free radicals in turn may directly damage DNA through production of 8-OXO-D-guanine which also leads to depurination.

The receptor independent hypothesis involving estrogen metabolites is supported experimentally by studies demonstrating the mutagenic potential of estradiol on estrogen receptor negative Michigan Cancer Foundation-10 (MCF-10) breast cancer cells *in vitro* and the transformation of these cells to cancer *in vivo*.[50-51] Experiments in castrate estrogen receptor knock out animals bearing the Wnt-1 oncogene demonstrate a dose dependent acceleration of breast tumor onset and an increase in tumor incidence.[52] In women, menopausal hormone therapy as well as plasma estrogen levels are associated with an increased incidence of ER-breast cancer, as well as an enhanced rate of ER+ tumors.[53-55]

Mammograhic density

Among all other risk factors, breast density represents the most powerful for predicting breast cancer risk (Fig. 29.4).[56-57] Increase in breast cancer risk from lowest to highest breast density category is on the order of fivefold depending upon the age of the patient (Fig. 29.4) with greater relative risk in older women. Mammographic density, when added to the factors used in the Gail predictive model, increases the power of prediction (albeit only modestly) and thus is independent of several other risk factors.[58-59] The cause(s) of increased breast density are poorly understood but are partly related to genetic factors as shown by twin studies.[60] Recent data suggest that areas of increased breast density contain higher levels of aromatase than nondense areas suggesting a role for increased *in situ* estrogen production.[61] Exogenous estrogens increase and anti-estrogens reduce breast density. As evidence of the biological import of breast density, only those individuals with a reduction of greater than 10% with tamoxifen experienced prevention of breast cancer.[62] It should be noted that on a practical basis, menopausal hormonal therapy with estrogen plus a progestogen and its associated increase in breast density alters the sensitivity and specificity of breast cancer detection with mammography.[57]

Natural History of Breast Cancer

A variety of data suggest that *de novo* breast cancers take many years to grow to a size large enough to be clinically detectable by mammography or by palpation (Fig. 29.5). The doubling time of tumors approximates 200 days in postmenopausal and 160 days in premenopausal women.[63] Mammographic thresholds for clinical detection vary as a function of breast density and age but average 1.16 cm for 50- to 80-year-old women. Palpation thresholds are larger and are estimated to approximate 2.0 cm. *De n*ovo tumors must undergo 30 doubling times to reach the detection threshold and on average this would require 16.4 years (Fig. 29.6A). Only *de novo* tumors with a doubling time of 50 days or less would reach the detection threshold over a period of 5 to 7 years and only 15% of tumors have doubling times less than 50 days. Accordingly, most newly diagnosed breast tumors had been present for several years prior to detection.[63]

Based on these considerations, one would expect a large reservoir of occult, undiagnosed tumors in 50- to 80-year-old women. Eight autopsy studies have examined this possibility and reported that an average of 7% of women harbor occult, undiagnosed tumors, 6% of which were DCIS and 1% invasive (Table 29.1). Assuming an average doubling time of 200 days and detection threshold of 1.16 cm, the time to reach detection for tumors of each doubling category are shown in Figure 29.6B. Tumors having undergone 5 doublings would take 13 years to reach detection whereas

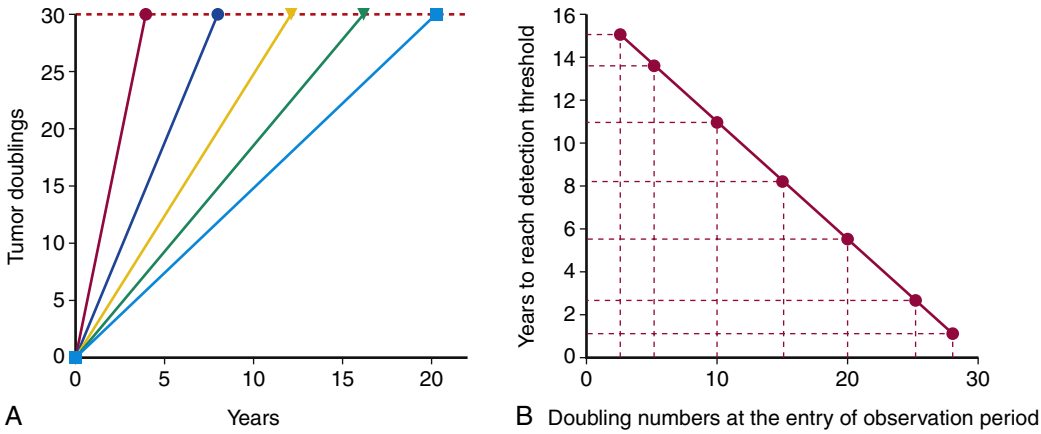


FIGURE 29.6 **A,** The time to reach the detection threshold at 30 tumor doublings (i.e., 1.16 cm) with effective doubling time iterations represented by the various colors

50 day effective doubling time
100 day effective doubling time
150 day effective doubling time
200 day effective doubling time
250 day effective doubling time

B, The time to reach the detection threshold of 30 tumor doublings (i.e., 1.16 cm) for tumors with iterations representing the given number of doublings at the beginning of the observation period. *(Adapted from Santen RJ, Yue W, Heitjan DF. Modeling of the growth kinetics of occult breast tumors: role in interpretation of studies of prevention and menopausal hormone therapy,* Cancer Epidemiol Biomarkers Prev, *21[7]: 1038–1048, 2012.)*

Table 29.1 Cancers Found at Autopsy

Report	Number of Cases	Incidence (%)
1962 Ryan	200	0
1973 Kramer	70	4.3
1975 Wellings	67	1.9
1984 Nielsen	77	14.3
1985 Alpers	101	8.9
1985 Bhathal	207	12.1
1987 Bartow	221	0
1988 Nielsen	109	14.7
Total cases	1052	6

Data from Ryan JA, Coady CJ. Intraductal epithelial proliferation in the human breast — a comparative study, *Can J Surg* 5:12–19, 1962; Kramer WM, Rush BF Jr. Mammary duct proliferation in the elderly. A histopathologic study, *Cancer* 31:130–137, 1973; Wellings SR, Jensen HM, Marcum RG. An atlas of subgross pathology of the human breast with special reference to possible precancerous lesions, *J Natl Cancer Inst* 55:231–273, 1975; Nielsen M, Jensen J, Andersen J. Precancerous and cancerous breast lesions during lifetime and at autopsy. A study of 83 women, *Cancer* 54:612–615, 1984; Alpers CE, Wellings SR. The prevalence of carcinoma in situ in normal and cancer-associated breasts, *Hum Pathol* 16:796–807, 1985; Bhathal PD, Brown RW, Lesueur GC, Russell IS. Frequency of benign and malignant breast lesions in 207 consecutive autopsies in Australian women, *Br J Cancer* 51:271–278, 1985; Bartow SA, Pathak DR, Black WC, et al. Prevalence of benign, atypical, and malignant breast lesions in populations at different risk for breast cancer. A forensic autopsy study, *Cancer* 60:2751–2760, 1987; and Nielsen M, Thomsen JL, Primdahl S, et al. Breast cancer and atypia among young and middle-aged women: a study of 110 medicolegal autopsies, *Br J Cancer* 56:814–819, 1987.

those having undergone 25 doublngs would take only 2.5 years (Fig. 29.6B). Using these statistics (i.e., 200 day doubling time, 7% prevalence of occult tumors, and mammographic detection threshold of 1.2 cm) a recently developed model was used to predict the population incidence of breast cancer (Fig. 29.7). Comparison of model-predicted with population-incidence data demonstrated excellent concordance and served to validate the model. The natural history of occult tumors becomes quite important when interpreting data regarding risks of breast cancer from exogenous hormone therapy.

Exogenous Hormones and Breast Cancer Risk

In premenopausal women, use of oral contraceptives for 10 or more years increases the relative risk of breast cancer by approximately 10% to 20%.[27,64-66] This increase affects very few women since the age-related absolute incidence of breast cancer is minimal in women taking oral contraceptives. The median age in the largest study of oral contraceptive use was 26 years old.[66] Based on Surveillance Epidemiology and End Results (SEER) population incidence data for 25- to 30-year-old women (approximate incidence 12/100,000 per year), use of an oral contraceptive would result in only 1.2 to 2.4 extra breast cancers per 100,000 women per year.

The concept that menopausal hormone therapy (MHT) increased the risk of breast cancer was initially controversial as it was based exclusively on conflicting observational studies.[55,67] However, the large CGHFBC study (Collaborative Group on Hormonal Factors in Breast Cancer Study) served to clarify the factors responsible for the differing conclusions among the various observational reports, as outlined below[68]:

1. The relative risk of breast cancer from MHT is small and large studies with a long duration of follow-up are required to minimize type I and type II statistical errors.
2. The risk of breast cancer increases linearly with duration of MHT use. Accordingly, comparisons of "ever users" with "never users" are invalid since duration of estrogen use is not considered.
3. The increased risk of breast cancer *imparted* by MHT dissipates within 4 years of cessation of therapy.

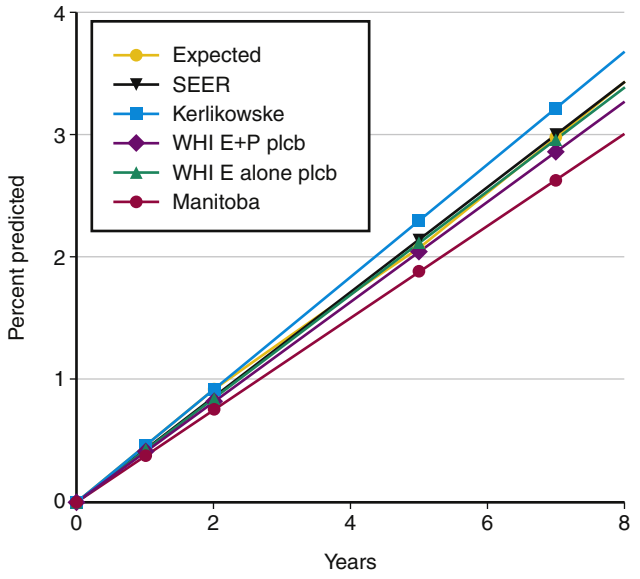


FIGURE 29.7 The observed percent incidence of breast cancer from the SEER, BCSC, Manitoba, WHI E+P placebo arm and WHI E alone placebo arms are compared with the predicted incidence based on the OTG model. SEER, surveillance epidemiology and end results; BCSC, Breast Cancer Surveillance Consortium; Manitoba, breast cancer mammographic screening data from Manitoba, Canada; WHI E+P, placebo arm of the Women's Health Initiative conjugated equine estrogen plus medroxyprogesterone acetate study; WHI E, same study but conjugated equine estrogen alone; OGT, occult tumor growth model. *(Reprinted with permission from Santen RJ, Yue W, Heitjan DF. Modeling of the growth kinetics of occult breast tumors: role in interpretation of studies of prevention and menopausal hormone therapy,* Cancer Epidemiol Biomarkers Prev *21[7]: 1038–1048, 2012.)*

Therefore, only women using MHT within 4 years of study might be found to be at increased risk.

4. Breast cancer risk also diminishes over a 4-year period following the menopause, presumably as a reflection of decreased estrogen and progesterone levels. As a result, analyses of observational studies need to match users vs. nonusers as to time following menopause.
5. The increased risk of breast cancer from MHT is higher in lean women. Inclusion of a large proportion of obese women in a single study might then obscure associations between MHT use and breast cancer risk.

When the CGHFBC took these confounding factors into account, the pooled observational data were relatively consistent and suggested an increased risk of breast cancer in women using estrogen plus a progestogen (P) over 5 years or taking estrogen alone long-term for 10 or more years.[68]

WHI ER+P study: The large, prospective, randomized, Women's Health Initiative (WHI) trial in postmenopausal women first published in 2002 strongly supported the observational data on E+P.[69] Nearly 16,000 postmenopausal women with an average age of 63 enrolled in the estrogen plus progestin arm of the WHI study and received either placebo or conjugated equine estrogens (0.625 mg) plus medroxyprogesterone acetate (2.5 mg) for 5 years. At study termination after 5.2 years, the relative risk was reported as 1.26 (RR 1.26, 95% CI 1.00 to 1.59). The absolute increase in risk (i.e., attributable risk) was small with 4 cases per 1000 women treated for 5 years.

In the original WHI report, it was noted that 74% of the participants were never users of MHT, 19.7% were past users, and 6.4% were current users. Among the never users, breast cancer risk was not increased (RR 1.09, CI 0.86 to 1.40), a statistic confirmed in a subsequent publication in 2006 (Fig. 29.8A and B). In another analysis, the risk of MHT was shown to be higher for women who initiated treatment within 5 years of menopause (deemed a short gap time) as opposed to those who initiated after a long interval (long gap time).

Effect of Specific Progestogens

The WHI E+P study utilized only one progestogen, medroxyprogesterone acetate (MPA), and did not address whether different progestogens might have lesser effects on breast cancer risk. Several observational studies examined the effects of various types of progestogen as well as differences between combined continuous and sequential regimens.[69,70] The Million Women Study suggested that all types of progestogen are associated with an increased risk of breast cancer and that a class effect of progestogens is responsible. An interesting but as yet unconfirmed finding, however, is that progesterone itself appears to be associated with a lower risk of breast cancer than synthetic progestogens. The French component of the European Prospective Investigation into Cancer and Nutrition (EPIC) study reported on 2354 cases of invasive breast cancer among 59,216 French postmenopausal women followed for an average of 8.1 years. This study reported a relative risk of 1.08 (CI 0.89 to 1.31) for estradiol in combination with micronized progesterone.[71] In contrast, the relative risk (RR) for estradiol combined with synthetic progestogens in that study was 1.69 (1.50 to 1.91), similar to risks reported in other epidemiological studies. This finding requires confirmation before general acceptance is warranted.

An understanding of the physiological basis for the association of progestogens with breast cancer rests on data indicating that progestogens are mitogenic on breast tissue in contrast to their anti-mitogenic effects on the uterus.[72] While data from cell cultures or animal studies are conflicting, the weight of evidence from patients suggests that progestogens are mitogenic in breast. Mammographic studies demonstrate that estrogen/progestogen combinations increase breast density to a greater extent than estrogen alone or placebo.[73] Histological examination demonstrates enhanced cell proliferation and percent of the breast containing glandular tissue as a function of duration of progestogen usage.[72] Increased proliferation would be expected to increase both initiation and promotion of breast cancer in a manner similar to that thought to occur with estrogens.[74]

Confounding Factors

Several commentators have pointed out factors confounding the interpretation of the WHI studies: (1) the frequency of dropouts (30%) and drop-ins (7%) (i.e., patients randomized to placebo and then deciding to take MHT during the study), (2) the exclusive use of MPA as the progestogen, (3) the average age of participants was 63 years old, (4) the fact that 26.5% of women had used prior hormone therapy and then underwent washout before starting MHT, (5) the

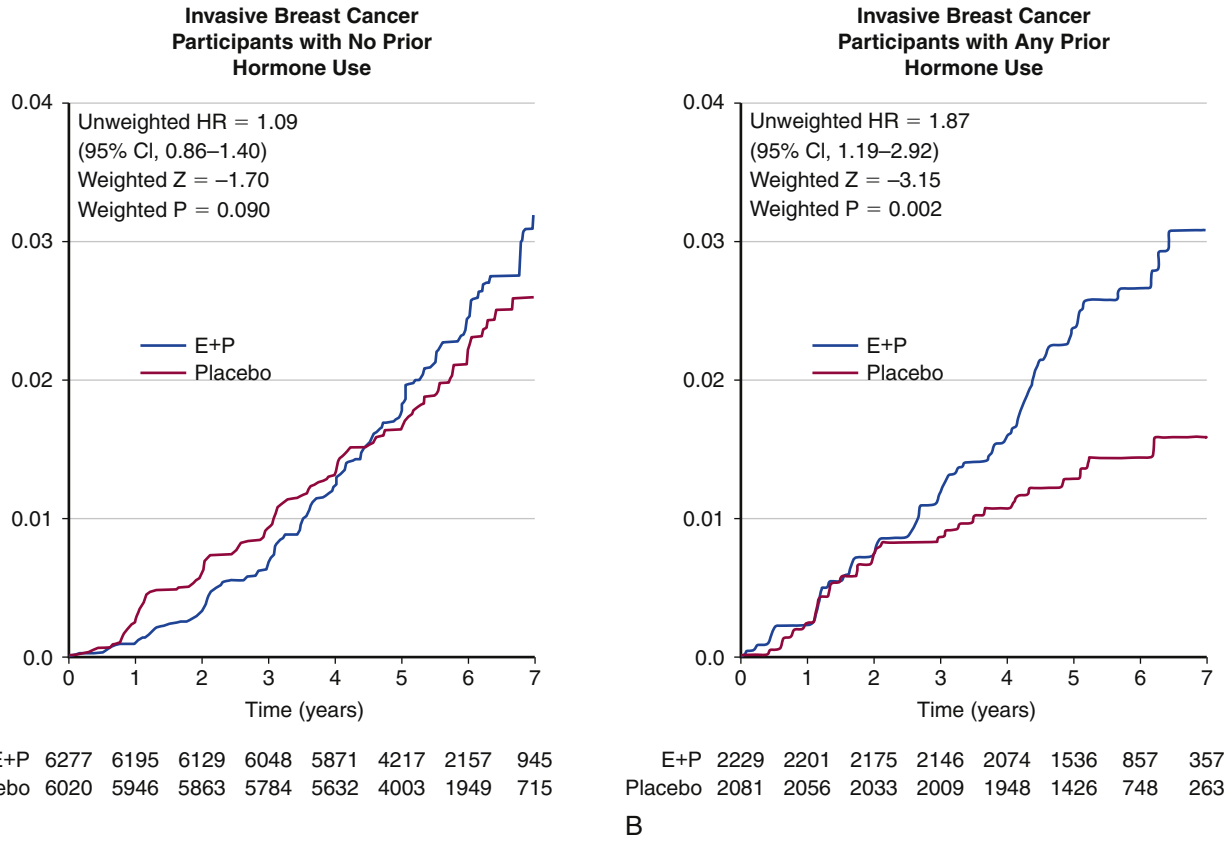


FIGURE 29.8 Data from the Women's Health Initiative trial comparing placebo to hormone therapy in a group of women who had never used hormone therapy previously **(A)** and in another group who did **(B).** HR, hazard ratio; CI, confidence intervals. *(Adapted from Anderson GL, Chlebowski RT, Rossouw JE, et al. Prior hormone therapy and breast cancer risk in the Women's Health Initiative randomized trial of estrogen plus progestin.* Maturitas *55[2]:103–115, 2006.)*

paucity of women with menopausal symptoms, (6) the fact that only 3.5% of the subjects were in the 50- to 54-year-old age group which represents women who usually consider initiation of MHT use and, (7) limitation of an increase in breast cancer to women who had previously used MHT and then stopped before randomization (Fig. 29.8A and B). Despite these issues, the WHI remains the only large randomized trial and the data on E+P and breast cancer risk appear valid. Further, the conclusions are congruent with the findings of the majority of properly conducted observational studies as reported in a recent meta-analysis.[45]

WHI E Alone Study

Another arm of the WHI compared placebo with conjugated equine estrogen alone in women who had previously undergone a total abdominal hysterectomy.[75] Three other, smaller randomized controlled trials (RCTs) of estrogen alone have also been reported in the past 10 years including the WEST (Women's Estrogen for Stroke Trial), ESPRIT (European/Australasian Stroke Prevention in Reversible Ischemia Trial), and EPAT (Estrogen in the Prevention of Atherosclerosis Trial) studies 1.[67] Data pooled from the four RCTs reported a paradoxical reduction in breast cancer risk (RR 0.79, CI 0.61 to 1.01) that approached but did not reach statistical significance. In the 11.8 year follow-up of the WHI E alone trial (i.e., 7 years of E alone and 4.8 years of subsequent observation), the reduction in breast cancer risk did reach statistical significance in the total group (RR 0.77, CI 0.62 to 0.95) and to an even greater extent in compliant patients (sensitivity analysis RR 0.69, CI 0.49 to 0.95) (Fig. 29.9).[76] The protective effect was confined to those without a family history of breast cancer or benign breast disease.[76]

The observational trials reporting an increased risk of breast cancer with long-term estrogen use and the WHI study reporting a reduced risk would seem paradoxical.[76-78] A plausible explanation for this discrepancy is the "Gap Time," the duration between onset of menopause and start of MHT. Women starting many years after onset of menopause (i.e., "long gap") experienced a significant reduction in breast cancer risk (RR 0.58, CI 0.36 to 0.93), whereas those starting immediately after menopause ("short gap") did not (RR 1.12, CI 0.39 to 3.21 reported initially and 0.89, CI 0.66 to 1.20 in a later analysis).[76,79] Data from the Nurses' Health Study suggest that the increased risk only occurs in women using estrogen alone for more than 10 years, particularly in lean women [i.e., body mass index (BMI) less than 25] (Fig. 29.10).[77,78] As discussed below, the reduction in risk in the "long gap" group may have resulted from a pro-apoptotic effect of estrogen.

An important issue is often overlooked in discussions of the WHI E alone trial. Forty-eight percent of women had received MHT previously but were eligible for study entry after a 3-month "wash out" period. Fifty-two percent had never taken menopausal hormone therapy and are termed "hormone naïve." Only the "hormone naïve group"

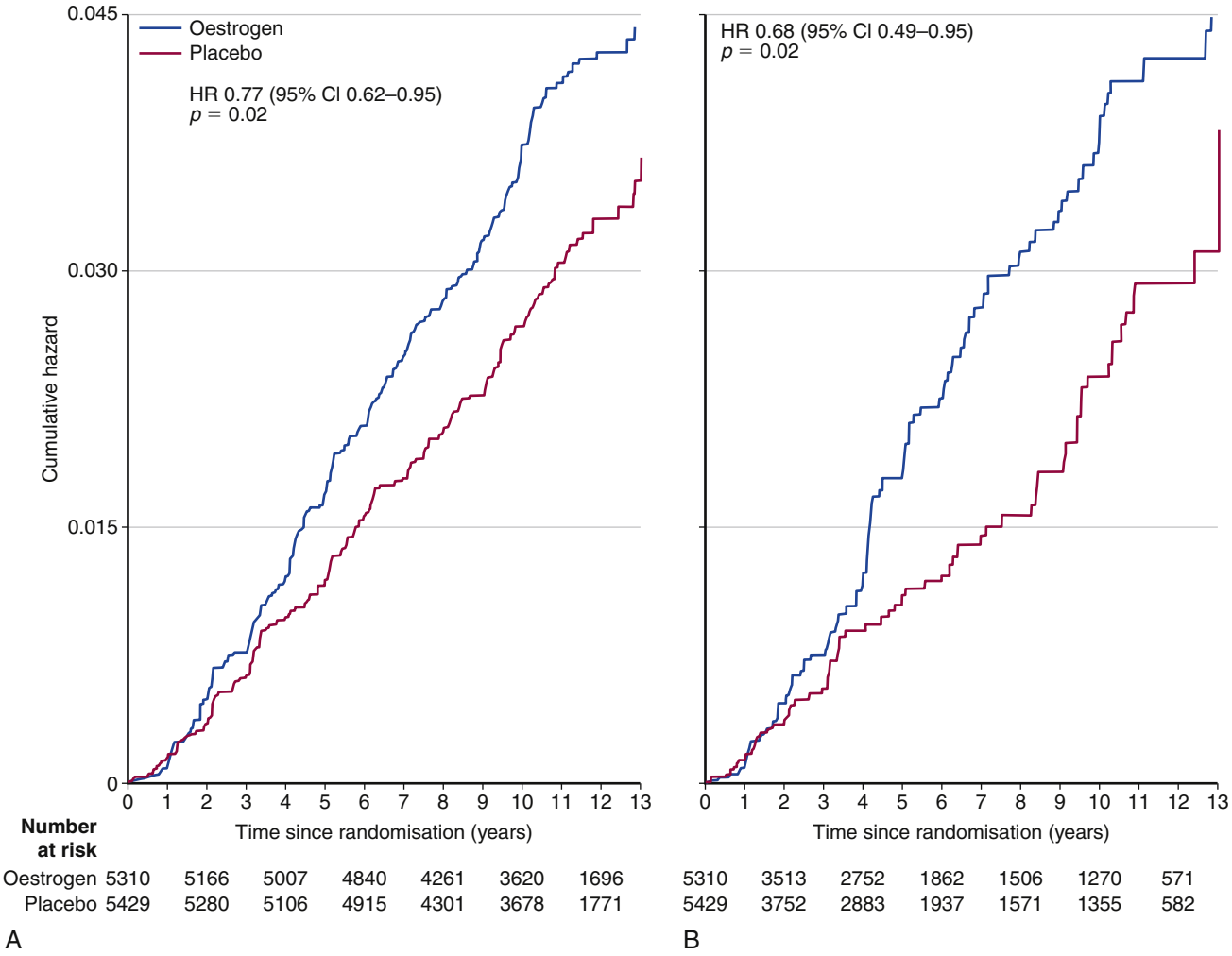


FIGURE 29.9 Kaplan–Meier estimates of cumulative hazards of invasive breast cancer in the WHI randomized trial of conjugated equine estrogen with the intention to treat principle **(A)** and with adjustments for adherence **(B).** WHI, Womens Health Initiative; HR, hazard ratio; CI, confidence intervals. *(Reproduced with permission from Anderson GL, Chlebowski RT, Aragaki AK, et al. Conjugated equine oestrogen and breast cancer incidence and mortality in postmenopausal women with hysterectomy: extended follow up of the Women's Health Initiative randomized placebo-controlled trial,* Lancet Oncology *13:476–486, 2012.)*

experienced a decrease in breast cancer risk (RR 0.65, CI 0.46 to 0.92). The 48% of women entering after cessation of MHT and a wash out period experienced no reduction (RR 1.02, CI 0.70 to 1.50), a response similar to that in the "short gap" patients.[69]

In the 11.8-year follow-up in the WHI, women receiving E alone experienced a lower overall mortality (30 deaths versus 50 deaths, RR 0.62 95%, CI 0.39 to 0.967) than those on the placebo arm.[76] Six deaths directly related to breast cancer were observed in the E alone group and 16 in the placebo arm (RR 0.37, CI 0.13 to 0.91). In contrast, the 11-year follow-up of the WHI E+P arm reported a slight increase in breast cancer death rate in the MHT arm (i.e., 0.63 extra deaths per 1000 women over 5 years). All-cause mortality was also slightly higher in the E+P group (1.07/1000/5 years), perhaps related to the average age of 63 in those entered.

Estrogen Paradox. Comparison of short-term estrogen use, which may decrease breast cancer risk by 30% (Fig. 29.9), with long-term administration for 10 to 20 years (Fig. 29.10), which appears to increase risk by 41% to 77%, suggests an "estrogen paradox."[77,78] One potential explanation for the "estrogen paradox" is that estradiol can exert two separate mechanistic effects based on timing: estradiol induced apoptosis *short-term* and the initiation-promotion of new cancers *long-term*. Recent studies demonstrate that estradiol can induce apoptosis in breast tumors that have been deprived of estradiol over a prolonged period.[80,81] In addition, high-dose estrogen administration to women with advanced breast cancer induces tumor regression in approximately 40% of ER positive patients.[82,83] At entry to the WHI estrogen alone study, the average age of the women was 63 years old, 12 years beyond the average age of menopause. Accordingly, women who had never previously taken hormone therapy were in a state of long-term estradiol deprivation, 12 years on average. If these patients had occult undiagnosed tumors (discussed later in the chapter), estrogen alone might have caused apoptotic cell death in them.

Occult Tumor Reservoir. Modeling studies examined the implications of the 7% of occult breast tumors found

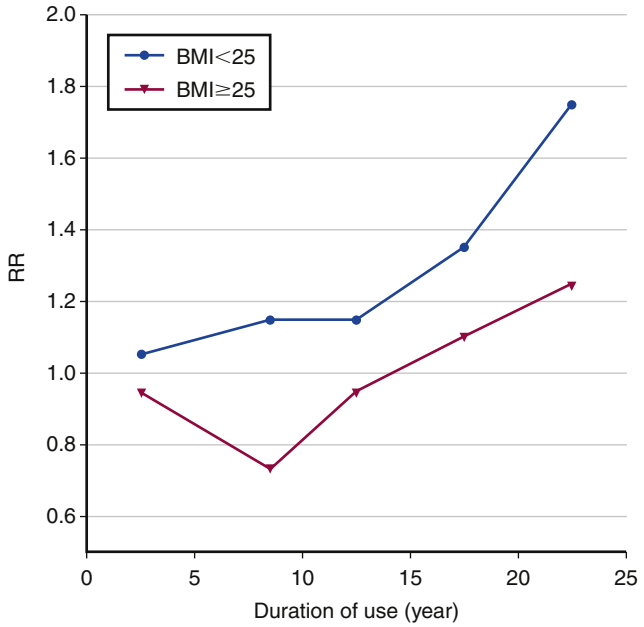


FIGURE 29.10 Relative risk (RR) of breast cancer as observed in the Nurses Health Study as a function of BMI. One line represents women with a BMI of less than 25 kg/m^2. And the other 25 kg/m^2 or greater. Each point on the line represents the mean RR of breast cancer for women taking E alone as MHT for 2 years to more than 20 years. MHT, menopausal hormone therapy. *(From Santen RJ, et al. Postmenopausal hormone therapy: an endocrine society scientific statement,* J Clin Endocrinol Metab *95[supplement 1]:S1–66, 2010.)*

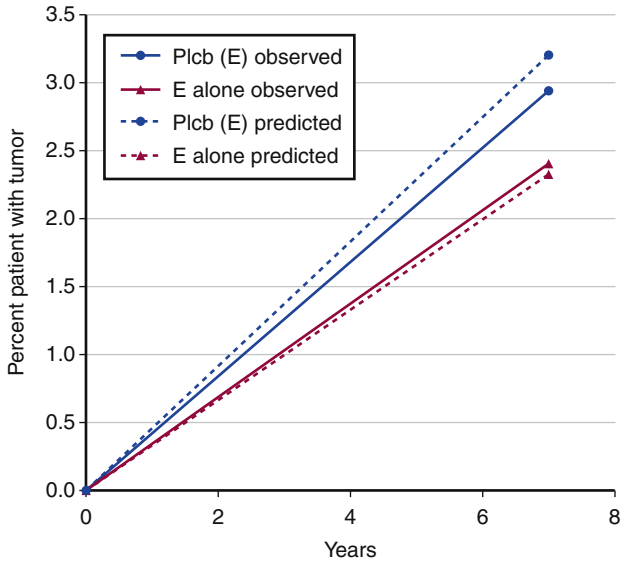


FIGURE 29.11 The incidence of breast tumors predicted in the placebo and hormone arms of the WHI E-alone trial over a 7.2-year period based on the OTG model. In comparison are shown the observed data in the placebo and hormone treated arms of the WHI E alone trial. WHI E, Womens Health Initiative study conjugated equine estrogen (E) alone. *(Reprinted with permission from Santen RJ, Yue W, Heitjan DF. Modeling of the growth kinetics of occult breast tumors: role in interpretation of studies of prevention and menopausal hormone therapy,* Cancer Epidemiol Biomarkers Prev, *in press 2012.)*

at autopsy. A pro-apoptotic effect of estradiol would be expected to reduce the size of those tumors such that they would not be detected over the 5 years of follow-up (Fig. 29.11). This could explain the significant reductions of breast cancer in subgroups of women in the WHI and Nurses Health studies. This short-term estrogen effect might then be superseded by a procarcinogenic effect of estradiol if the patients received this hormone over a prolonged period of up to 25 years (Fig. 29.10).[77,78,84] The "estrogen paradox" hypothesis is speculative and will require prospective, randomized, controlled trials over a longer period of time for confirmation.

Decreasing Breast Cancer Incidence in the United States

Breast cancer incidence decreased by 6.7% after the first publication of the WHI studies as documented by SEER population data in the United States.[85] ER positive tumors decreased by 14.7% (95%, CI 11.6 to 17.40) and ER negative by 1.7% (95%, CI −4.6 to +8.0). This finding was surprising since breast cancer incidence had been gradually increasing until 1999 and then plateaued until 2003. Ravdin and colleagues suggested that this might reflect the substantial cessation of use of MHT in women noted in 2002 and 2003 after publication of the WHI study.[85] A county by county analysis from California indicated that the steepest decline in breast cancer incidence (22.6%) occurred in counties with the greatest drop in MHT use, whereas the smallest decrease (8.8%) was observed in those with the lowest fall-off in use.[86] Decreased screening mammography could not explain these results since a large mammography screening study demonstrated nearly identical results.[87] One mechanistic explanation for these findings is that a subset of the occult breast cancers might regress in response to cessation of estrogen plus progestogen use and therefore not grow to the threshold of diagnosis over the next 2 to 3 years (Fig. 29.11). A study modeling the growth of occult tumors attributed the changes in breast cancer rate to an increase in doubling time from 200 days to 150 days in the women using E + P and a reduction in incidence rate upon cessation as doubling times fell back to 200 days.[63] If this explanation proves correct, one might expect a rebound in breast cancer incidence over the ensuing years.

Relative, Absolute, and Attributable Risks from MHT

A full understanding of the magnitude of risk from MHT requires knowledge of the precise meaning of statistical terms. Epidemiologists use *relative risk analysis* as a tool which provides substantial power to determine statistical significance of differences between groups. However, the term "relative risk" is misleading to patients since absolute or actual risk may be quite small in magnitude when relative risk is high. The lay press, patients, and many physicians confuse the terms relative, absolute, or attributable risk. The specific methods used to describe the endpoints reported in published studies of MHT confound the process of benefit and risk assessment. Various publications have described relative, absolute, or excess risks and examined

time frames ranging from 1 to 20 years. The initial WHI study and observational studies primarily reported relative risks (RR), a statistic relating the percent increment in an event in one population to that in another.[67,69] Other studies report absolute risk which describes the number of events in a population of given size. Relative risk provides meaningful information only when absolute risk is high but not when low. Excess (or attributable) risk subtracts the absolute risk in an untreated control group from that in a treated population. For an individual woman, excess risk or benefit is a more meaningful statistic since it represents an estimate of what she can expect to experience as a result of taking MHT.

Benign Breast Disease and Risk of Breast Cancer

Benign breast lesions with an enhanced rate of proliferation predict an increased incidence of breast cancer over time (Fig. 29.12).[88] Hyperplastic ductal lesions are often multicentric suggesting that some type of underlying abnormality is present which predisposes to such lesions. This has been called a "field defect" or more recently, a "mutator phenotype."[89] The multifocal nature of the associated benign hyperplastic lesions is most apparent in breast tissue from women with cancer. Examination of tissue adjacent to an invasive breast cancer or in the contralateral breast reveals one or more additional hyperplastic lesions in approximately 40% of patients.[90] The nature of the *field defect* or mutator phenotype has not been specifically identified but hypothetically could represent a single mutation of a gene controlling local estrogen production, cellular proliferation, DNA repair, metabolism of procarcinogens to carcinogens or other cellular events.

Available data suggest that benign breast lesions are frequently adenomas which then progress to frank neoplasms upon acquisition of additional mutations—80% to 90% of hyperplastic lesions contain DNA mutations similar to those in the contiguous tumors. Extensive molecular-genetic studies have now described progression of abnormalities in the spectrum of breast lesions.[89]

A major consideration for women who present with breast problems is whether they have a higher than normal risk of developing breast cancer. Certain breast lesions such as fibrocystic changes are associated with no increased risk of subsequent breast cancer unless a strong family history is present. Other lesions, characterized by the presence of increased cellular proliferation such as usual ductal hyperplasia, hyperplastic elongated lobular units, and solitary or multiple papillomas impart a small (i.e., less than twofold) relative increase in risk (Fig. 29.12).[88,89,91] With atypical ductal hyperplasia (ADH), the relative risk (RR) is 3.88 overall and 10.35 in those with multifocal (i.e., 3 or more) lesions with calcifications. In younger women, ADH imparts a 6.75 RR (i.e., women younger than 45 years old).[91] The relative risk of development of invasive cancer is increased tenfold to twelvefold when ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS) are present.

Proliferative lesions can progress to invasive cancer as evidenced by the increase in ipsilateral breast cancer in women harboring these lesions. However, risk of contralateral breast cancer is also increased when the period of observation extends over more than 10 years, suggesting an underlying defect affecting both breasts. The Mayo Clinic study of nearly 15,000 women established that only lesions with increased proliferation impart an increased risk of breast cancer unless a strong family history is present.[88] This clarified a prior study which did not distinguish proliferative from non-proliferative lesions and concluded that low category breast disease, including fibrocystic changes conveys an increased risk.[92] The anti-estrogen, tamoxifen, reduces the number of ADH lesions in high risk women and the risk of breast cancer.[42]

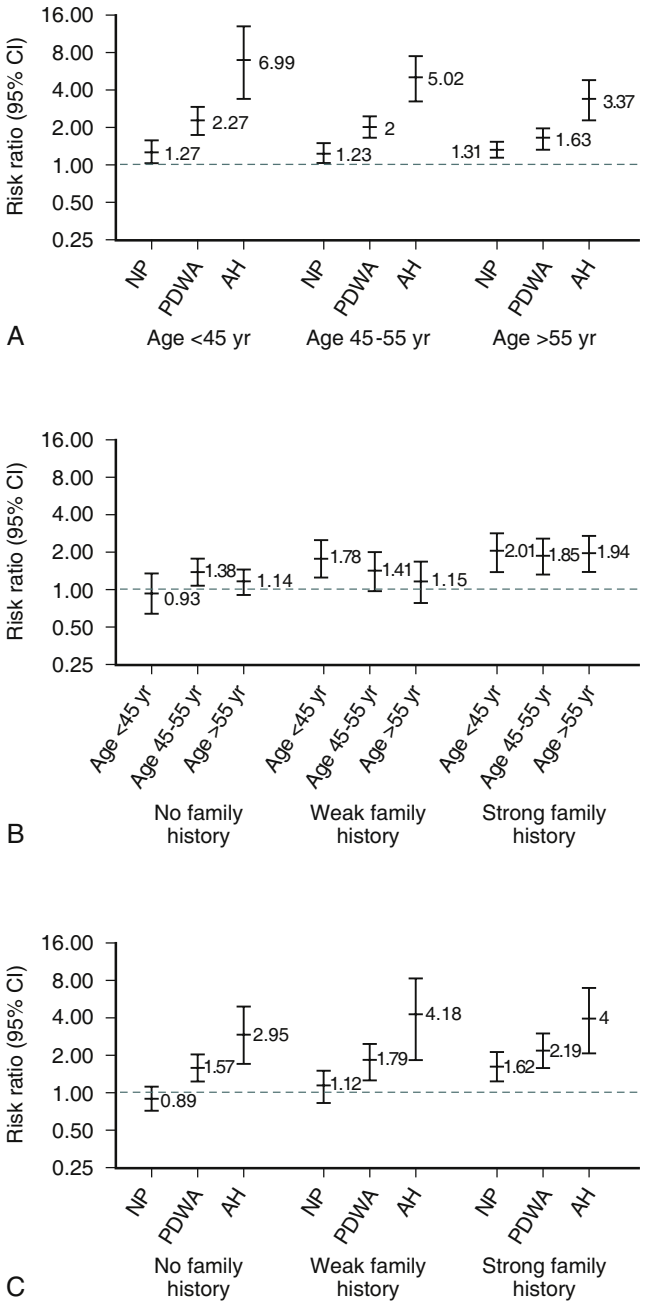


FIGURE 29.12 Risk of breast cancer related to various benign breast lesions and family history of breast cancer. AH, atypical hyperplasia; NP, nonproliferation; PDWA, proliferating duct without atypia *(From Hartman LC, Sellers TA, Frost MH, et al. Benign breast disease and the risk of breast cancer,* N Engl J Med *353[3]:229–237, 2005.)*

Estimating Breast Cancer Risk

To aid in assessing breast cancer risk, a questionnaire developed by Gail, utilizes answers to seven questions to calculate the 5-year and lifetime risk of developing breast cancer.[93] This model has recognized deficiencies in that it does not consider breast density, plasma estradiol levels, bone density, body mass index, weight gain in adulthood, second degree relatives with breast cancer, proliferative lesions of breast other than ADH, alcohol intake, or birth control pill and MHT use.[13] Nonetheless, several prospective studies validated the Gail model in high risk (National Surgical Adjuvant Breast Project [NSABP] prevention study) and in average risk women (the Nurses Health Study). The ratio of observed to expected cancers using this tool was 1.03 (95% confidence limits of 0.88 to 1.21) in the high risk patients and 0.94 (95%, CI 0.89 to 0.99) in average risk women and both were highly statistically significant. This risk tool is available as the "RISK DISK" from the National Cancer Institute. When family history is the primary predictor of breast cancer, the Claus model has been validated and widely applied.[93]

Newer models are being developed to enhance the power of risk prediction. Although only validated in one prospective study, the Tyrer/Cuzick model appeared to out-perform the Gail model in a population with a high familial breast cancer component.[94,95] This new model combines the factors utilized in the Claus and Gail models as well as a history of MHT use and appears quite promising as a new risk prediction tool. As mentioned above, addition of mammographic density to the Gail model improves risk prediction but to only a minor extent.[58,59,96] However, precise quantitation of breast density is infrequently utilized and a practical method (such as the "Risk Disk") is not yet available to integrate breast density into the Gail model to make the necessary calculations. Critical reviews of all of the various risk detection models are available.[95] It should be noted that analysis of data regarding occult, undiagnosed tumors leads to the conclusion that the breast cancer risk models are actually identifying women harboring occult, undiagnosed tumors rather than those at high risk of *de novo* tumors.[63]

Breast Cancer Risk and Clinical Decisions

Knowledge about underlying breast cancer risk influences advice given by healthcare providers and choices made by patients. Women known to be at high risk of breast cancer will frequently choose a surrogate for MHT (discussed later in the chapter) to treat menopausal symptoms. Those at low risk will usually chose estrogen or an estrogen and progestogen combination to relieve symptoms. Those at intermediate risk of breast cancer have several options including use of a selective estrogen receptor modulator (SERM), other alternatives to estrogen, watchful waiting, or MHT. The Endocrine Society has constructed a Menopause Map using these considerations to aid in the decision making process (http://www.hormone.org/menopausMAP/).

As a working guide, categories are arbitrarily defined based upon use of risk prediction models: with high risk ≥3% chance of breast cancer in 5 years; intermediate risk = 1.5% to 3% , and low risk ≤1.5%. Those classified as high risk include patients with a family history of breast cancer (particularly if associated with ovarian cancer), prior history of atypical hyperplasia (AH) or lobular carcinoma *in situ*, and age over 60 years old when combined with early menarche, late menopause, or first live birth. Intermediate risk patients have some risk factors but not others. Low risk patients are under 60 years of age; have a late onset of menarche, early menopause, early age of first live birth, and no family history of breast cancer; and lack predisposing breast lesions. Since no formal risk tool incorporates breast density, bone density, nor plasma estradiol levels into its assessment, physicians must take these factors into account in advising patients.

Prevention of Breast Cancer

Depending upon risk category, women may wish to take tamoxifen or raloxifene to prevent breast cancer. Both raloxifene and tamoxifen are now approved for this use in the United States. Evidence supporting anti-estrogens for prevention is substantial. A meta-analysis of the five large prevention trials demonstrated 42% reduction in breast cancer risk with tamoxifen when compared to placebo.[97] The most definitive trial, NSABP P-1, involved 13,388 women randomized to receive either placebo or 20 mg of tamoxifen daily[42] (Fig. 29.13). Eligibility into the study required an intermediate or high risk of developing breast cancer, defined in the study as a 1.67% or greater chance of developing a new breast cancer over a 5-year period. The rate of breast cancers in the tamoxifen group was reduced by 50% compared to the placebo group (Fig. 29.13). Considered separately, the risk of invasive breast cancer was reduced by 49% and noninvasive

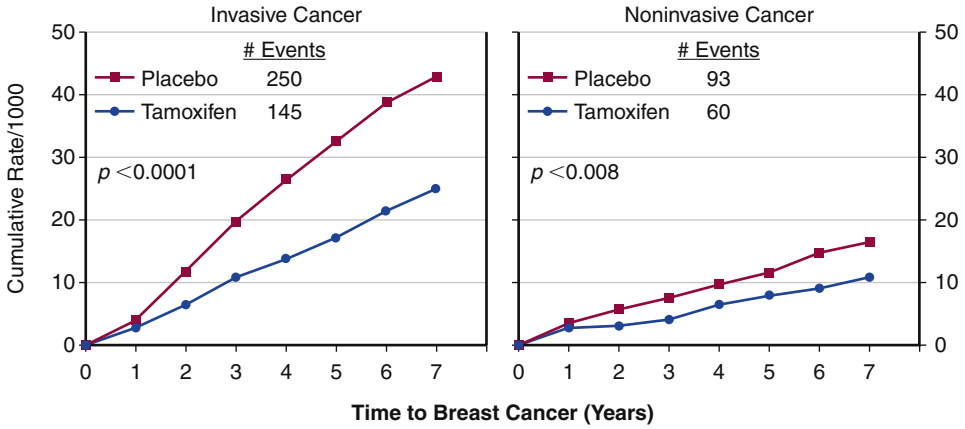


FIGURE 29.13 Results of the NSABP-P1 breast cancer prevention trial with tamoxifen versus placebo. *Left panel* is the effect of tamoxifen on invasive cancer and *right panel,* on noninvasive cancer. *(From Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study (per pub med),* J Natl Cancer Inst *97 [22]:1652–1662, 2005.)*

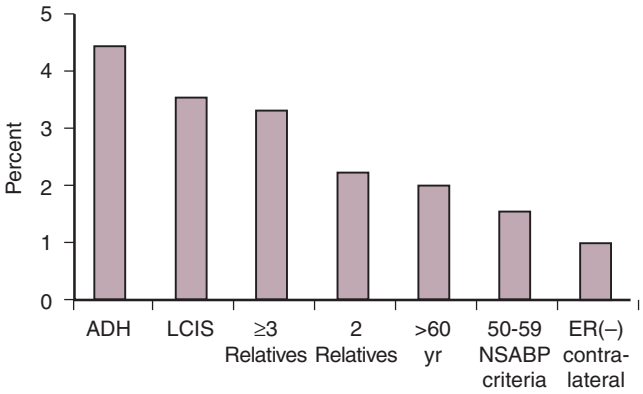


FIGURE 29.14 Absolute benefit from tamoxifen expressed as percentage of women whose breast cancer was prevented as a function of the underlying risk factor present. ADH, atypical ductal hyperplasia; ER, estrogen receptor; LCIS, lobular carcinoma *in situ;* NSABP, National Surgical Adjuvant Breast and Bowel Project. *(From Santen R. Endocrine-responsive cancer. In Kronenberg HM, Melmed S, Polonsky KS, Larsen PR [eds].* Williams Textbook of Endocrinology, *11th ed., Philadelphia, Saunders, 2008, Figure 42.88.)*

by 50%. This effect occurred in women of all ages studied (younger than 49 years old, 50 to 59 years old, 60 to 69 years old, and older than 70 years old) and in those with LCIS, ADH, and a family history of breast cancer. The benefits of tamoxifen related to the underlying risk of breast cancer and the specific risk factor present (Fig. 29.14).

Tamoxifen for treatment of breast cancer is considered well tolerated and safe. However, for use in otherwise normal women, infrequent side effects and toxicity become more important. Up to 40% of women starting on tamoxifen do not continue it because of perceived side effects including depression and mood changes.[98-102] A majority of eligible women choose not to start tamoxifen for breast cancer prevention.[103]

To compare risks and benefits in a meaningful way, the NSABP data are expressed as the number of women per 100 with a specific benefit or adverse event after 5 years of study. With respect to benefits, new invasive breast cancers were prevented in 1.7 of 100 women, noninvasive cancers in 0.67, and bone fractures in 0.50 for a total of 2.87 in 100 women benefitted after 5 years. Risks of tamoxifen are primarily related to its estrogenic effects on uterus, a prothrombotic effect, and adverse effects on the lens of the eye. To validly estimate actual risks to a patient, one must correct for the underlying risks in the population under study. For that reason, analysis of adverse events includes determination of *attributable risk* and involves subtracting the underlying rate from the total observed on tamoxifen. This indicates an excess of 1.5 of 100 women who developed cataracts, 0.69 with endometrial cancer (nearly exclusively in postmenopausal women), 0.27 with CVA, 0.25 with deep venous thrombosis, and 0.23 with pulmonary embolus or a total of 2.94 per 100.[104] The new endometrial cancers were predominantly Stage I and pulmonary emboli nonfatal. Neither risk nor benefit was observed regarding cardiovascular effects but the number of patients was not sufficient for statistical significance.

The increased incidence of uterine cancer in patients on tamoxifen presents a clinical management problem. Substantial study has examined how best to detect this cancer early.[105] Transvaginal ultrasound was initially proposed as a means of assessing both endometrial hyperplasia, as a precursor lesion, and early cancer. A prospective study demonstrated a mean increase in endometrial thickness from 3.5 ±1.1 to 9.2 ±5.1 mm in response to 5 years of tamoxifen. In 20% of women, endometrial thickness exceeded 10 mm or appeared suspicious for neoplasm. In this subset of women, biopsies revealed atrophy in 73%, polyps in 17%, hyperplasia in 7.7%, but endometrial cancer in only one patient.[106] This and other studies concluded that endometrial thickness does not usually indicate hyperplasia but rather, the presence of edema and dilated myometrial glands. It should be noted that nonprospective studies report a higher prevalence of benign polyps (5% to 55%) and hyperplasia (8% to 16%) but these are likely to be overestimates from selection bias.[107,108] Based upon these studies, recommendations for screening include yearly gynecological exam and endometrial sampling and ultrasound only for those with signs of abnormal vaginal bleeding.[105,106]

Another SERM, raloxifene, appears to prevent breast cancer in postmenopausal women without increasing the risk of endometrial cancer. After initial positive findings regarding raloxifene, the study of tamoxifen and raloxifen (STAR) trial compared raloxifene with tamoxifen in a "head to head" prevention trial in 18,000 postmenopausal women with a predicted risk greater than 1.67% at 5 years.[43,109] Both agents prevented invasive breast cancer by an estimated 50% but raloxifene exhibited a superior toxicity profile with 30% fewer thrombo-embolic events ($p = 0.01$); 38% fewer endometrial cancers ($p = 0.07$); 21% fewer cataracts ($p = 0.002$); an 84% reduction in uterine hyperplasia ($p < 0.01$), and 55% fewer unplanned hysterectomies. No differences between agents were observed in cardiovascular events, strokes, or fracture rates. A surprising finding was that raloxifene did not appear to prevent noninvasive breast cancer whereas tamoxifen has previously been shown to do so.[43] Longer term follow-up of the STAR trial demonstrated that tamoxifen was superior to raloxifene in preventing invasive breast cancer as well (Fig. 29.15).[110] This later study clearly demonstrates the superiority of tamoxifen over raloxifene for breast cancer prevention albeit with a higher risk of endometrial cancer with tamoxifen. Two other SERMs, arzoxifene and lasofoxifene, also prevent breast cancer but have not been approved for this indication.[111,112]

Trials of the aromatase inhibitors in the adjuvant setting uniformly demonstrate a greater reduction of contralateral cancers with the aromatase inhibitors (AIs) than with tamoxifen and suggest that AIs would be superior for prevention as well.[113,114] A recent study reported the prevention of breast cancer in postmenopausal women at high risk of breast cancer (i.e., Gail scores less than 1.66) with the steroidal AI, exemestane.[115] A total of 4560 women were randomized to the AI or placebo. At a median follow-up of 35 months, a 65% reduction in breast cancer was observed with the AI (HR 0.35 95%, CI 0.18 to 0.70). Minimal quality of life differences were observed. This agent is not currently approved for use in prevention.

Guidelines for Breast Cancer Prevention

Premenopausal women with a 5-year risk of breast cancer greater than 1.67% over 5 years are candidates for

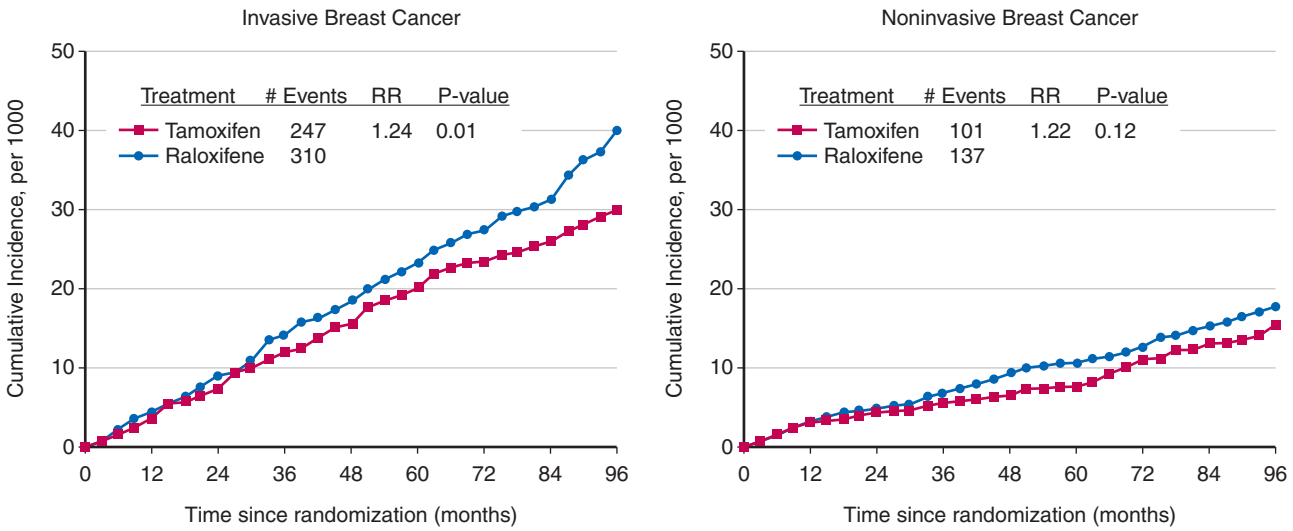


FIGURE 29.15 Comparison of tamoxifen with raloxifene for prevention of breast cancer as reported in the STAR trial update. *Left panel,* Invasive breast cancer. *Right panel,* Noninvasive breast cancer. *(From Vogel VG, Costantino JP, et al. Update of the National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) P-2 Trial,* Cancer Prevention Research *3(6):696–706, 2010.)*

tamoxifen unless they are at increased risk for DVT or pulmonary emboli. Post-menopausal women with similar breast cancer risk are candidates for tamoxifen if they no longer have a uterus and lack a predisposing risk for DVT or pulmonary emboli. Raloxifene may be preferred over tamoxifen in women with a uterus. Lesser efficacy must be balanced against improved uterine safety when raloxifene is being considered. The decision to take tamoxifen or raloxifene should be made by the patient in partnership with her healthcare provider and based upon a full discussion of individual risks and benefits expressed in absolute and not relative terms.[116,117] Prevention strategies for women carrying BrCA1 or BrCA2 mutations often involve more aggressive steps such as bilateral oophorectomy or mastectomy but tamoxifen may also be effective in these patients.[19,118]

Critical Assessment of Breast Cancer Prevention Strategies

Estimates indicate that between 20 and 100 women (depending upon underlying risk) need to be treated with tamoxifen to prevent one breast cancer. Available data demonstrate only a reduction in newly diagnosed breast cancers but effects on overall survival are not yet known. Recent modeling data suggest that the primary effect of tamoxifen is to reduce the growth of pre-existing, occult tumors and not to prevent *de novo* tumors.[63] Tumors whose growth was blocked by tamoxifen during 5 years of administration might re-grow later. For this reason, data on overall survival (to be available from the ongoing IBIS 1 trial) will be critically important.[97]

Future studies will need to utilize additional factors to select women at higher risk of developing breast cancer.[13] A study of raloxifene examined women with factors suggesting high exposure to estrogen long-term (i.e., increased bone mineral density, high BMI and high plasma estradiol levels). Results indicated that raloxifene was more effective in preventing breast cancer in women with high than with low long-term estrogen exposure.[119] A critical need at present is to develop a more powerful breast cancer risk model that incorporates genetic factors, breast density, levels of estrogen and its metabolites, history of fracture, waist hip ratio, and obesity as well as the factors utilized in the Gail and Claus risks prediction models.[13]

Hormonal Treatment of Breast Cancer: Background

Biological Subtypes of Breast Cancer

A major advance in the field of breast cancer research utilizes cDNA array analysis to assess the molecular and biological properties of individual tumors.[120,121] This methodology enabled a new biological classification of breast cancer based upon the degree of expression of key discriminant genes. Five common subtypes of breast cancer are generally agreed upon including luminal A, luminal B , basal, ERB-B2+, and tumors with normal breast gene expression. As shown in Figure 29.16, each subtype has a different prognosis. Data from separate molecular signature methods are largely concordant.[122-126] Additional subtypes such as Claudin low are being identified.

Methods such as Blue Print are being developed to limit the number of genes to be measured to classify subsets of tumors.[127] The differential biological properties, responses to therapy, and survival rates which characterize each of these subtypes justify the conclusion that individual breast cancers represent at least five and probably more disease entities. On a practical basis, cDNA array data are not always available clinically and testing is expensive. For these reasons, recent emphasis has focused on approximating the biological subtypes by immuno-histochemical methodology. Table 29.2 provides criteria for determining subtypes based on ER, PgR, HER-2, and Ki67 measurements.[128]

Stem Cell Composition

Emerging data suggest that tumor stem cells represent an important component of the cellular composition of mammary tumors.[129] With continuing potential for replication,

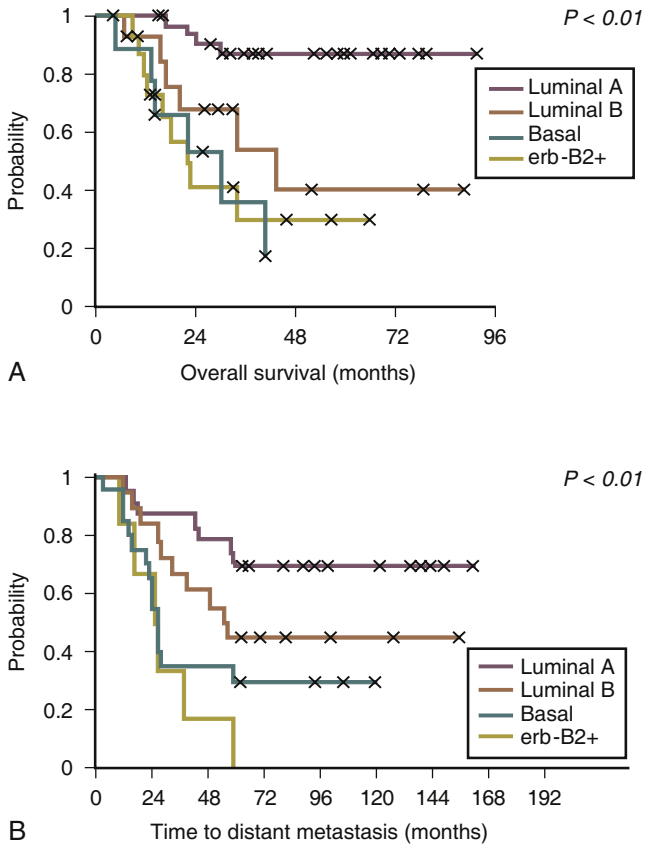


FIGURE 29.16 cDNA analysis of breast cancer tissues with categorization into Luminal A, Luminal B, Basal, and ERBB2 and the probability of overall survival and time to distant metastasis. The normal subtype is not shown. Luminal A, Luminal B, Basal, ERBB2, subtypes of breast cancer based on molecular signatures. *(Data reproduced from Sotiriou C, Ney SY, McShane LM, et al. Breast cancer classification and prognosis based on gene expression profiles from a population-based study,* Proc Natl Acad Sci USA *100(18):10393–10398, 2003.)*

stem cells may repopulate tumors after the more differentiated cells are destroyed by endocrine or chemotherapy. Tumor stem cells appear to have enhanced migratory and invasive potential and could play a major role in metastasis and colonization of tumors at distant sites.[1] Major effort is currently being directed toward stem cell targeted therapies to eliminate the ability to self-propagate.[130,131]

Prognostic Factors

Approach to the patient with breast cancer requires knowledge of the degree of aggressiveness and natural history of the type of tumor present. Figure 29.17 illustrates the prognostic power of various biological factors by comparing the effects of individual parameters on 5-year survival. The most powerful clinical and pathologically based prognostic parameters include nodal status, tumor grade and size, proliferative indices, and ERα content. This information can be integrated and used to determine prognosis during follow-up. A web based tool called adjuvant online (adjuvantonline.com) has been developed to calculate prognosis in individual women and to guide patients and their physicians in the choice of various therapeutic options.[132,133]

Other prognostic factors include HER2/neu content, degree of aneuploidy, and expression of certain oncogenes

Table 29.2 Surrogate Definitions of Intrinsic Subtypes of Breast Cancer

Intrinsic subtype	Clinico-pathological definition
Luminal A	Luminal A ER and/or PgR positive HER2 negative Ki-67 low (<14%)
Luminal B	Luminal B (HER2 negative) ER and/or PgR positive HER2 negative Ki-67 high Luminal B (HER2 positive) ER and/or PgR positive Any Ki-67 HER2 over-expressed or amplified
Erb-B2 over-expression	HER2 positive (nonluminal) HER2 over-expressed or amplified ER and PgR absent
Basal-like	Triple negative (ductal) ER and PgR absent HER2 negative

ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor-2; Ki-67, name of monoclonal antibody to detect proliferation. Named after the city of Kiel, Germany.

or coactivators such as cyclin-D1, A1B1, MAP kinase, Ras, human epidermal growth factor receptor (HER) III, and IV, heregulin, c-Src, 3-hydroxy-3methyl-glutaryl-CoA (HMG-CoA) reductase and ornithine decarboxylase (ODC) levels.[42,134] ERβ may also serve as a prognostic marker but interpretation is confounded by the presence of five ERβ isoforms and only ERβ 1 and 2 have been extensively studied.[135-139]

A risk score incorporating many of the above factors provides a more powerful means of assessing aggressiveness than use of single components. For example, a commercially available method, OncotypeDx^{R}, assigns a relative weight to the expression of each of 21 genes quantitated by polymerase chain reaction (PCR).[123,140-142] Women with node negative, ER + breast cancers are categorized into groups with low, intermediate, or high risk of recurrence. As evidence of its biological significance, the low-risk group experienced a 6.8% recurrence rate at 10 years versus 30.5% in the high-risk group. When patient age and tumor size are added to the model, only the cDNA score remains statistically significant upon multivariate analysis. The Mammoprint method is similar and involves the expression of 70 genes.[143,144] Both the Oncogene Dx^{R} and Mammoprint^{R} methods have been FDA approved in the United States and employed clinically. Other methodologies are being actively studied but are as yet not ready for clinical use. One involves measurement of circulating tumor cells in the blood of patients with breast cancer and the other assesses micrometastases in lymph nodes and bone marrow.[134,145]

Treatment of Established Breast Cancers

In the United States in 2011, 229,060 new cases of breast cancer were diagnosed and 39,920 died of this disease.[146] One approach to improve patient outcome is earlier diagnosis. Mammographic screening and digital mammography have increased the sensitivity of detecting early disease.[147]

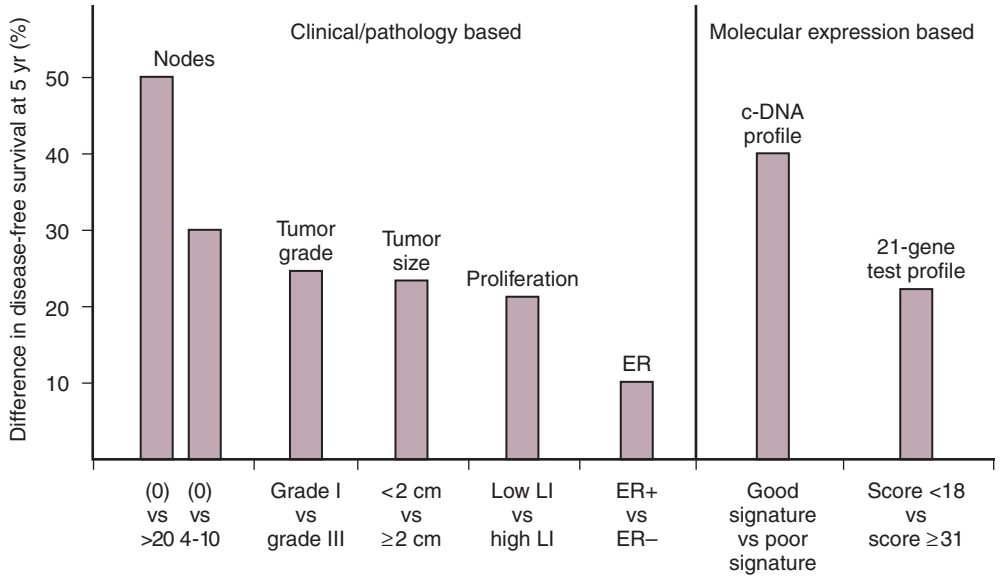


FIGURE 29.17 The prognostic value of several parameters related to patients with an initial diagnosis of breast cancer. All values are presented as the percent difference in disease free survival at the 5-year interval. The method of presentation allows one to determine the increased numbers of women per 100 who would be alive without recurrence at 5 years if they have a favorable prognostic factor compared with those with an unfavorable factor. ER, estrogen receptor; LI, labeling index. *(From Santen R. Endocrine responsive cancer. In Kronenberg HM, Melmed S, Polonsky KS, Larsen PR [eds]. Wiliams Textbook of Endocrinology, 11th ed, Philadelphia, WB Saunders, 2008.)*

Tumors generally must exceed 2 cm to be detected by palpation versus 1.1 cm by mammography and less than 1 cm for MRI. Earlier detection and use of adjuvant therapy likely explain the 12% decline in breast cancer death rate in the United States and Western Europe over the past 10 years. Adjuvant hormonal therapy is effective and associated with minimal side effects and toxicity compared to chemotherapy.

Hormones and Tumor Growth

The mechanisms whereby estradiol stimulates breast cancer growth are complex and involve direct regulation of genes involved in stimulation of proliferation and blockade of apoptosis. In addition, induction of growth factors through secondary actions and cross talk between growth factor and estrogen pathways at both upstream and downstream levels contribute to tumor growth.[148,149] Membrane initiated, extra-nuclear effects of estradiol on growth factor mediated mitogenic pathways transduce these signals through nuclear actions.[48] Hormonal treatment strategies utilize agents that abrogate the effects of estrogen on these extra-nuclear and nuclear pathways and thus inhibit growth and induce apoptosis.

Hormonal Therapies

Anti-estrogens, blockade of estrogen synthesis with aromatase inhibitors, suppression of ovarian function with GnRH analogues or use of additive androgens, progestins, and estrogens all represent proven hormonal therapy.[150] Patients who initially respond to hormonal therapy eventually relapse but frequently respond subsequently to second or third line therapies. The responses to sequential hormonal therapies suggest an adaptive process whereby tumors do not become totally resistant to hormonal therapy but develop a transitional state during which alternative means of blocking hormonal pathways causes tumor regression. As an example, patients relapsing following oophorectomy or tamoxifen treatment commonly respond secondarily to inhibitors of estrogen production (aromatase inhibitors).[150]

Predictive Factors

Hormone Receptors

Measurement of ERα and PR receptors provides the most robust means of predicting who will respond initially to hormonal therapy. Approximately 70% of breast tumors in postmenopausal women are ERα positive and 50% in premenopausal. Fifty to 75% of patients with ER+ and PR+ tumors respond to hormonal therapy versus 30% to 50% with ER+ and PR− tumors. Absence of ERα in the tumor predicts that fewer than 5% to 10% of women will experience benefit and, if both ERα and PR are negative, an even lower percent.[150]

With prior use of quantitative tissue binding assays for ER, the percentage of patients responding increased as a function of ER concentration.[150] As an example, responses to tamoxifen increased as a function of ER level with a statistically significant ($p = 0.002$) positive trend.[151] Data from the Oncotype DX assay provide confirmation regarding the role of ER quantitation. The absolute level of ER mRNA, which varies over more than a 300-fold range, correlates linearly with improved responses to hormone therapy. These data suggest that the concentration of receptor provides more useful information than using the standard dichotomized, negative or positive designation.[152] Quantitative receptor measurements also provide additional information about chemotherapy responsiveness as initially

suggested by Lippmann and colleagues. Low ERα levels, in addition to predicting nonresponsiveness to hormonal therapy, also predict a high likelihood of responding to chemotherapy.[153,154]

Standard clinical determinations of receptor content involve immuno-histochemical (IHC) analysis which has largely replaced ligand binding assays. Current criteria require that only 1% of cells be ERα positive to score the tumor ER+.[155] An alternative approach is to semi-quantitate the level of ER in tumors using the Allred scoring system which classifies tumors on the basis of proportion of cells positive and intensity of staining using a scale of 0 to 8.[156] As opposed to the ligand binding assays, IHC techniques detect only ERα but not ERβ. While ERβ may confer prognostic and predictive information, data are not yet sufficient to recommend its routine clinical assessment.[136,157]

An initial problem with ERβ measurements was the variable reliability of specific antibodies and differences in methodology but these problems have been largely solved.[138] However, the presence of five isoforms with differing biological import provide additional difficulties in the interpretation of ERβ data.[135,136] In addition, certain tumors make ERβ variant proteins which can hetero-dimerize with full length ERα or ERβ and exert dominant negative effects.[158] Additional studies are needed before ERβ measurements will be considered clinically useful.

Biological Parameters

Studies over the past several decades provide strong evidence that biological parameters also predict the potential effectiveness of hormonal therapies. Older age, long disease free survival, high degree of tumor differentiation, metastases to bone and skin and prior response to endocrine therapy predict a higher likelihood of responses to hormonal therapy.[150]

Signaling Pathway Signatures

Factors suggested as being predictive of responses to hormonal therapy but not fully validated for clinical use include ER coactivators and corepressors, members of the EFG-R family, and protein profiling signatures.[159,160] Gene array signatures have also been suggested as possible predictive tools but are not fully validated. The OncotypeDx^{R} risk score showed a nonsignificant ($p = 0.06$) trend as a predictive marker with low-risk score patients deriving substantial benefit from tamoxifen and those with high-risk scores minimal responses.[159]

Hormone Resistance

***De Novo* Resistance.** Approximately 40% of women with ER+ tumors fail to respond to initial hormonal therapy. Several new techniques have been employed to identify women with *de novo* resistance and to understand the mechanisms involved. DNA expression signatures have provided a degree of insight but these are not deemed sufficiently established at present to apply clinically[128,161-164] (NCCN Breast Cancer Panel, 2006 http://www.nccn.org/).

Acquired Resistance. Women who initially respond to hormonal therapy nearly uniformly relapse after an average of 12 to 18 months due to acquired resistance.[150] Recent concepts hold that breast cancer cells are inherently plastic and capable of adapting to any given hormonal therapy with utilization of one of several alternative signaling pathways. Sequential second and third line hormonal therapies can then block these alternative pathways and induce additional responses.[165-171]

Multiple individual factors and integrative pathways may be involved in resistance in individual tumors as outlined in Table 29.3 and described in detail in several reviews.[148,149,172,173] Most information has been gained from study of breast cancer cells grown *in vitro* or as xenografts and exposed long term to tamoxifen or estrogen deprivation. Grouped according to the general mechanistic categories involved in acquired resistance, these include (1) loss of ERα or its functionality, (2) conversion of tamoxifen from an estrogen antagonist to agonist, (3) enhanced extra-nuclear effects of ERα, (4) up-regulation of non-ERα-mediated cell proliferation pathways, (5) signaling pathway modifications resulting in reduced apoptosis, (6) changes in the breast tumor microenvironment, (7) development of hypersensitivity to estrogen, and (8) miscellaneous metabolical and cellular changes. As these individual components frequently work in concert through pathway cross-talk, research emphasis currently focuses on integrative signatures to assess these interactions and to determine which pathways predominate.

A few specific examples from recent data highlight the complexity of changes resulting in acquired resistance. Truncation of the estrogen receptor into a 36 Kd isoform results in an anti-estrogenic ER.[174] Enhanced expression of the ER coactivator AIB1 results in tamoxifen resistance.[148] Up-regulation of signaling pathways involving MAP kinase, PI-3 kinase (also named protein kinase B), AKT, mammalian target of rapamycin (mTOR), vascular endothelial growth factor (VEGF), HER-2, and fibroblast growth factor (FGF) result in proliferative and angiogenesis signals independent of ER alpha. Scaffold proteins such as 14-3-3-ξ, when up-regulated, result in coordinated overexpression of the MAP-kinase, EGF-R, AKT, and HER-2 pathways.[175] Alterations of BCL Xl, caspase 9, BIK, BCL-2, and Bcl-2 homologous killer (BAK) can dampen apoptosis and thus add to increased tumor cell survival. Tumors adapting to estrogen deprivation therapy become hypersensitive to lower amounts of circulating estrogen which then "drive" cell proliferation.[176-178] Alterations of the microenvironment mediated by the integrins, Crk-associated substrate (CAS), adhesions molecule, and immune cell function can contribute to hormone resistance.[148]

Concepts regarding resistance developed from experiments in breast cancer cell cultures and xenografts frequently translate into clinically important concepts. As examples, HER-2 and AIB1 over-expressing tumors exhibit resistance to tamoxifen in patients.[148] The estrogen hypersensitivity concept likely explains secondary responses to aromatase inhibitors in patients relapsing after oophorectomy since the third generation aromatase inhibitors lower estradiol levels substantially below these achieved by oophorectomy.[179] Addition of growth pathway inhibitors to hormonal therapies, as a means to counteract resistance, provide a degree of additional clinical benefit as reviewed below.

Table 29.3 Acquired Hormone Resistance

General Category	Specific Alteration
Loss of ERα or loss of ERα function	• DNA methylation • ↑ Receptor degradation • Alternative splicing with ERα 36 receptor with antagonistic properties • ↑ Micro RNA regulation ERα • Corepressor and coactivator modulations • Antagonism of ERα by ERβ variants • Altered FoxA1 pioneer factor function
Enhanced estrogen agonistic activity of tamoxifen	• ↑ AIB1 co-activator function • ↓ NCOR corepressor function • ↑ PELP • ↑ HER-2 • ↑ IGF-1/IGF-1 R • ↑ EGF-R • ↓ miR 342 • ↑ 14-3-3
Enhanced non-genomic actions of ERα; enhanced cell proliferation through non-ERα mediated mechanisms	• ↑ Growth factor mediated pathways (EGF, IGF-1/insulin, PAK-1, IKK, SRC, c-myc, AP-1, NFκβ, PTEN mutations, HER-2, MAP-K, PI-3K/AKT, mTOR, VEGf, FGF) • ↑ Cell cycle mediators (cyclin D1, cyclin E), NKκβ • ↓ Cell cycle repressors (p21, p27, RB, micro RNA 221, 222)
Reduced apoptosis	• ↓ BCLXL, ↓ caspase 9, ↓ BIK, ↓ BAK, ↓ BCL-2
Hypersensitivity to estradiol	• ↑ ERα concentration • ↑ MAP-K • ↑ PI-3-K • ↑ mTOR • ↑ HER-2
Change in micro environment	• Altered integrin function • Altered stromal, immune cell function • Altered cellular matrix adhesion molecules • CAS activation
Alteration of metabolical pathways	• ↓ HMG-CoA reductase

ER, estrogen receptor; NCOR, nuclear receptor corepressor; PELP, proline, glutamic acid, leucine rich protein; HER-2, human epidermal growth factor receptor-2; IGF-1, insulin like growth factor-1; IGF-1R, insulin like growth factor-1 receptor; EGF-R, epidermal growth factor receptor; MIR 342, micro RNA numner 342; 14-3-33, name of scaffold protein; PAK-1, P21 activated kinase 1; IKK, inhibitor of nuclear factor K_β kinase; SRC, short for sarcoma, proto-oncogene encoding a tyrosine kinase; C-MYC, a regulator gene that codes for a transcription factor; AP-1, adaptor protein 1; NFKB, nuclear factor kappa light chain enhancer of activated B cells; PTEN, phosphatase and tensin homolog; HER-2, human epidermal growth factor receptor-2; MAP-K, mitogen activated protein kinase; PI-3K, phosphoinositol-3-kinase; AKT, also known as protein kinase β; mTOR, mammalian target of rapamycin; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; RB, retinoblastoma protein; P21,27, cell cycle inhibitors named for molecular weight; Cyclin E, cell cycle stimulating protein variety E; BCL-XL, BAK, BCL-2, BIK, family of evolutionary proteins; HMG-CoA reductase, 3-hyroxy-3 methyl-glutaryl-CoA reductase.

Hormonal Therapies for Breast Cancer

Background

Decisions regarding hormonal therapy must take into account age and menopausal status; tumor size, invasiveness, biological aggressiveness, receptor content, and metastatic sites; and prior responses to hormone therapy. *In situ* cancer is viewed differently than invasive. The treatment of ductal carcinoma *in situ* involves local excision with preservation of wide tumor free margins and radiotherapy for selected lesions. Hormonal therapy, used for ER+ lesions, reduces local recurrences and prevents new ipsilateral and contralateral tumors.

Patients with invasive breast cancer undergo lumpectomy or mastectomy and, if warranted, radiotherapy. Most patients then receive adjuvant therapy to destroy occult cancer cells at loco-regional and distant metastatic sites. Adjuvant therapy refers to the treatment used after breast surgery and before signs of recurrent disease in an attempt to eradicate micro-metastases or occult, non-resected local tumor cells. Upon tumor recurrence, the disease is called advanced and first, second, and third line hormonal or chemotherapies are then utilized in sequence. A recently introduced approach, neo-adjuvant hormonal therapy, involves use of hormonal therapy for 3 to 4 months prior to surgery for patients with large tumors. The goal is to reduce the size of the lesion so as to make lumpectomy a feasible alternative to mastectomy.

Mechanisms and Efficacy

The molecular mechanisms underlying the use of hormonal therapy and the efficacy of specific approaches will be reviewed in the next section. Practical recommendations about specific sequences based on current guidelines are then covered in a later section of this chapter.

Tamoxifen

Mechanisms of Action

In premenopausal women, tamoxifen exerts anti-estrogenic effects on breast which are similar to those induced by surgical removal of the ovaries with resultant estrogen deprivation. The estrogen agonistic properties of tamoxifen in premenopaual women are minimal. In postmenopausal women, tamoxifen acts an anti-estrogen on breast tissue but exerts estrogen agonistic effects on uterus, vagina, bone, pituitary, and liver.[150,180-182] For this reason, tamoxifen is classified as a selective estrogen receptor modulator or SERM as is its close relative, toremifene. Attempts to determine the

mechanistic reasons for the divergent actions of tamoxifen on various tissues in premenopausal and postmenopausal women have led to a better understanding of the complexity of ER mediated transcriptional regulation. (Reviewed briefly here and covered in more detail in Chapter 11.)

Tamoxifen binds to ERα in its ligand binding domain (AF 2) which then facilitates the binding of the anti-estrogen-ER complex to specific estrogen response elements on DNA (EREs).[183] Conformational changes in the ER binding pocket at helix 12 induced by anti-estrogens hinder binding of coactivators to the ER and facilitate binding of corepressors. The continued presence of corepressor in the complex is thought to explain the anti-estrogenic properties of tamoxifen. The relative amounts of corepressor and coactivator in certain tissues and the presence of other factors regulate whether tamoxifen acts as an agonist or antagonist. As examples, up-regulation of HER and AIB1 appear to enhance the estrogen agonistic properties of tamoxifen.[171] Additional estrogenic effects are mediated by membrane initiated (extra-nuclear) actions at the cell membrane as well as by tethering of the ER to binding sites on c-Jun, SP-1, IGF-R, PI-3 kinase, HER-2-Neu, c-Src.[184-187]

Metabolism of Tamoxifen

The metabolical conversion of tamoxifen to 4-OH tamoxifen and then to endoxifene is thought to be critical for the efficacy of tamoxifen since these two metabolites are much more active than the parent compound.[188] Based on the fivefold to tenfold higher blood levels of endoxifen than 4-OH tamoxifen in women taking this medication, it has been proposed that endoxifen is the major active metabolite. Conversion to endoxifen is mediated by the cytochrome p450 enzyme, CYP 2D6. Initial reports suggested a poorer prognosis in tamoxifen treated patients with low levels of CYP2D6 activity. More recent reports have not confirmed this finding and definitive conclusions await further studies.[188-192] In the meantime, clinical guidelines NCCN Breast Cancer Panel, 2006, (http://www.nccn.org/) suggest avoiding certain SSRIs such as fluoxetine and paroxetine that can block cyp2D6 and thus endoxifene formation in women being treated with tamoxifen. Based on the conflicting clinical evidence, routine measurement of CYP2D6 is not recommended (NCCN Breast Cancer Panel, 2 http://www.nccn.org/006).

Overall Efficacy

Until recently, the overall benefit of tamoxifen was usually reported as the relative improvement in frequency of defined events such as tumor recurrence or survival. In the neo-adjuvant, adjuvant, and advanced disease settings, tamoxifen results in a relative benefit of approximately 50%. Absolute benefit rate has now been widely accepted as a more relevant statistic. This is defined as the absolute number of women per hundred treated who benefit from taking tamoxifen. The absolute benefit increases as a function of the event rate even though the relative 50% benefit does not change. The most important response parameter is disease recurrence which is reduced by tamoxifen (Fig. 29.18).

Efficacy in Ductal Carcinoma in situ *(DCIS)*

Randomized trials demonstrate the benefit of tamoxifen in women with DCIS.[193] In a large NSABP clinical trial, 13% of women treated with lumpectomy, irradiation, and placebo experienced a new tumor event over 5 years.[193] One third of these new events involved appearance of a contralateral breast cancer; one third a new ipsi-lateral tumor and one-third, local recurrence of the original tumor. Tamoxifen reduced these events in absolute terms by 5% with equal benefit in preventing new contra-lateral and ipsi-lateral tumors and recurrence of the original tumor. A later post-hoc analysis reported that only women with ER+ tumors benefitted from this approach.[194] A 13.6-year follow-up confirmed the durability of the responses to tamoxifen.[180] The beneficial effects of tamoxifen appear to be limited to those who had not received radiotherapy after excision.[195]

Adjuvant Therapy for Invasive Cancer

Tamoxifen is active only in women whose tumors are ER+ and/or PR+. A recent overview analysis provided a synopsis of our knowledge regarding adjuvant therapy with tamoxifen.[151] Use of tamoxifen for 5 years resulted in a reduction of cancer recurrence of 50% for the first 5 years and 39% for years 5 to 9. Breast cancer mortality at year 15 was reduced relatively by 30% which represented an absolute improvement of 9%. Premenopausal or postmenopausal women with positive lymph nodes at the time of diagnosis experienced a 10.9% absolute prolongation of recurrence free survival at 5 years in response to tamoxifen. A lesser effect (5.6%) was observed in those with negative nodes[196] (Fig. 29.18A). Tamoxifen also caused an absolute 1% to 5% reduction of contralateral breast cancers at 5 years depending upon underlying risk. More importantly, tamoxifen causes an increase in overall survival, suggesting that this drug may cure a fraction of patients. Longer follow-up, however, will be needed to verify this possibility (Fig. 29.18B and C).

Direct comparative studies indicate superiority of 10 years versus 5 years of tamoxifen therapy.[197] Previously, the optimal duration of use was generally agreed to be 5 years. Although usually only administered for 5 years, benefit carries over long-term ("carry-over effect"). The most recent update reported that the relative risk (RR) of disease specific mortality with tamoxifen versus placebo was 0.66 for years 0 to 4, 0.68 for years 5 to 9, and 0.68 for years 10 to 14 ($p < 0.001$) (Fig. 29.18B and C).[151]

Advanced Invasive Disease

Nearly half of women with ER+ advanced disease benefit from tamoxifen. Responses last 12 to 18 months on average before relapse.[150] Efficacy is limited to those with ER+ and/or PR+ tumors.[196] Selected studies indicate that the presence of HER 2 is associated with a decreased response to tamoxifen,[198,199] but not all data support this conclusion.[200] One large study [anastrozole, tamoxifen, alone and in combination (ATAC)] also suggested that ER+ and PR− tumors also respond less well to tamoxifen but a similar trial (Breast intergroup-BIG-1-98) did not confirm this observation.[113,114,201] Women with disease in soft tissue and bone experience the highest percentage response to tamoxifen whereas those with extensive liver metastases, brain metastases, or lymphangitic spread to lung appear to be relatively tamoxifen resistant even when the tumors are ER+.[150]

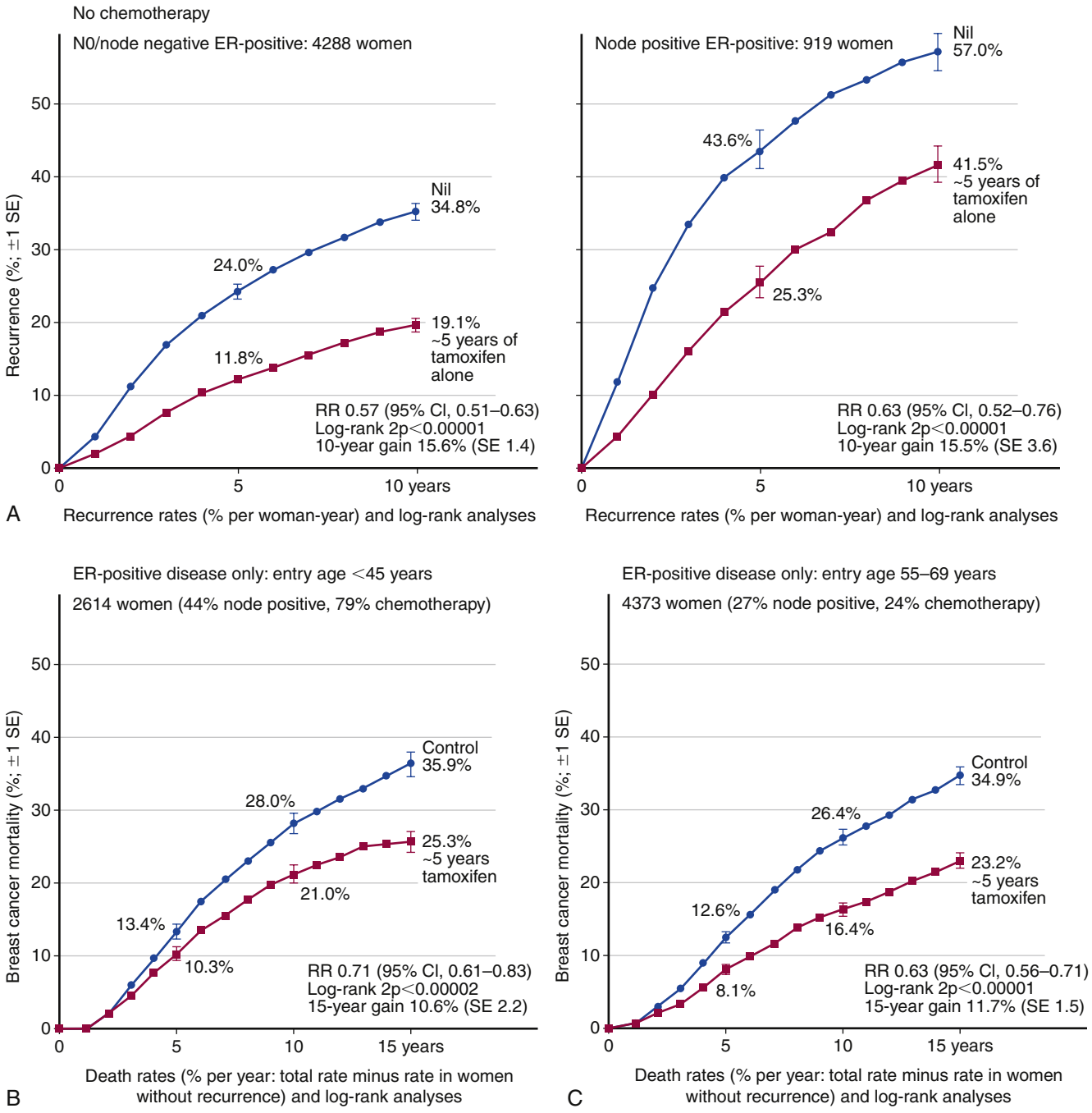


FIGURE 29.18 Effect of tamoxifen as adjuvant therapy of breast cancer on recurrence. Women receiving no adjuvant hormonal therapy (nil) are compared to those taking tamoxifen for 5 years. Women are then followed for up to 15 years. **A,** *Left panel,* Node negative ER+ disease. *Right panel,* Node positive ER+ disease. **B,** Effect of tamoxifen in node positive ER+ women less than 45 years of age on breast cancer specific mortality. **C,** Effect of tamoxifen in women ages 55 to 69 years old with ER+ tumors regardless of lymph node status. ER+, estrogen receptor positive; RR, relative risk. *(From Early Breast Cancer Trialist's Collaborative Group Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen; patient–level meta-analysis of randomized trials,* Lancet *378:771–784, 2011.)*

Inhibitors of Estradiol Synthesis

Mechanisms

The aromatase enzyme catalyzes the rate limiting step in the conversion of androgens to estrogens.[202] One steroidal aromatase inhibitor, exemestane, and two nonsteroidal, anastrozole and letrozole, are highly potent and specific and approved in the United States and Europe. Both subclasses of inhibitor reduce aromatase to 1% to 2.5% of baseline activity in postmenopausal women,[203] substantially reduce plasma estradiol levels, suppress tissue concentrations of this steroid in breast tumors and are devoid of estrogen agonistic properties. Only postmenopausal patients benefit from aromatase inhibitors since interruption of estradiol negative feedback with a reflex increase in luteinizing hormone (LH) and follicle stimulating hormone (FSH) results in override of aromatase blockade in premenopausal women.[204]

Efficacy

Adjuvant Therapy. The efficacy of these agents in the adjuvant setting has been examined by trials comparing AIs with tamoxifen. Two similar large trials, the ATAC (Anastrazole and Tamoxifen Alone and in Combination—ATAC trial), and the Breast International Group (BIG) 98 trials compared the effects of nonsteroidal AIs (letrozole or anastrozole) versus tamoxifen with endpoints of time to progression of disease, time to treatment failure, and overall survival.[113,114,205] A third trial utilized the steroidal AI, exemestane.[206] At 5 years of follow-up, all three trials demonstrated an absolute superiority of the AI of approximately 3%. The BIG-98 study reported an 18% (HR 0.82 95%, CI 0.70 to 0.95) significant increase in overall survival with letrozole but only after correcting for selective crossover of tamoxifen patients.[207] Improved overall survival was not seen in the ATAC trial. On the basis of these data, the AIs have now been approved in the United States for use in the adjuvant setting. The third generation aromatase inhibitors (AIs) anastrozole, letrozole, and examestane have now been proven superior to tamoxifen in direct head to head trials.[110-114,208,209]

Sequential Use. Several studies are currently comparing the sequential use of AIs or tamoxifen during adjuvant therapy.[188,210-214] The IES study (International Exemestane Study) compared the effect of 5 years of tamoxifen with 2 to 3 years of tamoxifen with crossover to exemestane at 2 to 3 years.[215] This study showed a reduction in new cancer events in the AI compared to the tamoxifen group. The ABCSG-ARNO trial was similar but used anastrozole rather than exemestane. Switch to the AI resulted in a 3% improvement in event free survival and in overall survival.[213] However, with extended follow-up (median 71 months), the BIG-98 trial reported similar disease free survivals for the tamoxifen to lerozole sequence and the letrozole to tamoxifen sequence.[216] This finding was supported by the TEAM trial.[217] A meta-analysis of these studies reported benefit of the sequential use of an AI after tamoxifen.[218]

A four-arm study reported the comparison of 5 years of tamoxifen, 5 years of letrozole, and the switching from one to the other at 2 to 3 years. The estimated disease free percentages for the letrozole monotherapy, letrozole followed by tamoxifen, and tamoxifen followed by letrozole arms was respectively 87.9%, 87.6%, and 86.2%. These data suggest that either sequence is relatively equivalent (Fig. 29.19). However, nonsignificant trends favor the initial use of an AI in the patients with positive nodes.

Extended Use. Extended therapy with 5 years of letrozole after an initial 5 years of tamoxifen increased disease free survival and overall survival in women with lymph node +, ER+, and PR+ tumors.[219] In a separate subgroup analysis of this study, the group with positive nodes also reported improved overall survival.[188,220,221] The NSABP B-33 and ABCSG-6a trials reported similar findings.[188]

Advanced Invasive Disease. Five large, multicenter, multinational, randomized trials directly compared the AIs with tamoxifen.[222-228] All followed similar trial designs and included postmenopausal patients with locally advanced or metastatic breast cancer. No women had received adjuvant tamoxifen within 12 months and none were known to be ER negative. All trials demonstrated the superiority of the AIs in clinical efficacy with incremental responses ranging from 2% to 13%. The differences were statistically significant in all but one trial in which the receptor status was unknown in 55% of patients.[222] A recent meta-analysis of these data reported the superiority of AIs over tamoxifen with odds ratios (OR) for overall response of 1.56 (95% CI 1.17-2.07) and for clinical benefit with an odds ratio of 1.70 (95% CI 1.24 to 2.33).[208] In this analysis, the trend toward improved overall survival did not reach statistical significance (OR 1.95 95%, CI 0.88 to 4.30) but a Cochrane review did report significant survival benefit.[229] These data allow the conclusion that the AIs are superior to tamoxifen for objective response rate and clinical benefit.

Comparison Among AIs. A comprehensive, direct head to head comparisons of the three approved AIs: letrozole anastrozole, and exemestane in the neoadjuvant setting have shown that each of these agents have similar efficacy even though letrozole is more potent in suppressing estrogen levels.[230,231]

Side Effects and Toxicity of AIs versus Tamoxifen. AIs are associated with unique side effect and toxicity profiles compared to tamoxifen (Table 29.4). When analyzing these data, it should be kept in mind that adherence to the various therapies has not been complete and the side effect profile can contribute to lack of compliance.[188] Pooled data indicate that the AIs are associated with deep venous thromboses (DVT) and endometrial carcinoma (EC) less commonly than with tamoxifen.[232] On the other hand, the AIs are associated with an increased odds of developing bone fractures (OR 1.47 95%, CI 0.34 to 1.61) and cardiovascular disease (OR 1.26, 95% CI 1.10 to 1.43), particularly in more advanced stage 3 and 4 disease.[232-234]

Combining observations from all trials, it appears that nausea, hot flashes, and GI distress were comparable with tamoxifen or an AI. Comparisons of aromatase inhibitors with placebo (or with tamoxifen) reveal an increase in hot flashes, osteoporosis, and myalgia with aromatase inhibitors. Surprisingly, women receiving placebo or tamoxifen also experience these same side effects quite commonly. For example in the placebo arm of the extended letrozole trial,[235] arthralgias occurred in 16.6%, hot flashes in 40.5%, and myalgias in 9.5%.[235] Cholesterol levels are slightly higher in patients taking aromatase inhibitors as opposed to tamoxifen but this may reflect the lipid lowering properties of tamoxifen. If cholesterol levels do rise in response to the AIs, the increase is minor.[236] Generally considered to be unique to the AIs is a form of arthritis-arthralgia shown recently in GWAS studies to be associated with the inflammatory cytokine, interleukin (IL) 17 receptor.[237]

Fulvestrant

Clinical and experimental data suggest that long-term exposure to tamoxifen might induce tumors to undergo adaptive mechanisms and cause the agonistic properties of this SERM to predominate.[171] Based upon these observations, anti-estrogens were developed that were relatively devoid of agonist properties and were originally called "pure

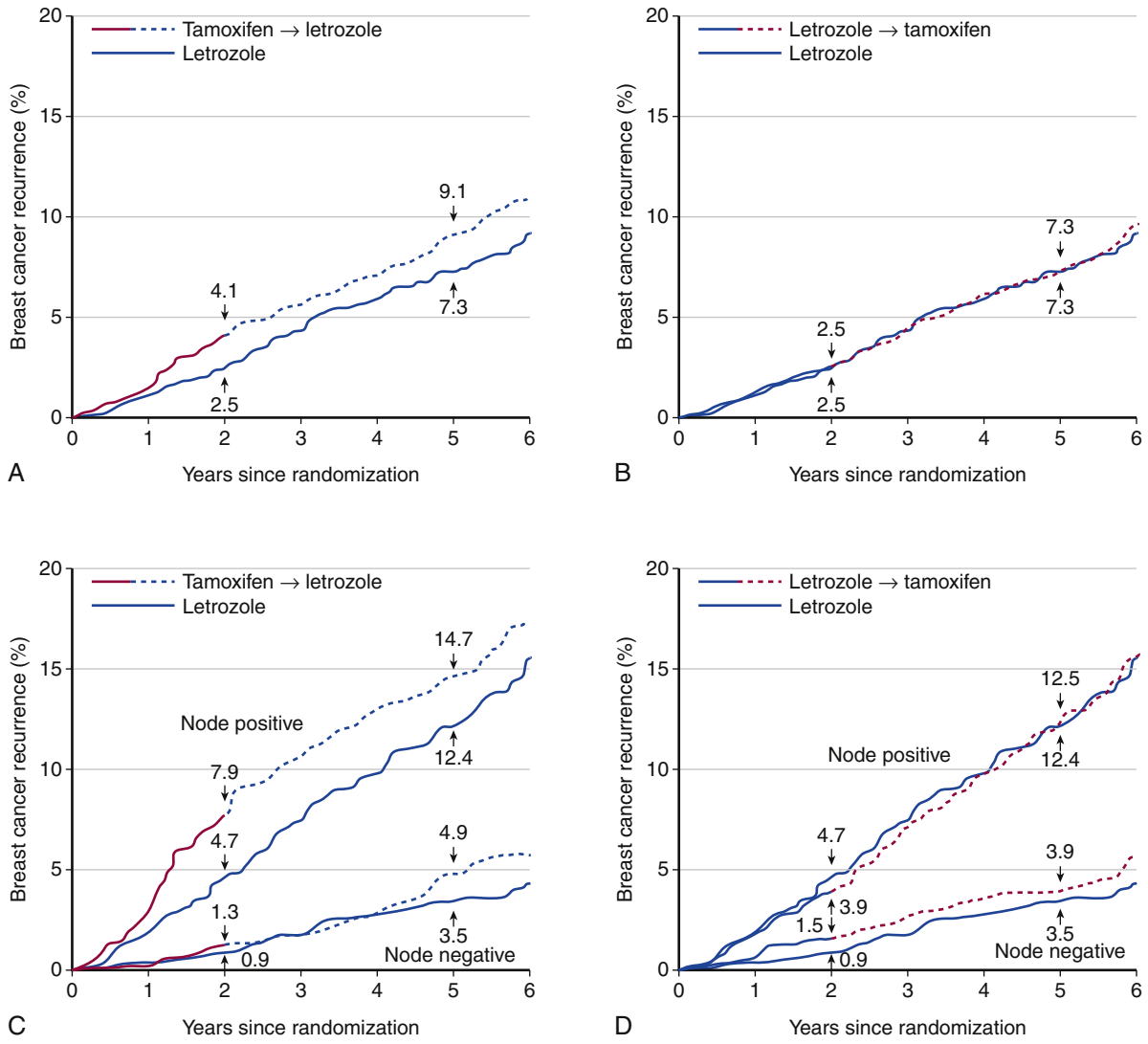


FIGURE 29.19 Results of the Breast International Group 98 (BIG 98) trial with four arms; tamoxifen for 5 years, letrozole for 5 years, tamoxifen for 2 years with switching to letrozole and letrozole for 2 years with switching to tamoxifen. Panels **A-D** illustrate the results. *(From The BIG-98 Colaborative Group Letrozole therapy alone or in sequence with tamoxifen in women with breast cancer,* N Engl J Med *361:766–776, 2009.)*

Table 29.4 Toxicity and Side Effects—AIs vs. Tamoxifen

	CV	Cerebro-V	VTE	Bone Fracture	EC	Hot Flashes	Arthralgia	Myalgia
AI*	4.2%	1.4%	1.6%	7.5%	0.1%	33.5%	22.5%	8.4%
TAM*	3.4%	1.5%	2.8%	5.2%	0.5%	37.8%	16.6%	7.0%
AI vs. TAM	OR 1.26 (1.10-1.43)	OR 1.01 (0.81-1.26)	OR 0.55 (0.46-0.64)	OR 1.47 (1.34-1.61)	OR 0.34 (0.22-0.53)	NA NA	NA NA	NA NA
Statistical Significance	$P < 0.001$	$P = 0.93$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = < 0.05$†	$P = < 0.05$†	$P = 0.05$†

*Letrozole vs. Tamoxifen Breast International Group 1-98 trial.
†Based on 95% confidence intervals not touching 1.0 relative risk line.
CV, cardiovascular disease (myocardial infarction, angina, cardiac failure); Cerebro-V (CVA or transient ischemic attack); VTE, venous thrombotic episode; EC, endometrial carcinoma; AI, aromatase inhibitor; TAM, tamoxifen.

anti-estrogens." Fulvestrant, the FDA approved drug in this class, largely lacks estrogen agonist effects. It has two separate mechanisms of action: (1) it inhibits E2 mediated transcription by favoring binding of corepressors to the ER complex and (2) increases the rate of degradation of the ER.[238] Because of its effect to reduce the concentration of the ER, fulvestrant has now been termed an "Estrogen Receptor Down-Regulator or SERD."

A comparative study in 458 women with advanced disease demonstrated similar efficacy in patients treated with fulvestrant or the aromatase inhibitor anastrozole in patients progressing after prior endocrine treatment. Clinical benefit

(i.e., complete objective response, partial objective response, and stable disease for 6 months) occurred in 44.5% receiving fulvestrant versus 45% receiving the AI, anastrozole. Another study compared tamoxifen with fulvestrant as first line therapy of advanced breast cancer and found similar efficacy with respect to all parameters examined.[239] Finally, patients initially treated with fulvestrant and relapsing continue to maintain responsiveness to aromatase inhibitors or to tamoxifen.

After the introduction of fulvestrant, the concept arose that higher doses were needed to achieve maximum efficacy. Initial trials comparing standard to loading dose regimens to high dose therapy reported similar results with each approach.[240,241] Later studies of high dose fulvestrant reported a longer time to progression with this SERD versus anastrozole (23.4 months for fulvestrant versus 13.1 months for anastrozole, $P = 0.0496$).[242] Another study in metastatic disease also showed that high dose fulvestrant was superior to lower dose (HR 0.80, 95% CI 0.68 to 0.94).[243] Two studies examined the combination of fulvestant with an AI with one reporting positive results and the other not.[244,245] Based upon these data, the appropriate role of fulvestrant in the clinical armamentarium remains to be fully defined.

Surgical Oophorectomy. Prophylactic oophorectomy in premenopausal women represented the first adjuvant endocrine therapy for breast cancer. While initially thought to be ineffective, meta-analyses demonstrate clear benefit with a 6% absolute survival advantage at 15 years for patients less than 50 years of age who are lymph node negative and a 12.5% survival advantage for node positive patients (Fig. 29.20). Since receptor status was unknown in patients in these early trials, a large fraction of receptor negative patients were likely included and the results probably underestimated results for patients with receptor positive tumors. In the advanced disease setting, surgical oophorectomy induces clinical benefit in approximately 50% of ER+ and/or PR+ patients.[150]

Medical Oophorectomy. Medical ovarian suppression with GnRH analogues effectively replicates the hormonal effects of surgical oophorectomy (see chapter 31 for description of GnRH analogues and mechanism of action). Two studies indicate that the GnRH analogues also produce clinical effects similar to those induced by surgical oophorectomy in the advanced disease setting.[246,247] With respect to adjuvant therapy, studies have examined the effects of chemotherapy vs. Med OOX rather than comparing Med OOX to no treatment. The Zoladex Early Breast Cancer Research Association Study (ZEBRA) demonstrated equivalence of the GnRH agonist, goserelin, to standard CMF (cytoxan, methotrexate, 5-fluoro-uracil) chemotherapy on overall survival in ER+ patients.[248]

Ongoing studies are addressing two questions. The first is whether chemotherapy followed by complete ovarian deprivation is superior to chemotherapy followed by partial ovarian deprivation. Davidson and colleagues showed the superiority of CAF (cytoxan, doxorubicin, fluorouracil) followed by Med OOX plus tamoxifen versus CAF alone or CAF plus Med OOX.[249] The complete estrogen blockade strategy (Med OOX plus tamoxifen) improved time to

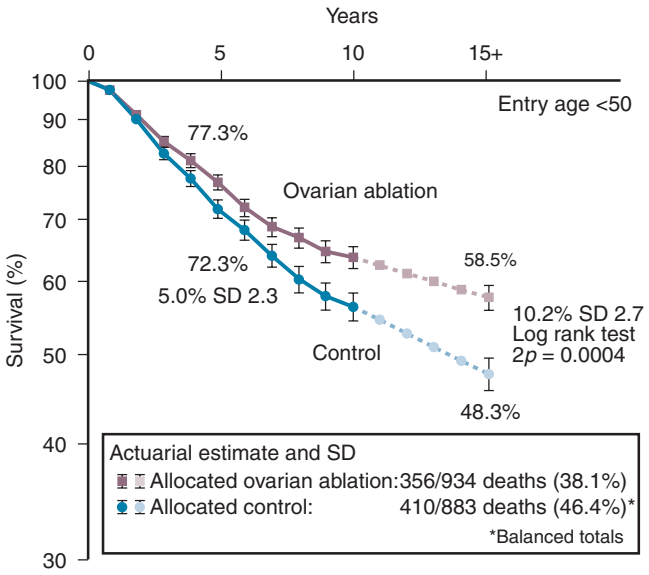


FIGURE 29.20 Survival data in patients undergoing a prophylactic oophorectomy in comparison with a group of women not receiving this therapy. *(From Santen R. Endocrine-response cancer. In Kronenberg HM, Melmed S, Polonsky KS, Larsen PR [eds].* Williams Textbook of Endocrinology, *11th ed, Philadelphia, WB Saunders, 2008, Figure 42.18.)*

treatment failure and disease free survival but not overall survival when compared to Med OOX without tamoxfen. One component of the SOFT trial is also addressing this point by comparing tamoxifen alone versus medical oophorectomy plus tamoxifen, versus medical oophorectomy plus an AI in patients who had received standard chemotherapy.[250] A recent study indicated that the AI plus medical oophorectomy (Med OOX) was not as effective as Med OOX plus tamoxifen.[251]

The second question addresses whether AIs are superior to tamoxifen for complete ovarian deprivation when given following chemotherapy and Med OOX. The TEXT Trial is addressing this issue by comparing Med OOX plus tamoxifen with Med OOX plus exemestane. Finally, to determine if chemotherapy is required at all when using complete estrogen deprivation, the PERCHE study will compare a chemotherapy/complete estrogen deprivation arm with one using only complete estrogen deprivation. Other studies will evaluate treatment effects in young patients with cancer since they are known to have a worse prognosis than older premenopausal women. The ongoing trials (SOFT, TEXT, PERCHE) which examine the individual roles of Med OOX, an AI, chemotherapy, and the combination of these agents should provide information regarding this subset of patients.

Chemical Castration. Chemotherapeutic agents destroy granulosa cells in the ovary and may result in transient or permanent amenorrhea and menopausal symptoms in premenopausal women (Fig. 29.21).[252] Complete ovarian destruction, as evidenced by the onset of amenorrhea, is more common in women over age 40.[252] Of interest is the return of menses in up to 20% of women over 40 when an aromatase inhibitor is started.[253] The effect of chemical castration was not initially felt to be clinically important. However, recent data suggest that adjuvant chemotherapy in premenopausal women exerts its anti-tumor effects on

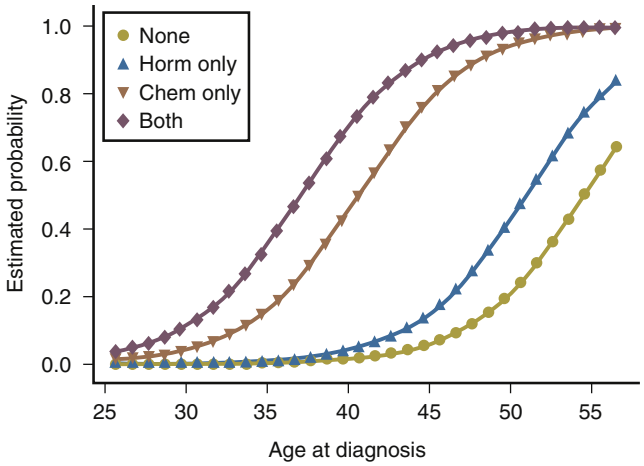


FIGURE 29.21 Cumulative frequency analysis of development of amenorrhea as a function of age in women with breast cancer in comparison with no treatment. The effect of chemotherapy on ovarian function reduces the age of menopause by an average of nearly 20 years. *(Taken from the data of Goodwin PJ, Ennis M., Pritchard KI, et al. Risk of menopause during the first year after breast cancer diagnosis,* J Clin Oncol *17[8]:2365–2370, 1999.)*

neoplasms with high ER levels through an ovarian ablative effect to a substantial degree.[254]

As a test of the contribution of chemical castration to efficacy, several trials have compared adjuvant chemotherapy to Med OOX with and without tamoxifen in premenopausal women.[250,255,256] The chemotherapy and hormonal therapies appear to produce comparable antitumor effects in women with moderate or high levels of estrogen receptor (ZEBRA trial).[250] The International Breast Cancer Study Group (IBCSG) trial VII compared CMF with goserelin as adjuvant therapy in premenopausal women. In those with ER+ tumors, disease free survival was 81% in both groups at 5 years.[257] These results suggest that at least part of the benefit of chemotherapy in premenopausal women results from "chemical castration."

Further support of the chemotherapy castration hypothesis comes from studies showing that chemotherapy is less effective in patients without complete cessation of menses and that addition of Med OOX in this setting provides improved responses.[258,259] It would appear then, that in women younger than age 40, addition of medical oophorectomy to chemotherapy would be beneficial. On the other hand, addition of Med OOX to chemotherapy in women older than 40 years old with ER positive tumors might not since chemical castration will be complete. In younger women at high risk of recurrence, chemotherapy followed by hormonal therapy is more efficacious than chemotherapy alone. These and other observations suggest that effects of chemotherapy are partially due to a reduction of hormone secretion and partially to cytotoxic effects.

Hormone Additive Therapy. Androgens or estrogens are generally considered inferior to use of tamoxifen and aromatase inhibitors as hormonal treatment.[150] Until recently, high dose estrogen had been used sparingly because of the increased side effect profile versus tamoxifen. However, a 20-year follow-up of a randomized comparison of tamoxifen with diethylstilbestrol (DES) reported a significant survival advantage in patients receiving the estrogen and lack of cross resistance between these two therapeutic approaches.[83] This surprising study suggests reconsideration of use of high dose estrogen in selected patients.[82] A series of preclinical studies demonstrated the likely mechanism for the beneficial effects of estradiol, namely the stimulation of apoptotic tumor cell death by estrogens.[80,81,260] A recent review reports a 30% response to use of high-dose estrogen in women refractory to multiple hormonal agents.[82]

Emerging Therapies. Neo-adjuvant therapy represents the use of an antitumor agent prior to surgery in an attempt to shrink the tumor sufficiently to allow lumpectomy rather than mastectomy.[181] The concept of neo-adjuvant chemotherapy has been adopted into clinical practice whereas neo-adjuvant hormonal therapy is now undergoing clinical trial. A nonrandomized trial demonstrated an 81% reduction on tumor volume with letrozole versus 75% with anastrozole and 48% with tamoxifen.[261] More recently, a randomized trial involving 324 patients compared letrozole 2.5 mg daily with tamoxifen 20 mg daily in women with ER+ tumors of greater than 2 cm in size.[262] Letrozole caused a 55% rate of objective response (CR and PR) versus 36% for tamoxifen ($p < 0.001$). Breast conserving surgery was chosen in 45% of patients receiving letrozole and 35% receiving tamoxifen ($p < 0.001$). Approximately 50% of women had a sufficient reduction in size of tumor to allow lumpectomy. Of interest is the fact that Her 2 positive tumors responded better to the AI (i.e., 88% response) than to tamoxifen (i.e., 21%).[199]

A similar trial [Immediate Preoperative Arimidex, Tamoxifen, or Combined with Tamoxifen (IMPACT)] confirmed the greater ability to perform lumpectomy in women receiving the AI (46% versus 22%, $p = 0.03$), but objective tumor responses in the AI and tamoxifen arms were similar.[263] Current guidelines consider neo-adjuvant endocrine therapy still to be experimental but this approach is becoming more widely used with emergence of these two studies. Nonetheless, comparison of groups of patients treated conventionally and with neo-adjuvant endocrine therapy with analysis of survival will be required to confirm the major utility of this approach.

Responses to neoadjuvant therapy might provide predictive information regarding long-term hormonal responsiveness or even overall survival. Emerging evidence suggests that a reduction of the Ki67 proliferation index might provide the best parameter to predict long-term outcome whereas apoptotic indices appear not to be informative.[264] cDNA array changes might also provide useful predictive information but verification of this possibility requires additional study.[265]

Anti-Growth Factor Therapy. Preclinical data suggest that tumors exposed to tamoxifen or to aromatase inhibitors adapt by up-regulating growth factor pathways. An attractive hypothesis is to administer growth factor pathway inhibitors concomitantly with endocrine therapies to inhibit the development of resistance. Several studies in xenograft models have proven the principle that this strategy can work and several clinical trials of this concept are underway. Limited studies suggest a progression free survival benefit of adding either trastuzamab or lapatinib to an AI in postmenopausal women with HER-2 and hormone receptor

positive metastatic breast cancer.[266,267] Two studies examined the effects of mTOR inhibitors in combination with AIs with one reporting positive results and the other negative.[268,269] The most recent study, breast cancer trials of oral everolimus-2 (Bolero-2), examined the mTOR inhibitor everolimus in women relapsing on an aromatase inhibitor and found an extended progression free survival.[269a] Results from addition of the EFG-R inhibitor gefitanib to hormonal therapy have been mixed with no consistent benefit.[270-272]

Recommended Approaches to Hormonal Treatment of Breast Cancer

Specific guidelines regarding hormonal therapy are based on several factors: subtype of breast cancer (i.e., Luminal A and Luminal B, Table 29.2) endocrine response category, menopausal status, and age of the patient. General principles regarding hormone therapy provided in this section attempt to consolidate two independent sets of recommendations: those of the 2011 St. Gallen expert panel and the NCCN guidelines.[128,273] It should be noted that the St. Gallen guidelines recently adapted an approach based on subtypes of breast cancer. Because of the simplicity of the approach, the St.Gallen guidelines are described here but these generally conform to the NCCN guidelines.

Adjuvant Treatment of Localized Breast Cancer (DCIS)

Consideration should be given to use of tamoxifen for 5 years for prevention of recurrence and contralateral breast cancer in both premenopausal and postmenopausal women with ER+ ductal carcinoma *in situ*.

Invasive Breast Cancer

Neoadjuvant Endocrine Therapy

This represents an option to be considered for postmenopausal patients with ER or PR positive disease. AIs are to be preferred based on trial data demonstrating greater efficacy than with tamoxifen. Treatment should be continued for 4 to 8 months with the primary goal to enable breastsparing excision with elimination of the need for mastectomy.

Adjuvant Therapy

Luminal A Tumors. The majority of patients can be treated with hormonal therapy alone. Unfavorable nodal status or other factors may suggest inclusion of chemotherapy (Table 29.5). Small tumors up to 0.5 cm with negative lymph nodes are so favorable that endocrine treatment may not be warranted.

In premenopausal women, evidence supports the use of tamoxifen, ovarian suppression with a GnRH analogue, tamoxifen plus ovarian suppression with a GnRH analogue, or surgical oophorectomy as adjuvant therapy. The St. Gallen panel recommends either tamoxifen alone or combined with ovarian suppression with consideration of surgical oophorectomy under selected circumstances.[274]

In postmenopausal women, current recommendations suggest the use of either AIs or tamoxifen as preferred initial adjuvant therapies for postmenopausal women with ER and/or PR+ tumors larger than 1 cm unless there are contraindications. If the lymph nodes are positive, the tumors are ER+ PR−, or the patient has a propensity for thromboembolism, an AI would generally be considered preferable.[275]

Table 29.5 Selection of Endocrine ± Chemotherapy

Clinico-Pathological Subtype	Type of Therapy
Luminal A	Endocrine therapy alone unless high nodal status or other indicator of high risk (e.g., Oncotype Dx score)
Luminal B (HER-2 negative)	Endocrine therapy with or without cytotoxic chemotherapy depending on level of endocrine receptor expression, high risk (e.g., Oncotype Dx score, nodal status), perceived risk, and patient preference
Luminal B (HER-2 positive)	Cytotoxics + anti-HER-2 agents + endocrine therapy (preference for aromatase inhibitors supported by selected data); no data are available to support omission of cytotoxics in this group

HER-2, human epidermal growth factor receptor-2, Oncotype Dx, 21 gene molecular diagnostic score to predict prognosis.

Luminal B/HER2− Tumors

In premenopausal women, endocrine therapy is the same as in the Lumina A patients. The inclusion and type of cytotoxics depends on level of endocrine receptor expression, perceived risk, and patient preference. Use of the OncotypeDx or Mammoprint signatures might be considered as a means to decide about chemotherapy.

In postmenopausal women, endocrine therapy is the same as in the Luminal A patients. The inclusion and type of cytotoxics may depend on level of endocrine receptor expression, perceived risk, patient preference, or molecular signature as above.

Luminal B/HER2+ Invasive Tumors

In premenopausal women, endocrine therapy is the same as in the HER2− patients. Adjuvant therapy with trastuzamab should be added in those with a tumor greater than 1 cm or positive lymph nodes and considered in those with smaller tumors. No evidence is available that supports the omission of cytotoxics in this group.

In postmenopausal women, the same endocrine therapy is chosen as in the luminal B and HER2− patients. The same considerations with respect to tumor size are applicable with respect to adding adjuvant trastuzamab as in premenopausal women. No evidence is available that support the omission of cytotoxics in this group.

Duration of Adjuvant Hormonal Therapy in Women with Luminal A and B Tumors

Premenopausal at diagnosis: Hormone therapy is recommended for 5 years. In selected patients, an aromatase inhibitor may be considered for an additional 5 years if the patient has become postmenopausal and no further endocrine therapy if the patient is still premenopausal.

Postmenopausal at diagnosis: The NCCN lists five possible approaches and does not specifically prefer one over another. These include (1) an AI for 5 years, (2) tamoxifen for 2 to 3 years and then switch to an AI for 2 to 3 years to complete the 5 years or to extend the AI to a total of 5 years, (3) an AI for 2 to 3 years with switch to tamoxifen to complete the 5 years, (4) tamoxifen for 4.5 to 6 years with an AI for an additional 5 years, and (5) tamoxifen for 5 years for those with a contra-indication to an AI, who decline its use, or are intolerant. When trastuzumab is used in HER2+ patients, the minimum duration should be 1 year.

Treatment of Advanced Disease

Prior Endocrine Therapy Less Than One Year Ago

Both Luminal A and B tumors are considered for hormone therapy with attention to menopausal status. Premenopausal women with intact ovarian function who had received tamoxifen previously should receive ovarian ablation or suppression therapy plus endocrine therapy as for postmenopausal women. The postmenopausal women relapsing after less than 1 year of therapy are considered to be hormone refractory and chemotherapy would usually be the preferred the next step.

Prior Endocrine Therapy More Than One Year Ago

Both Luminal A and B tumors are considered for hormone therapy. In premenopausal women, the tumor is considered possibly hormone responsive and ovarian ablation or suppression plus endocrine therapy with an AI if tamoxifen was previously used as for postmenopausal women is preferred. In postmenopausal women relapsing after more than 1 year of endocrine therapy, the preferred choices are either an AI, tamoxifen (or toremifene), or a selective estrogen receptor down regulator such as fulvestrant. If visceral disease is present in any of these categories, initial chemotherapy should be considered.

Sequential Endocrine Therapies

An individual responding to one hormone therapy can be considered to possibly benefit from a second, third, or even fourth line endocrine therapy. These include steroidal aromatase inhibitors, nonsteroidal inhibitors, fulvestrant, tamoxifen or toremifene, megestrol acetate, fluoxymesterone, and ethinyl estradiol or estradiol (estrace). After the use of second line endocrine therapy, no data are available to know which third and fourth line therapies are preferable.

Use of Chemotherapy

After the patient fails to respond to a sequential hormone therapy, the next choice is chemotherapy. Discussion of the current choices of chemotherapy is beyond the scope of this chapter. The interested reader is referred to the NCCN guidelines.[273]

Strategies Undergoing Evaluation

In premenopausal women, conclusive data regarding the superior efficacy of complete estrogen deprivation with Med OOX plus tamoxifen or an AI are lacking and the subject of ongoing study. Nonetheless, the St. Gallen Panel accepted the concept of complete estrogen suppression as reasonable for very young patients, especially in the intermediate and high-risk groups. This approach was also considered appropriate alternative therapy for premenopausal women at any age at high-risk, especially if chemotherapy did not induce complete ovarian failure. The Panel did not recommend GnRH analogues plus aromatase inhibitors outside of clinical trials.

Long-Term Quality of Life in Breast Cancer Survivors

With earlier diagnosis of breast cancer, an increasing percentage of women survive breast cancer long term. Two thirds of these women are menopausal at the time of diagnosis and half of the premenopausal women undergo permanent ovarian failure as a result of chemotherapy. Menopausal hormone therapy (MHT) is generally thought to be contraindicated in these women since estrogens may cause re-growth of residual tumor tissue after surgery or cause a second primary.[67] Data from *observational* studies, however, do not provide evidence of a deleterious effect. *Prospective* studies of the safety of MHT in this setting are conflicting as the Habits trial reports an increase in risk of recurrent breast cancer with MHT,[276] whereas the Stockholm trial does not.[277] The differences in results may relate to the increased use of tamoxifen (51%) in the Stockholm trial versus the Habits trial (22%) but this conclusion requires experimental confirmation. Until this issue is more definitively resolved, it is prudent to avoid estrogens in breast cancer survivors if alternatives to estrogen are effective.[67]

Several agents are available to substitute for estrogen. These include bisphosphonates to prevent or treat osteoporosis, statins to prevent heart disease, venlafaxine and gabapentin to diminish the number and severity of hot flashes,[278-280] vaginal moisturizes and lubricants without estrogen for symptoms of urogenital atrophy, and SSRIs for depression thought to be associated with estrogen deficiency. This approach does not protect against Alzheimer disease or improve cognitive function but the recent WHI study demonstrated that MHT increased rather than decreased the risk of dementia.[281] Use of low dose vaginal estrogens should only be used with caution and informed consent of the patient because of systemic absorption.[282] In general, these alternative approaches are not as effective as exogenous systemic estrogen. If alternatives to estrogen are not satisfactory, women might choose to receive MHT after a full discussion of the risks and benefits and with informed consent.

Breast Cancer in Men

The incidence of breast cancer in men is 100-fold lower than in women with 2140 new cases diagnosed in 2011 and 450 deaths.[146] The incidence of male breast cancer has increased over the past 8 years from 1450 cases per year in 2004 to 2140 in 2011. Median age at diagnosis is 65 to 67 years old and 35% occur in men 70 years old and older.[283] Approximately 90% are invasive ductal carcinomas but DCIS (ductal carcinoma *in situ*) is not uncommon.

Known risk factors include family history; clinical disorders associated with reduced testosterone production

or estrogen excess such as orchitis, orchiectomy, undescended testes, testicular injury, obesity, exogenous estrogen administration, and Klinefelter syndrome; radiation exposure; Jewish ancestry; BrCa1 and BrCa2 carriers, low levels of physical activity; prior chest wall irradiation, and benign breast disease.[284] Suggestive risk factors include the Cowden, Lynch, and Li-Fraumeni syndromes; mutations in PTEN, T53, and PALB2; and cirrhosis. Inconclusive factors reported in the literature to be possibly associated include mutations of the androgen receptor gene, the Chek 2 gene, prostate cancer, gynecomastia, occupational exposures, electromagnetic frequency exposure, high temperatures, diet, and alcohol.[285]

Male breast cancer typically presents as a painless, firm mass often associated with nipple retraction or ulceration, fixation of skin to underlying muscle, and palpable axillary nodes.[286] Diagnosis is then made by mammography and biopsy of the lesion. At presentation of breast cancer, 37.5% of men have regional lymph node involvement versus only 29.2% of women with this disease. Tumors are larger at diagnosis in men than in women and 41.7% of men have tumors localized to their breast versus 50.5% of women. The percent ER and PR positive is higher in men (90.6% and 81.2%) than in women (76% and 66.7% respectively). Two percent to 15% express HER2, a figure lower than in women. The 5-year survival rate is 63% and 10-year, 41%.[283]

Modified radical mastectomy is the usual surgical approach. Surgical assessment of the axillary nodes is an essential component of primary therapy. Sentinal node biopsy, as in women, can be considered but available data in support of this approach in men is limited due to the infrequency of male breast cancer. Adjuvant radiotherapy, particularly in men with positive nodes, is usually recommended but survival benefit is unknown. As 90% of men have ER + tumors, tamoxifen is chosen as the initial adjuvant therapy. Upon recurrence, if sufficient time has elapsed since adjuvant use, tamoxifen induces an 80% response rate and is the preferred first line therapy. Second line and later therapies can involve megestrol acetate, GnRH agonist analogues, and androgen. Aromatase inhibitors have been effective in a few patients but orchiectomy or GnRH suppression is required to prevent reflex rises in testosterone which can abrogate the estrogen suppressive effects. HER2 targeted therapy and chemotherapy have been effective in inducing remissions in selected patients. No randomized, controlled trials are available to accurately assess relative efficacies of various therapies for male breast cancer.[287]

Prostate Cancer

Incidence

Carcinoma of the prostate is the most common solid tumor and second leading cause of death from cancer in American men. Approximately 241,740 will be diagnosed with prostate cancer and 28,170 men will die from this disease in the United States in 2012.[146] The incidence of detected prostate cancer has increased exponentially over the past 2 decades but with a more gradual increase in the number of disease related deaths. Increased detection reflects not only an aging population but also the use of more sensitive screening techniques such as the measurement of serum prostate specific antigen (PSA),[288] first introduced in the mid-1980s. With the utilization of PSA, the incidence of newly diagnosed cancer of the prostate increased 18% per year from 1989-1992, compared to a 6% per year increase during the years 1985 to 1989. This increase subsequently leveled off to 14% per year between the years 1992 to 1995.[289] With its widespread use, PSA has increased the detection rate of clinically localized prostate cancer almost twofold compared to the use of digital rectal exam (DRE) alone.[290]As a result of PSA screening at diagnosis, the percentage of patients with low-risk disease, defined as clinical stage T1/T2a, biopsy Gleason score of 6 or less, and a serum PSA less than 10 ng/mL has increased.[291] In 1989, 31% of men at diagnosis met the criteria for low-risk disease versus 47% in 2002. Controversy has arisen as to whether or not these patients should undergo definitive treatment.

The global incidence of prostate cancer varies substantially. In Europe, the overall incidence rate was 93.4 per 100,000 in 2008 and tended to be lowest in the southern and eastern regions and highest in northern and Western Europe.[292] For example, the lowest and highest recorded incidence rates in 2008 were in Ukraine (27.7 per 100,000) and Ireland (183.1 per 100,000), respectively. Although the incidence of premalignant prostate lesions and latent prostate cancer is approximately the same in Japan and China as in the United States, the rates of invasive cancer differ markedly.[293,294] Additional mutations and promotional factors necessary to convert latent to invasive cancer apparently occur less commonly in the Japanese and Chinese living in Asia. In contrast, the initial mutations leading to latent cancer occur at the same rate. These observations suggest that environmental or dietary factors may influence later steps in the mutational process or factors that promote tumor growth. In support of these concepts, prostate cancer incidence increases when men migrate to a more western environment. Japanese men living in Hawaii have an incidence rate higher than those in Japan but lower than Caucasians living in the United States.[295]

Endocrinology of Prostate Cancer Growth

DHT, the 5 alpha-reduced product of testosterone (T), binds to the AR with a 2.5-fold higher affinity than T itself and serves as the major regulator of prostatic tumor growth. Direct effects of androgen itself, indirect effects induced by stimulation of growth factors, or a combination of these two mechanisms may mediate androgen induced proliferation.[296] Approximately 7000 μg of testosterone are secreted daily by the testes of which 500 μg are converted into DHT in various peripheral tissues (Fig. 29.22). The adrenal gland provides an additional 5% of the androgen produced in adult men. Testosterone as well as pre-androgens such as androstenedione, dehydroepiandrosterone (DHEA), and DHEA-sulfate originate in the adrenal and are also converted in peripheral tissues into DHT. In addition to peripheral conversion, a large fraction of the DHT present in benign and malignant prostatic tissue is produced locally in the prostate gland from circulating precursors. Therefore, approximately 40% of intraprostatic DHT originates from steroids of adrenal origin.

Prostate cancer cells contain ARs that bind DHT and transmit proliferative signals in androgen dependent prostate cancer. As opposed to the use of receptor assays in breast cancer, measurement of the AR does not provide

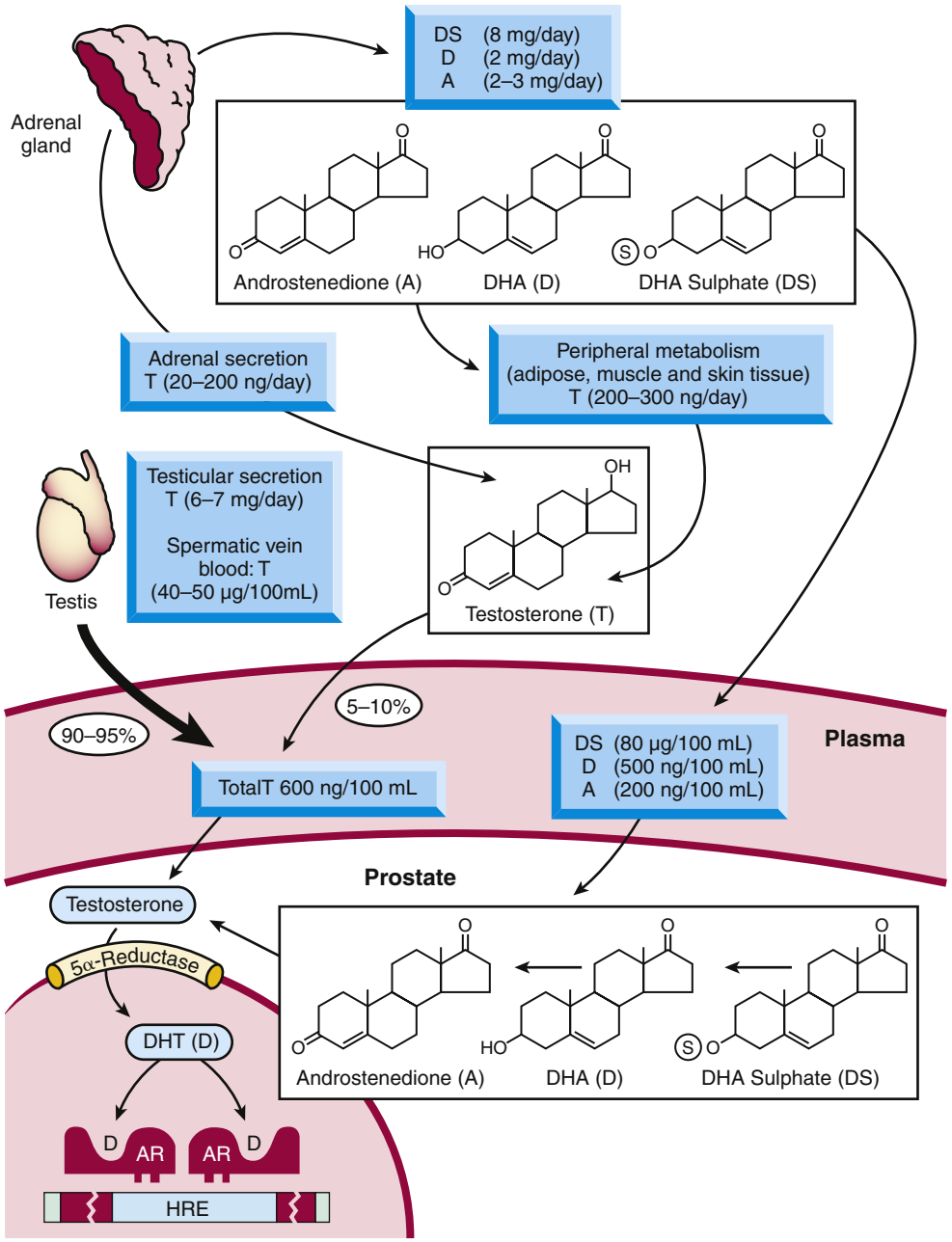


FIGURE 29.22 Diamgrammatic representation of the sources of hormones available to prostate tissue. *(From Santen R. Endocirne responsive cancer. In Kronenberg HM, Melmed S, Polonsky KS, Larsen PR [eds].* Wiliams Textbook of Endocrinology, *11th ed, Philadelphia, WB Saunders, 2008.)*

predictive information regarding hormone responsiveness. Mutations of the AR occur but the frequency in early stage prostate cancer is quite low.[297,298] However, in metastatic and androgen-independent tumors, the incidence of mutations is much higher (up to 40%).[299] The frequency and type of mutation appears to be influenced by selective pressure, namely that exerted by anti-androgens. For example, men treated with flutamide exhibit higher levels of mutations compared to men not receiving this anti-androgen.[300] The majority of these mutations occur at AA 877, the same site mutated in LnCAP cells which allows flutamide to become an androgen agonist. This finding helps explain the occurrence of flutamide withdrawal responses observed in men with prostatic cancer (discussed later in the chapter).[301-303]

Men with prostate cancer respond to androgen deprivation for varying time periods. Typically, after an initial response to androgen blockade therapy, tumors will progress and this will usually be in concert with an increase in serum PSA. Secondary responses to blockade of adrenal androgens occur as evidenced by a 50% or greater decrease in PSA levels and often by pain reduction and improvement of fatigue but rarely by objective regression of tumor mass or healing of bone lesions.

Several theories might explain secondary hormonal responses and evolution into completely androgen independent growth, but none are definitively proven. Nonmutually exclusive mechanistic theories include development of hypersensitivity to residual androgens, enhancement of androgen receptor action by phosphorylation of key sites in the receptor, and ligand independent activation of the receptor via growth factors or mutations. A concept that underlies all of the proposed theories is that growth factor

pathways can become up-regulated and/or activate the AR independently of ligand in tumors leading to androgen resistance.[304] For example, the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway regulates activity of the AR through phosphorylation of the receptor itself as well as downstream targets resulting in resistance to apoptosis and increased cell survival.[305,306] Altered downstream targets of PI3K/AKT include mTOR, p70S6 kinase, and 4E-BP1.[307,308] In addition, mutations or inactivation of PTEN are found commonly in advanced prostate cancer. PTEN is a phosphatase that dephosphorylates and inactivates PI3K. With mutational inactivation of PTEN, an associated up-regulation of phosphorylated, activated AKT occurs as well as downstream activation of mTOR. Antibody-based approaches demonstrate that loss of PTEN is highly correlated with activation of AKT and phosphorylation and activation of S6, a substrate of p70S6 kinase. For this reason, use of agents to block mTOR, such as temsirolimus and everolimus, are currently being examined in prostate cancer.[309-312] MMP-9 is also up-regulated in the process of development of resistance and could serve as an additional target for therapy.[313]

Etiology

Primary nonmodifiable risk factors for prostate cancer development include age, race, and family history.[314] The Surveillance Epidemiology and End Results (SEER) Registry reports that the incidence of prostate cancer increases with age from 9.2/100,000 between ages 40 to 44 years old to 984.8/100,000 at ages 70 to 74 years old.[315] Incidence is increased in African-American males with an age adjusted relative risk of 1.73 (95% CI 1.23 to 2.45). The mortality rate is nearly double compared to Caucasian males. Genetic factors are also important as the relative risk for a man with one first-degree relative with prostate cancer increases over twofold with even higher risk with multiple first-degree relatives.[316,317] Overall, men with prostate cancer report a positive family history 3.1 to 4.3 times more commonly than men without and twin studies have shown a genetic component in 42% (95% CI: 29, 50%).[20]

Specific genetic lesions resulting in prostate cancer development are uncommon and evidence is lacking regarding causality in potential loci. However, recent genome-wide association studies have identified 46 susceptibility loci which are currently being studied in order to better create risk prediction models.[318] Candidate genes based on linkage analysis include *BRCA2*,[319] *RNASEL* (an endoribo-nuclease), macrophage scavenger receptor 1 gene *(MSR1)*, AR (androgen receptor), CYP17 (cytochrome p450 enzyme responsible for the 17 hydroxylation of steroids), and SRD5A2 (the predominant form of 5-alpha reductase in the prostate).[320] Several epidemiological studies have reported a correlation between the number of polyglutamine repeats (CAG) on the *AR* and incidence of prostate cancer but others have not confirmed this finding.[320] Data from the Utah pedigrees identified another high-risk gene on chromosome 17p called prostate cancer susceptibility gene encoding a metal dependent hydrolase (ELAC/HPC2) in exon 1 of the *AR* gene.[321,322] BRCA2 but not *BRCA1* was associated with prostate cancer in Ashkenazi Jews whereas most series in unselected populations do not show this association.[323]

A recent preliminary finding suggested that fusions between two genes could activate carcinogenic pathways, a concept analogous to the fusion of the bcr-abl genes in leukemia. Based upon outlier cDNA studies, fusions between the TMPRSS2 and the ETS genes EGR and ETV1 were commonly found in prostate cancer (i.e., in more than 90% of the 57% of tumors that over express *EGR* or fusion between TMPRSS2 and ETS gene [ETV1]) and may play an etiological role.[324]

Modifiable risk factors that have been proposed include diet, body mass index, smoking, and physical activity. The significant differences in age standardized global incidence rates provide highly suggestive evidence that dietary and environmental factors play major roles in risk. Differential ingestion of high fat diets, red meat, green tea, or soy products provide potential explanations for the divergent rates of prostate cancer among different populations.[325-327] Several case-control studies examining western diets have shown a positive association with prostate cancer risk but these studies are confounded by potential recall bias and do not prove causality.[328-330] Others suggest that components of vegetables such as lycopene or antioxidants such as vitamin E and selenium may reduce the risk of prostate cancer. However, results examining dietary supplements have been mixed, possibly due in part to the difficulty in accurately recording the true intake of vitamins and minerals. Surprisingly, studies including the *Cancer Prevention Study* and the *National Institutes of Health-American Association of Retired Persons Diet and Health Study* both demonstrated that multivitamin users were at higher risk for fatal prostate cancer compared to those men who did not consume multivitamins.[331,332] One study, the *Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study* which supplemented alpha-tocopherol (50 mg/day) to elderly Finnish smokers, did demonstrate a decreased risk of prostate cancer.[333] However, the Selenium and Vitamin E Cancer Prevention Trial enrolling over 35,000 men, did not find an association between prostate cancer risk and either Vitamin E or Selenium.[334] In summary, prospective studies of dietary factors and supplements have not confirmed beneficial effects of any of these components.[335,336]

Chronic or recurrent inflammation may play a role in the development of prostate cancer. Prostatitis, diagnosed by symptoms, occurs in 9% of men between 40 and 79 years old.[320] Inflammatory cells release oxidants that could result in DNA damage and act as initiators of prostate cancer. A lesion called "proliferative inflammatory atrophy" has been proposed as a precursor to prostatic intraepithelial neoplasia (PIN) and prostate cancer.[337] Chronic inflammation is associated with proliferative inflammatory atrophy and signs of molecular stress such as high levels of glutathione-S-transferase A1 and cyclooxygenase-2 (COX-2). These observations suggest that inflammation increases oxidative stress which in turn causes DNA adducts which could then contribute to prostate cancer development.[320]

Hormonal factors and particularly circulating androgens likely play a role in the initiation and promotion of prostatic cancer. Men surgically orchiectomized early in life, historically Chinese Emperor-serving eunuchs or Italian Castrati singers,[338] end up with nonpalpable or rudimentary prostates and in theory would rarely develop prostate cancer, although the latter has been difficult to document in the published literature.[339]

Ross and colleagues suggested that the lower frequency of prostate cancer in Japanese and Chinese populations may relate to a lower level of 5-alpha reductase activity than in their Caucasian counterparts, perhaps on a genetic basis.[340] This could lead to lower levels of dihydrotestosterone (DHT) in prostatic tissue and less androgen induced proliferation. However, direct isotopic kinetic measurements of 5-alpha reductase activity in Chinese versus Caucasian men demonstrated no differences in the levels of this enzyme, at least in peripheral tissues.[341]

Clearly, the most important advancement of our understanding of the relationship between androgens and the prostate came from the work of Charles Huggins, eventually leading to his award of the Nobel Prize in 1966. The novelty of Huggins' findings was that hormones stimulated prostate tumors to grow and also enhanced tumor cell survival.[342] Huggins demonstrated that orchiectomy controlled tumor growth, at least temporarily in men with advanced metastatic prostate and prolonged their survival. Subsequent studies on Dominican Republic pseudo-hermaphrodites and other males who congenitally lack the type II 5-alpha reductase enzyme helped confirm the importance of DHT in prostate growth and development.[343,344]

Prevention

A large randomized, controlled trial, the *Prostate Cancer Prevention Trial (PCPT)* tested the hypothesis that reduction of DHT formation with finasteride,[345] an inhibitor of type II 5-alpha reductase, would reduce prostate cancer incidence. The trial, initiated in 1993 and enrolling 18,882 men aged 55 or older with a normal digital rectal exam (DRE) and initial PSA of 3.0 or lower, included 9060 men in the final analysis. A biopsy was performed at the end of the study (7 years) or for clinical indications (e.g., abnormal DRE or adjusted PSA of 4.0 or higher). At the end of 7 years of therapy, 4368 men receiving finasteride and 4692 receiving placebo were available for analysis. The finasteride group experienced a 24.8% reduction in prostate cancer diagnosis (95% CI: 18.6 to 30.6%; $P < 0.001$). However, tumors of Gleason grade 7 to 10 occurred in 15% more men receiving finasteride (280 of 757 tumors or 37%) than in those taking placebo (237 of 1068 tumors or 22%; $P < 0.001$). High-grade disease occurred in 6.4% of the finasteride group and 5.1% of the placebo group. Five deaths due to prostate cancer occurred in each study arm. Side effects in the finasteride group included reduced volume of ejaculate, erectile dysfunction, loss of libido, and gynecomastia ($P < 0.001$ versus placebo) but attributable risks of these side effects ranged only from 1% to 13%.

These results stimulated multiple follow-up studies to identify possible reasons for the finding of an increase in higher grade lesions. Possible explanations included pathological artifact, volume-grade artifact, or detection bias. One of the effects of finasteride is shrinkage of normal prostate volume to 50% of the original size. Therefore, it is theoretically possible that biopsy of larger prostates (i.e., men on placebo) would result in fewer high-grade cancers detected and that the true incidence would be similar between the two groups. Notably, this detection bias was demonstrated in retrospective reviews.[346,347]

If the increase in high-grade lesions were real and not artifactual, several mechanisms have been postulated. The results could be due to selection of androgen-independent clones induced by lower intra-prostatic DHT as has been observed in hypogonadal men with prostate cancer.[345,348] Polymorphisms of genes involved in estrogen metabolism that are associated with increased risk of prostate cancer could have been involved.[349] Since the Gleason grading system was created based on prostate tumors that were hormonally-naïve,[350] finasteride may have altered the diagnosis of high-grade cancer by modulating the influence of the epithelium.[351,352]

Despite the evidence that finasteride did decrease the incidence of low-grade prostate cancers, debate continues as to whether or not it should be used in prostate cancer prevention. Analysis of the lifetime implications, side effects (e.g., erectile dysfunction, loss of libido, and gynecomastia), and cost effectiveness of using finasteride for prostate cancer prevention does not appear to support its routine use.[353]

The reduction by dutasteride of prostate cancer events (REDUCE) trial[354] examined the possibility that blocking both type I and type II 5-alpha reductase might be superior to blocking only type II. Dutasteride, approved in 2002, blocks both type I and type II 5 alpha reductase subtypes and putatively might be superior for prevention of prostate cancer. Dutasteride in comparison with finasteride, induces a greater reduction in both serum[355] (94.7% versus 70.8%) and intra-prostatic[356] (greater than 90% versus 68% to 86%) DHT levels.[355,356] The REDUCE trial enrolled a total of 6729 men with the following characteristics: ages 50 to 75 years old, initial PSA of 2.5 to 10 ng/mL, and negative biopsies at the time of enrollment. Among the men randomized to 0.5 mg/day of dutasteride (n = 3305) or placebo (n = 3424), 659 and 858 developed prostate cancer within the 4-year study period demonstrating a 22.8% (95% CI: 15.2, 29.8) reduction in relative risk of prostate cancer in the treated group. Unlike the PCPT trial, however, no increased risk of high-grade cancers was noted with dutasteride.[357] Although the lack of an increase in high-grade cancer in the REDUCE trial would support that this finding was an artifact in the PCPT, these studies were designed differently. Most importantly, subjects in the REDUCE trial underwent a biopsy at the beginning of the study and were enrolled only if this was negative. Therefore, in the PCPT, some subjects may have harbored occult prostate cancer that went undetected until well into the study.

Epidemiological and secondary endpoint data suggested the possibility that other approaches such as supplementation of selenium, vitamin E, and lycopene might prevent prostate cancer but available data are not supportive. The *Selenium and Vitamin E Cancer Prevention Trial* (SELECT) randomized 34,887 men,[358] aged 55 years old or older (50 years old if African-American) with normal DRE and PSA of 4.0 ng/mL or less, to receive selenium, vitamin E, both agents, or placebo. No significant differences in prostate cancer incidence were observed and the study was stopped in 2008. Notably, extended follow-up through 2011 demonstrated an actual increased risk in prostate cancer development with vitamin E.[359]

Early Case Detection

DRE provided the only prostate cancer screening modality available prior to the PSA era and cancers then were

detected at more advanced stages. The introduction of serum PSA allowed detection of earlier stage disease. PSA is secreted into prostatic ducts under androgen influence and activation of PSA occurs upon cleavage of seven amino-terminal amino acids by the protease human kallikrein 2.[360] Although investigators have localized PSA to the periurethral glands of males and females as well as the apocrine sweat glands in males,[361] the vast majority of PSA is produced by prostatic acinar and ductal epithelium which secrete the protein into surrounding lumina. PSA circulates in the blood either free or complexed with the serum proteins, α2-macroglobulin or α1-antichymotrypsin.[362] A change in the serum concentration of PSA in an individual can signify an abnormal process such as cancer of the prostate. Alternatively, other benign entities can cause an increase in serum PSA such as benign prostatic hyperplasia (BPH), infection, inflammation (e.g., chronic prostatitis), ejaculation, urinary obstruction, and recent manipulation or "trauma" to the genitourinary tract (e.g., catheterization, prostate needle biopsy, or cystoscopy). A simple screening DRE, however, should not cause a significant elevation of serum PSA.

Although BPH results in a tenfold increase in prostatic tissue concentration of PSA, the presence of cancer leads to a tenfold increase in serum PSA secondary to epithelial membrane disruption.[363] The histological hallmark of prostate cancer formation is the disappearance of the basal cell layer and disruption of the luminal epithelial cell layer. This disruption allows PSA, normally secluded from the surrounding prostatic stroma, to "leak" into the serum through both lymphatic vessels and capillaries.

A serum total PSA value of 4 ng/mL or greater or an abnormal DRE are the accepted thresholds before proceeding to prostate needle biopsy, most commonly using transrectal ultrasound. Common practice involves obtaining biopsies of ultrasound detected-hypoechoic lesions as well as 12 cores from different areas of the prostate. As for sensitivity and specificity, the positive and negative predictive values of PSA will depend on the chosen threshold. Based on multiple investigations in patients with a normal DRE and a PSA value between 4 and 10 ng/mL, the positive predictive value (PPV) approximates 30%.[364-369] However, the PPV increases to approximately 60% when total serum PSA is greater than 10 ng/mL.[364,367,370]

Because lower stage prostate cancers can be indolent and of no clinical significance, screening with PSA has undergone considerable scrutiny. As shown in Figure 29.23 causes of death other than prostate cancer predominate as men age. This is particularly striking in tumors with low Gleason scores. For some clinicians, the decision to use a threshold of 4 ng/mL seems quite arbitrary. In order to improve its sensitivity, specificity, and overall performance as a biomarker in prostate cancer screening, multiple variations of PSA use have been introduced over time. These include PSA Density, the ratio of free to bound PSA in the bloodstream, PSA velocity, age-adjusted PSA, and PSA doubling time.

PSA Density

Due to concern that an elevated PSA may be the result of BPH and not prostate cancer (both diseases of "older" men), investigators advocated the use of PSA density to help distinguish between the two conditions.[371-374] PSA density is obtained by dividing the total serum PSA by total prostate gland volume (measured using transrectal ultrasound).[371,372] Although the optimal cutoff is unknown, men with BPH have a significantly lower PSA density compared to those with cancer. Benson and colleagues demonstrated that the mean PSA density was 0.04 in men with BPH and 0.58 in those with prostate cancer, respectively. No PSA density greater than 0.12 was seen in patients with BPH.[371,372] In addition, in patients with a PSA density greater than 0.10, 97% of subjects had prostate cancer.

Although, PSA density did seem initially promising and improved PSA screening specificity, subsequent studies have shown its use to miss an unacceptable amount of prostate cancer cases. A large multicenter trial demonstrated that, in men with PSA values between 4 and 10 ng/mL, 47% of those with prostate cancer would not be identified if the PSA density cutoff were 0.15.[290] This lack of sensitivity in PSA density may be secondary to additional sources of error including variability among ultrasound technicians as well as variability in sample analysis.[366] In addition to PSA density, one can measure Transition Zone PSA Density.[375] In patients with BPH, the primary site for prostate enlargement is the transition zone (TZ). Theoretically, by measuring the PSA density based on the TZ, the accuracy of using PSA density would increase. However, because measurement of TZ PSA density again requires the use of trans-rectal ultrasound and is even more subject to inter-technician variability, it has not gained widespread use.

Protein-Bound PSA

Serum PSA exists as either unbound (free) or bound to the protease inhibitors α1-chymotrypsin or α2-macroglobulin. Patients with prostate cancer have a higher proportion of α1-chymotrypsin bound PSA compared with men without prostate cancer.[376] To exploit this difference, assays were developed to detect the ratio of free to protein-bound PSA. Currently, a ratio of free PSA to total PSA less than or equal to 10% is indicative of cancer while a ratio greater than 20% is likely due to a benign cause such as BPH. The assay is most useful in patients with total PSA values between 4 and 10 ng/mL.[377] Unfortunately, many men exhibit free to total ratios between 10% and 20% thereby hampering the full utility of this assay.

PSA Velocity

The rate of serum PSA increase (PSA velocity or PSAV) can be used to detect prostate cancer. Although questions exist concerning its predictive value,[378,379] PSAV has been shown to be significantly different between men with and without cancer. Current recommendations are to proceed to biopsy if there is an increase in total PSA of 0.75 ng/mL or greater per year with at least three different measurements over an 18- to 24-month period.[378,380-383] Using the cutoff point of 0.75 ng/mL leads to a sensitivity of 72% and a specificity of 90% in men with occult prostate cancer and a PSA less than 10 ng/mL. Unfortunately, the utility of using PSA velocity as a screening tool may be limited because of potentially large intra-individual variability, specifically in the context of shorter time intervals and lower total PSA.[381,384,385] A number of men with occult prostate cancer, especially those with a total PSA less than 4 ng/mL, do not have a PSA increase of 0.75 or greater in 1 year.[386]

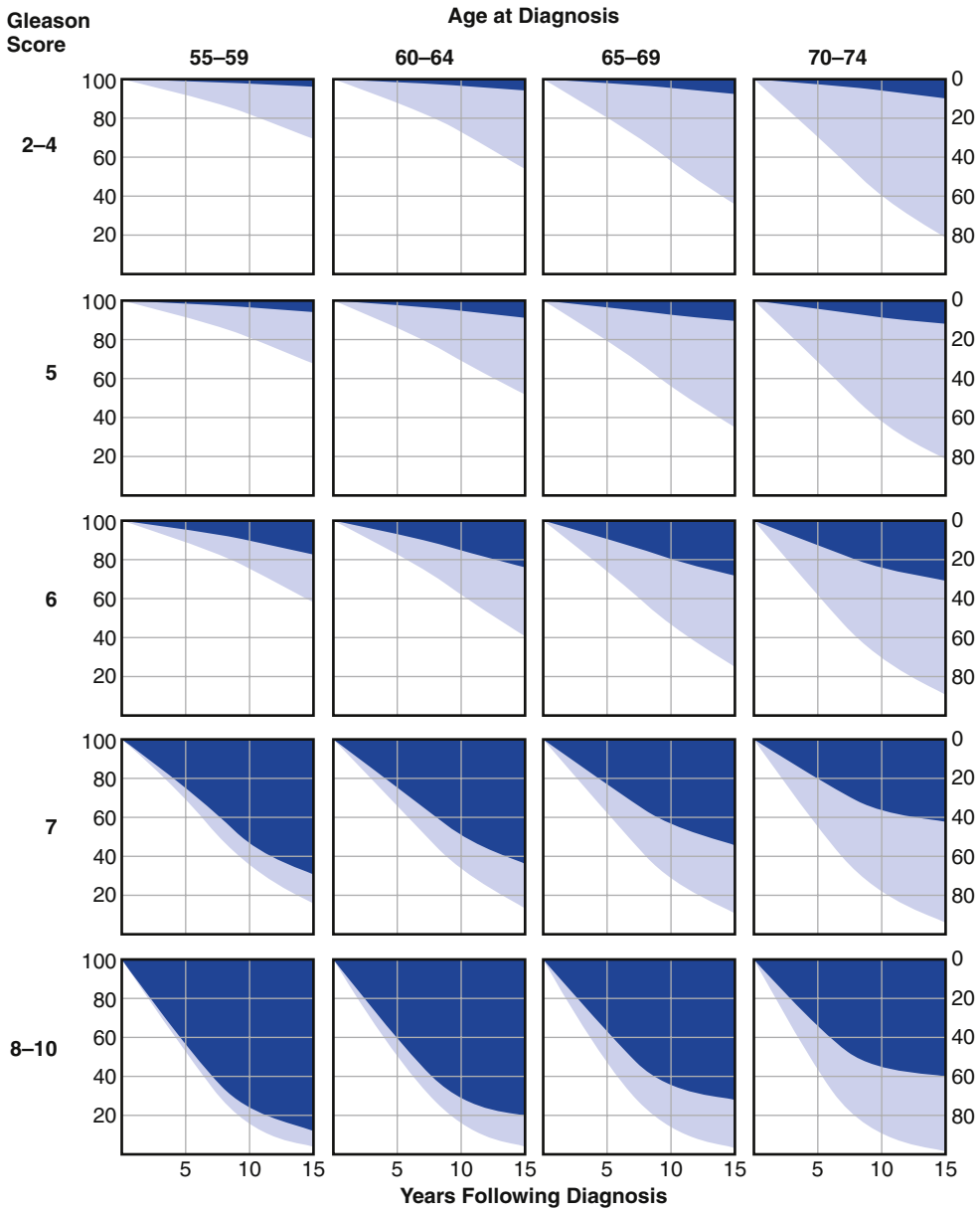


FIGURE 29.23 Total mortality *(shaded areas)* versus prostate cancer specific mortality *(solid areas)* in men of ages 55 to 74 years old at time of diagnosis and as a function of Gleason score. *(From Santen R. Endocrine responsive cancer. In Kronenberg HM, Melmed S, Polonsky KS, Larsen PR [eds].* Williams Textbook of Endocrinology, *11th ed, Philadelphia, WB Saunders, 2008.)*

Age Adjusted PSA

Normal serum PSA values increase with age. Therefore, an increase in serum PSA in a younger man due to prostate cancer may not reach the biopsy threshold of 4 ng/mL. Studies have shown that 22% of men with PSA values between 2.6 and 4 ng/mL have cancer of the prostate.[387] Therefore, one recommendation is to use age-specific PSA values to better capture younger men who have occult prostate cancer but a PSA value less than 4 ng/mL.[388,389] Theoretically, by creating age-specific thresholds, more younger men (age less than 60 years) with prostate cancer would be identified while more older men (age greater than 60 years) without prostate cancer would be spared a biopsy.[390,391]

Multiple studies examining this issue have demonstrated that while sensitivity would indeed be increased in younger men through the use of age-specific thresholds, the sensitivity would be significantly decreased in older men. Osterling and colleagues, comparing the use of age-specific PSA against the standard PSA greater than 4 ng/mL in men 50 years old or older, found that the positive predictive value increases from 37% to 42% with age-specific PSA. However, the detection rate of cancer decreased to 3.8% from 5.7%.[391,392] In another study examining 6600 men 50 years old or older, investigators found that by increasing the age-specific threshold, 47% of localized prostate cancer was missed, although there was a decrease of 44% in the number of unnecessary biopsies. Overall, while age-specific PSA does offer the advantage of decreasing the number of biopsies in older men without prostate cancer, the number of cancer cases missed significantly limits its value in prostate cancer screening.

PSA Guidelines

Various professional societies have provided guidelines for PSA screening, both for and against, but no general agreement exists.[393,394] Additionally, randomized trials have demonstrated conflicting results. The *European Randomized Study of Screening for Prostate Cancer* involved

Table 29.6 AJCC Prostate Cancer Stage Groupings

	Stage 1		Stage IIa					Stage IIb			Stage III	Stage IV		
Jewett-Whitmore stage	A1		A2, B0-2								C1-3	D1		D2
TNM stage	T1a-c N0M0	T2a N0M0	T1a-c N0M0	T1a-c N0M0	T2a N0M0	T2a N0M0	T2b N0M0	T2c N0M0	T1-2 N0M0	T1-2 N0M0	T3a-b N0M0	T4 N0M0	Any T N1M0	Any T Any N M1
Gleason score	≤6	≤6	7	≤6	≤6	7	≤7	Any	Any	≥8	Any	Any	Any	Any
PSA level (ng/mL)	<10	<10	<20	10-19.9	10-19.9	<20	<20	Any	≥20	Any	Any	Any	Any	Any

over 182,000 men from eight European countries and ages 50 to 74 years old at the time of study entry.[395] At a median follow-up time of 11 years, the authors demonstrated a significant reduction in cause-specific mortality (relative reduction of 21% in PSA-screened group) but no significant reduction in overall mortality. The *Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial*, with an updated follow-up 13 years after enrollment of 76,685 men between 55 and 75 years old, demonstrated no significant difference in mortality based on PSA screening.[396]

A common-sense approach suggests screening only when test results would dictate diagnostic and therapeutic decisions. The patient needs to understand the consequences of a positive test and be willing to proceed with further diagnostic and therapeutic measures if the PSA were positive. Accordingly, patients selected for screening should be those in whom definitive treatment or hormonal manipulations and not surveillance would be the likely choice if cancer were detected. Based upon this reasoning, informed consent of the patient is required before embarking on screening with PSA.

Repeat Biopsy

Men with borderline PSA values (2.6 to 4.0 ng/mL) often undergo prostate biopsy under ultrasound and, in 75% of those men biopsied, no evidence of prostate cancer is found. When to re-biopsy if no cancer is found remains an unresolved but important clinical question. A recent study analyzed the records of 24,893 men being followed as part of a community based screening study and identified 1011 with subsequent prostate cancer. Based upon these data, the authors recommended repeat biopsy in men with a PSA of 2.6 to 4.0 ng/mL if they had high-grade PIN, initial PSA of 3.6 to 4.0 ng/mL, abnormal DRE, a family history of prostate cancer or a PSA velocity of 0.1 ng/mL or greater.[397] The complication rate of ultrasound-guided biopsy is reasonably low but does increase slightly when 10 or 15 cores are taken rather than 6.

Clinical Staging

Clinical staging of prostate cancer provides a means of determining prognosis and is used for making treatment decisions. Two analogous systems have been used in the past but the TNM classification is now preferred (Table 29.6). Newer modalities, including Tesla and Endorectal MRI are now being used to better define tumor size and assess for extra-prostatic extension.[398,399]

Decisions Regarding Definitive Treatment

Risk Stratification

Logical choices among various therapeutic options require assessment of the likelihood that complications and death from prostate cancer will ensue. Several algorithms provide a means to assess risk; the most widely used is the Partin nomogram developed at Johns Hopkins Hospital and based on patients undergoing radical prostatectomy.[400] The Partin nomogram predicts the risk of extraprostatic extension, lymph node involvement, and seminal vesicle involvement based on pre-operative PSA, Gleason score at biopsy, and clinical T stage. The National Comprehensive Cancer Network (NCCN) has devised another practical risk stratification system (Fig. 29.24) which initially divides patients into clinically localized, locally advanced and metastatic disease; then into low, intermediate and high risk subgroups; and finally according to expected survival of less than or greater than 10 to 20 years. The purpose of the NCCN guidelines is to better enable the physician to guide appropriate therapies.[401] One underlying but nonetheless important principle in assessing patient risk is the hallmark spectrum of biological aggressiveness exhibited by prostate cancer. At one end of the spectrum are "latent" prostate cancers, defined as small tumors less than 0.5 g that will not progress or result in cause-specific death. On the other end of the spectrum are large bulky tumors with high Gleason grade which most always rapidly progress. A key consideration is that older men frequently die of other existing medical conditions and not prostate cancer itself (Fig. 29.23). In men with a limited life expectancy as a result of other medical conditions, the risks of definitive therapy for prostate cancer most likely outweigh any foreseen benefits. As a practical guide, the NCCN has established guidelines for initial assessment, staging, and recurrence risk for prostate cancer (Fig. 29.25).

Definitive Treatment Strategies in Localized Disease

Three competing strategies are recommended for management of localized prostate cancer: active surveillance, radical prostatectomy, and radiotherapy. A key question is whether radical prostatectomy prolongs disease-free (DFS) and overall survival (OS) when compared to surveillance. This issue has recently been examined in a randomized, controlled, clinical trial comparing radical prostatectomy to surveillance in men with clinically localized disease.[402] Six hundred and ninety-five men with localized prostate cancer, a life expectancy of greater than 10 years, and a mean age of 64.7 years

Recurrence Risk	Recurrence Risk	Recurrence Risk
Clinically Localized:	Clinically Localized:	Clinically Localized:
Very Low: • T1c • Gleason score ≤6 • PSA <10 ng/mL • Fewer than 3 prostate biopsy cores positive, ≤50% cancer in any core • PSA density <0.15 ng/mL/g		High: • T3a or • Gleason score 8–10 or • PSA >20 ng/mL
Low: • T1-T2a • Gleason score ≤6 • PSA <10 ng/mL	Intermediate: • T2b-T2c or • Gleason score 7 or • PSA 10-20 ng/mL	Locally Advanced: Very High: T3b-T4
		Metastatic: Any T, N1 Any T, Any N, M1

FIGURE 29.24 Risk stratification method of National Cancer Center Network (NCCN). PSA, prostate specific antigen.

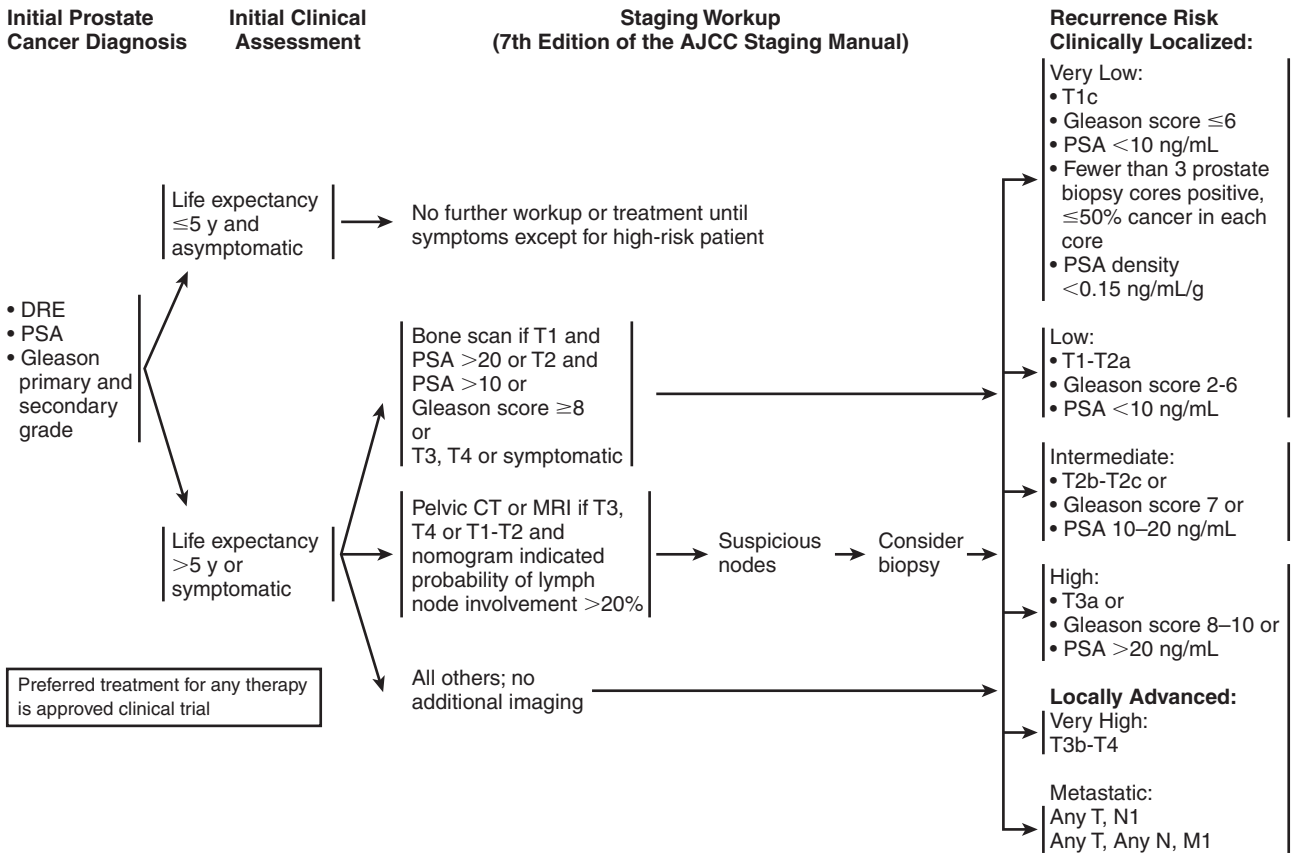


FIGURE 29.25 National Comprehensive Cancer Network (NCCN) guidelines (2012) for initial assessment, staging, and recurrence risk for prostate cancer.

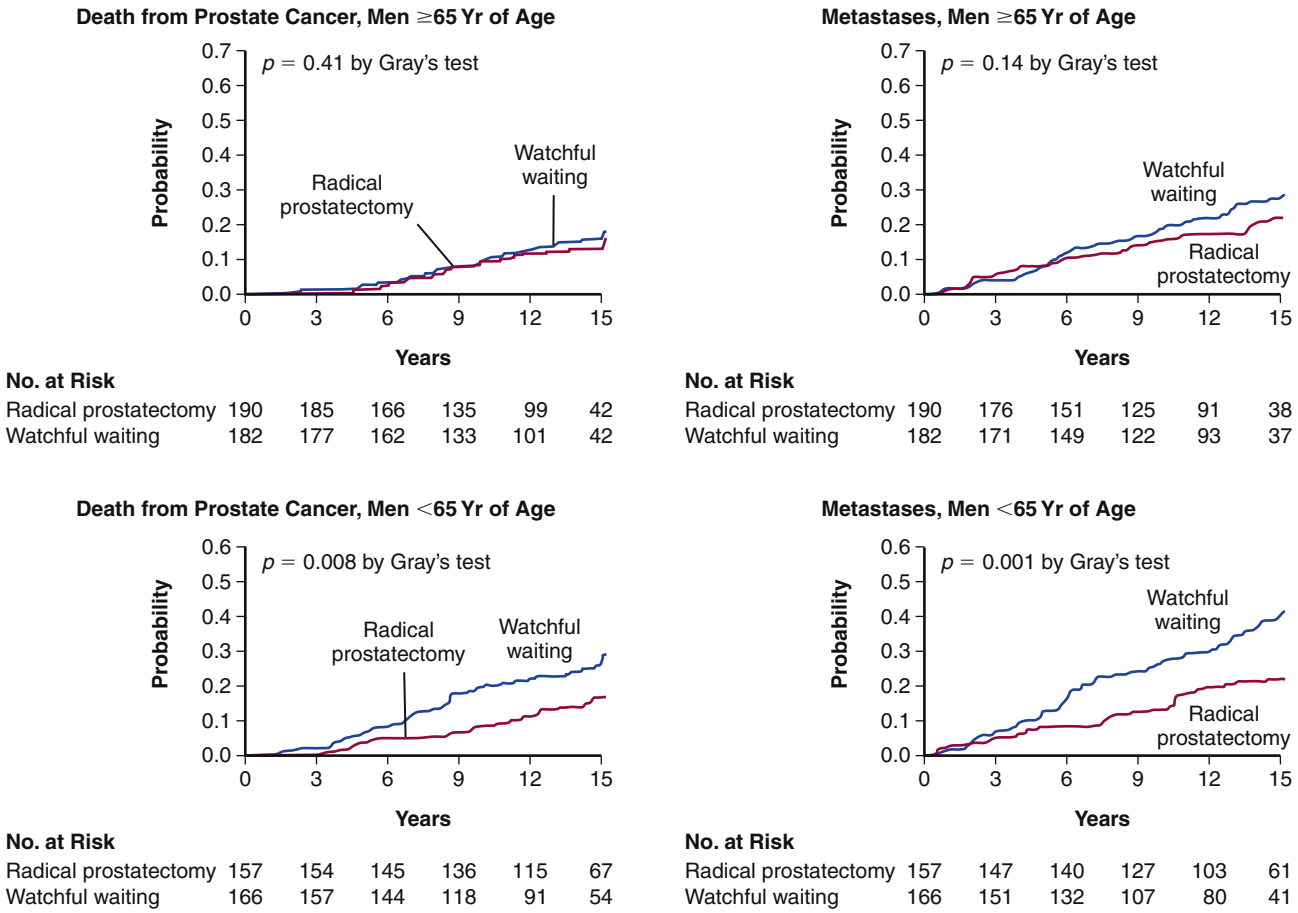


FIGURE 29.26 Death from prostate cancer in men treated with watchful waiting versus radical prostatectomy. Panels labeled as to specific subgroups examined. *(From Bill-Axelsen A, et al. Radical prostatectomy versus watchful waiting in early prostate cancer,* N Engl J Med *364[18]: 1708–1717, 2011.)*

were randomized and followed for a median of 8.2 years (Fig. 29.26). At the time of the initial study, 83 men in the surgery group and 106 in the surveillance group died of all causes ($p = 0.04$). Death was due to prostate cancer in 8.6% of the surgical group and 14.5% of the surveillance group. The difference in death rates became greater at the 10-year time period (5.3 percentage points) than at the 5-year time frame (2.0 percentage points). The risk of distant metastases was reduced to 1.7% from 10.2% by surgery and the rate of local progression from 25.1% to 19.1%. Further follow-up in 2008 and 2011 confirmed the reduction in death from prostate cancer in patients undergoing surgery.[403,404] Although this study does demonstrate improvement in both OS and DFS with surgery, the effect is rather small. Nonetheless, the decrease in incidence of metastases and local tumor progression can be considered clinically significant.[402,404]

Observational studies over several decades demonstrated the efficacy of radiotherapy (both delivered in the conventional external beam format and as brachytherapy) as definitive therapy in men with prostate cancer and as an alternative to surgical prostatectomy. Recent studies addressed whether neo-adjuvant or adjuvant androgen deprivation therapy enhances the overall efficacy of therapy in men with aggressive disease. Radiation Therapy Oncology Group (RTOG) trial 86-10 examined the role of neo-adjuvant endocrine therapy prior to radiation. The incidence of local progression at 5 years was 46% for patients with T2-4 tumors receiving adjuvant hormonal therapy and 71% for those receiving radiation alone ($p = 0.001$) but no difference in overall survival (OS) was noted. Studies examining neoadjuvant hormonal therapy prior to prostatectomy demonstrate no change in cause-specific survival but do show lower risk of positive margins at the time of surgery. Current clinical trials examining neoadjuvant abiraterone, an irreversible inhibitor of 17 α-hydroxylase/C17, 20 lyase (CYP17A1) that is currently approved for management of castration-resistant prostate cancer, are currently underway.

Radiotherapy with concomitant (adjuvant) androgen deprivation therapy has been the subject of two randomized trials.[405,406] A RTOG study involved patients with T3 and T4 disease given radiation therapy followed by medical castration with goserelin starting at the last week of irradiation.[407] Disease-free survival (DFS) at 5 years was 60% for the goserelin arm and 44% for radiation alone ($p < 0.0001$). Only the subset of men with Gleason grade 8 to 10 tumors experienced a survival benefit from 66% to 55% ($p = 0.03$). However, the 10-year follow-up demonstrated a survival benefit for all patients treated with adjuvant hormonal therapy on multivariate analysis (HR 1.3; $p < 0.001$). DFS was also improved (HR 2.2; $p = 0.0003$).[407] In a similar EORTC trial, the 5-year DFS was 85% in the adjuvant hormone treated group (goserelin) versus 48% in the radiation alone group ($p = 0.001$). Importantly, OS was significantly increased in the adjuvant hormonal group (79% versus 62%; $p = 0.001$).

The majority of data favor the use of adjuvant hormonal therapy in patients undergoing radiation therapy as definitive treatment for prostate cancer but only for high risk localized or locally advanced disease.[408,409] Analysis of pooled data from the RTOG neo-adjuvant and adjuvant trials concluded that the key consideration may be the necessity for long-term rather than short-term hormonal therapy in either neo-adjuvant or adjuvant therapy approaches.[409] In those men receiving short-term hormonal therapy (goserelin and flutamide for 2 months before and 2 months after radiotherapy), statistically significant improvements were observed for the endpoints of distant metastasis-free and no evaluable disease (NED)-free intervals but not for OS. Only long-term adjuvant therapy improved OS and only in the subset of men with Gleason Grade 7 to 10 tumors. These conclusions on long-term versus short-term adjuvant hormonal therapy were further supported by a nonrandomized Canadian trial.[410]

Brachytherapy, conformal radiation therapy and intensity modulated radiation therapy (IMRT) provide approaches that minimize side effects and allow for higher radiation doses to the cancer tissue itself.[411-413] Current guidelines suggest the use of conformal or IMRT in preference to standard dosimetry methods. IMRT allows dose intensification that may or may not eliminate the need for adjuvant hormonal therapy. Brachytherapy with implanted seeds provides another useful alternative.

Use of anti-androgen therapy is not indicated for clinically localized disease. The *Early Prostate Cancer (EPC)* program examined the use of 150 mg of bicalutamide in addition to standard treatment (e.g., radiation, prostatectomy, or surveillance) for clinically localized disease (71% of 2285 patients) or locally advanced but nonmetastatic disease (29%).[414] At a median follow-up time of 9.7 years, PFS was significantly improved in the bicalutamide group (HR 0.85; $p = 0.001$) although there was no significant improvement in OS. Patients with locally advanced disease treated with radiotherapy derived the greatest benefit in terms in OS. However, patients with localized disease did not benefit in terms of either PFS or OS.[414]

Locally Advanced or Metastatic Disease

Androgen deprivation represents the earliest form of systemic therapy for locally advanced and metastatic prostate cancer and observational studies have demonstrated its efficacy. Three methods have been historically used: surgical castration, high-dose estrogen, and GnRH agonist analogues. Surgical castration as well as high-dose estrogen in the form of DES has been used as treatment of prostatic cancer since the 1940s. In the VACURG studies, 5 mg of DES decreased the rate of recurrence of prostate cancer but increased the cardiovascular death rate. Following this observation, careful dose response studies indicated that 3 mg of DES daily minimizes the risk of cardiovascular disease acceleration and maximizes the beneficial effects on prostate cancer. Gynecomastia and impotence are the major side effects. However, DES is no longer available in the United States.

GnRH superagonist or antagonist analogues are now available which suppress testosterone to castrate levels and cause tumor responses equivalent to those induced by castration. Comparison of the various monotherapies was the subject of a meta-analysis involving 10 separate trials which concluded that orchiectomy,[415] GnRH agonist analogues, and DES produced equivalent survival in men and that no differences existed among the various GnRH analogues currently available. The decision between surgical castration and GnRH analogues (GnRH-A) represents a major question. While surgical orchiectomy produces a rapid decrease in serum androgen levels, it does not require patient compliance long-term, and is effective in inducing tumor regression in nearly 90% of patients, more than half of men in the United States prefer medical castration as a means to avoid surgery, most likely due to the psychological impact of castration.

Highly potent agonist analogs of GnRH have been approved for use in prostate cancer and effectively produce a "medical orchiectomy." These compounds paradoxically inhibit LH secretion by the pituitary suppressing testicular testosterone production. Initially, GnRH-A administration increases LH threefold to fourfold and testosterone twofold for 1 to 2 weeks. Thereafter, LH is profoundly suppressed and plasma testosterone levels fall from approximately 500 ng/dL to castrate levels of 15 ng/dL. Hormonal inhibition continues for up to 2 years of continuous therapy. The initial rise in testosterone causes a transient disease flare in 5% to 10% of patients, producing an objective increase in tumor size in approximately 3% of patients and a subjective increase in bone pain from metastases in the remainder. Although tumor flare is usually transient, severe reactions with spinal cord compression or death have been observed in the occasional patient. Based on this, it is necessary to administer an anti-androgen such as bicalutamide during the initial few weeks of GnRH-A therapy.

Newer GnRH receptor antagonists have been developed to block the interaction of GnRH with its receptor on the pituitary gland resulting in an immediate and substantial reduction in LH and testosterone levels without causing the flare phenomenon. In a phase III, randomized clinical trial,[416] degarelix, approved by the FDA in 2008 for patients with advanced prostate cancer, demonstrated equal effectiveness compared to leuprolide at suppressing testosterone to castrate levels. Notably, degarelix suppressed levels of both testosterone and PSA significantly faster and no patient experienced a testosterone surge compared to 81% of leuprolide-treated patients.

The major rationale for using a GnRH-A is to induce castrate testosterone levels without requiring surgical removal of the testes. Both testosterone and DHT levels in patients treated with either a GnRH-A or orchiectomy fall to a similar extent. Objective regressions occur as frequently as with orchiectomy and a meta-analysis study including 10 randomized controlled trials with 1908 patients,[415] provided evidence that medical and surgical castration exert equal clinical effects. Initially, a major problem with GnRH-A therapy was the requirement for daily subcutaneous administration with the possibility of noncompliance with daily injections and incomplete androgen suppression. Third-generation formulations are now available which allow injections at 3- to 6-month intervals or implants yearly. These biodegradable preparations appear highly effective, well tolerated, and acceptable to patients. Because some androgen responsive cells remain in prostate tumors after relapse on the GnRH agonists, continued GnRH-A treatment is advocated upon disease relapse and preferably for life.

Immediate versus Delayed Hormonal Therapy for Locally Advanced or Metastatic Disease

Historically, approximately 25% of men presented with either metastatic or lymph node positive disease. However, currently less than 5% of men present with synchronous metastatic disease.[417] Whether to initiate hormonal treatment immediately in these patients or to defer until symptomatic is a major question, especially in light of recent evidence demonstrating increased cardiovascular disease and mortality with use of androgen deprivation therapy.[418-421] Because cure with hormones is not possible, the two goals of treatment are to increase life expectancy and to relieve symptoms. If treatment were to be advocated in asymptomatic patients, the therapy should improve the length of patient survival. Rigorously controlled series from the Veterans Administration Cooperative Urological Research Group (VACURG) trials during the 1960s indicated no clear survival benefit from endocrine therapy in patients with stage C or D disease (i.e., advanced) at the time of diagnosis.

Based upon these findings, standard practice usually involved withholding hormonal therapy until symptomatic metastatic disease developed. However, two studies provide evidence favoring therapy immediately upon detection of metastatic disease. Messing and colleagues studied men with prostate cancer found to have positive pelvic lymph nodes at the time of radical prostatectomy.[422] The study randomized men into either an immediate therapy group (medical or surgical orchiectomy) or into a group observed until an indication for hormonal treatment occurred. Death due to prostate cancer occurred in 3/47 men in the immediate treatment group and 16/51 in the deferred group ($p < 0.01$). At a median follow-up of 11.9 years in 2006,[422] there was continued benefit of early androgen deprivation in OS, DFS, and CSS. In another study, 934 men with locally advanced prostate cancer or with asymptomatic metastatic disease were randomized to immediate vs. deferred hormonal therapy. An initial report concluded that there was an OS benefit from immediate therapy. Overall, 203 men died from prostate cancer in the immediate therapy arm and 257 in the deferred group ($p = 0.02$). However, on extended follow-up, the OS benefit was no longer statistically significant. Regardless, deaths due to prostate cancer were reduced to 241 in the immediate therapy arm versus 287 in the deferred arm ($p = 0.0019$).[423] In this group of elderly men, deaths from other causes occurred commonly and were not influenced by immediate hormonal therapy. Nonetheless, there were fewer cases of cord compression (10 versus 24); pathological fracture (14 versus 22), extraskeletal metastases (47 versus 62), and ureteral obstruction (49 versus 64) in the immediate treatment group.[423]

Another study,[424] randomized men not accepting radical prostatectomy to immediate or deferred androgen therapy and found no differences in overall pain free time and performance status. CFS tended to be longer in the immediate group ($P = 0.09$) but OS was identical ($P = 0.96$). Other evidence of efficacy of early therapy comes from nonrandomized studies from the Mayo clinic which demonstrated a statistically significant survival advantage for early endocrine therapy (castration) in men with stage D1 (T0-3, N1-2, M0) disease.[425] Seventy-three patients underwent either radical prostatectomy or radical prostatectomy combined with orchiectomy. An advantage for immediate adjuvant therapy was demonstrated as 5-year survival rates were 93% in the immediate orchiectomy group and 80% in the deferred orchiectomy group. In another study,[426] benefit was seen only in patients with diploid and not in those with tetraploid or aneuploid tumors. Finally, a subset analysis of men in the radiation therapy oncology group trial number 85-31 (RTOG 85-31) study examined a group of 139 men with capsular penetration or seminal vesicle involvement; 71 men received RT plus LH-RH agonist therapy and 68 RT alone.[427] Statistically significant improvement in PFS and freedom from biochemical relapse (i.e., PSA), was observed in the LH-RH group. However, there was no statistically significant difference in OS in this relatively small subset of men followed for a median of 5 years only.

A systematic review in 2002 concluded that early androgen deprivation therapy resulted in significant reductions in both disease free progression and complications due to progression.[428] PFS was significantly higher at years 1, 2, 5, and 10 (OR 3.99, 4.79, 3.15, and 3.49, respectively) in men treated with early androgen deprivation and OS at 10 years was also improved (OR 1.5; 95% CI 1.04, 2.16). Taken together, these data suggest that immediate hormonal therapy may be efficacious for asymptomatic men presenting with lymph node spread. Nonetheless, in elderly men with increased risk of dying of non-prostate cancer causes, deferred therapy appears to be a reasonable choice.

The American Society of Clinical Oncology (ASCO) critically analyzed these data and commented that all of the trials cited above were conducted before the routine use of PSA testing. The ASCO review was considered to be limited by the variability in the interventions as well as in the stages of the cancers of the patients enrolled. From this analysis, the guidelines concluded that data at the present time do not provide definitive evidence that early endocrine therapy is beneficial regarding improved survival of patients presenting with metastatic disease. Longer follow-up and additional studies sufficiently powered to detect an increase in overall survival are needed before definitive conclusions are warranted.[429,430]

Men who experience a rise in PSA after an initial fall to undetectable levels after radical prostatectomy might also benefit from hormonal therapy. Data indicate that a rapid rise in PSA suggests the presence of metastatic disease whereas a slow rise suggests local recurrence. In a single small study, 68% of men progressed to detectable clinical disease upon observation for a median of 19 months. With adjuvant hormonal therapy or radiotherapy, the rate of progression to clinically detectable disease decreased to 21%.[431]

Adverse Effects of Androgen Deprivation Therapy

Medical or surgical castration causes an abrupt reduction of androgen levels resulting in hot flashes in up to 80% of patients, 27% of whom report this to be the most troublesome adverse event. Bone density falls in men in response to declines in androgens and their aromatized estrogen metabolites and the risk of fracture increases starting 1 year after initiation of therapy. Either pamidronate or zoledronic acid, both potent intravenous bisphosphonates, can

Table 29.7 Secondary Hormonal Therapies for Recurrent Disease

Modality	Responders	Total Patients	Percent Responding	Clinical Setting
Flutamide	23	100	23%	First relapse after medical or surgical orchiectomy
Prednisone	21	101	21%	First relapse after medical or surgical orchiectomy
Flutamide withdrawal	29	138	21%	Relapse after combined androgen blockade or flutamide
Aminoglutethimide + hydrocortisone	14	29	49%	After anti-androgen withdrawal
Hydrocortisone	16	82	20%	After anti-androgen withdrawal
Ketoconazole Hydrocortisone	43	72	60%	After anti-androgen withdrawal
Megestrol l acetate	17	119	14%	After anti-androgen withdrawal
DES	71	243	29%	After anti-androgen withdrawal
Abiraterone	797	231	29% (PSA response); improved survival 14.8 months vs placebo 10.9 months ($P < 0.001$)	After anti-androgen withdrawal and docetaxel

The full version of this table, including references, can be found on the companion Expert Consult web site at www.expertconsultbook.com.

completely abrogate the effects of androgen deprivation on bone. Significant erectile dysfunction occurred in 78.6% and 73.3% of men after orchiectomy and GnRH-A therapy, respectively.[432] Other metabolical effects that occur as a result of androgen deprivation therapy include a decrease in hemoglobin and lean body mass, increase in total body fat mass and rises in total cholesterol and triglyceride levels. Fatigue and psychological distress tend to be more common in men receiving active therapy compared to those choosing to defer treatment. Finally, gynecomastia occurs in 1% to 16% of men treated with androgen deprivation therapy.

Secondary Hormonal Therapies for Recurrent Disease

Disease that recurs following medical or surgical castration can be treated with secondary hormonal therapies. Prior to the PSA era, the clinician had to rely on bone x-rays and soft tissue changes to document responses and these were rarely observed. Consequently, the efficacy of secondary hormonal therapies was controversial. More recently, clinicians have accepted a 50% decline in PSA as reflecting an objective response to therapy. Studies demonstrate that this PSA endpoint predicts a significantly prolonged median overall survival, objective progression free survival and time to pain progression.[433] Using PSA measurements, it has now been possible to show efficacy of a number of secondary hormonal therapies. Responses to anti-androgens, ketoconazole, abiraterone, flutamide, bicalutamide, aminoglutethimide, DES, and glucocorticoids alone range from 14% to 60%. Head to head comparisons are unavailable for these agents and relative efficacy cannot be compared[434] (Table 29.7).

Anti-Androgen Withdrawal

Experimental studies with LnCAP cells *in vitro* detected a mutation in the androgen receptor in this cell line that caused it to paradoxically respond to flutamide with increased proliferation. As a result of this observation, clinical studies examined whether tumors in some patients might adapt to flutamide by developing mutations allowing flutamide to behave as an androgen agonist. In support of this possibility, approximately 40% of men on flutamide experienced tumor regression after withdrawal (so called withdrawal responses). These data suggest that a first step in patients receiving flutamide, either as part of a complete androgen blockade regimen (discussed later in the text) or as secondary hormonal therapy, is to stop flutamide. These withdrawal responses occur with bicalutamide as well but are less common.[435]

Other Secondary Hormonal Therapies

Prior to the PSA era, objective regression, stabilization and/or symptomatic relief were reported with several agents (Table 29.7). These included a high dose formulation of DES (stilphostrol) and tamoxifen. A study using high dose tamoxifen (160 mg/m2/day) observed only a 3.3% rate of objective response based upon PSA in heavily pretreated men with prostate cancer.[436] Each of these agents could be considered for patients relapsing slowly after castration. Recent emphasis has focused on blockade of adrenal androgens that contribute to intra-prostatic DHT. Ketoconazole, an agent which blocks the 17α-hydroxylase/C17, 20 lyase complex was initially in combination with glucocorticoid replacement used for this purpose. A more recent development has been the drug abiraterone which irreversibly blocks the 17α-hydroxylase/C17, 20 lyase complex. Clinical trials have shown abiraterone to increase survival,[437,438] with minimal side-effects, in post-chemotherapy, hormonal refractory patients. In a phase II study by Morris and colleagues, 58 patients with progressive metastatic prostate cancer despite docetaxel therapy were treated with abiraterone (1000 mg daily) plus prednisone (5 mg twice daily). A greater than 50% decline in PSA levels was noted in 36% of patients, partial responses were seen in 18% of patients, and improved performance status was observed in 28% of patients. Based on initial data, phase III trials are underway but at present, abiraterone is now clinically approved for use in castrate men who have failed chemotherapy.

Other Nonhormonal Treatment Measures

Potent bisphosphonates effectively reduce the incidence of skeletal related events in men with hormone refractory

prostate cancer. A large, randomized phase III trial demonstrated that zoledronic acid significantly reduced the incidence of pathological fractures, spinal cord compression, the need to treat with radiotherapy for bone, and bone pain in men with prostate cancer. The dose used was 4 mg every 3 weeks for 24 months. The number with an SRE (skeletal related event) decreased from 49% for placebo to 38% with zoledronic acid ($P = 0.28$).[439] Based on these data, it is recommended that zoledronic acid be routinely used in patients with hormone refractory prostate cancer who have at least one bone metastasis. In patients with no lesions, routine bone scans should be used to detect new lesions and zoledronic acid started when the initial lesion is found.

Treatment of Hormone Refractory Recurrent Disease

At a certain point, the tumor becomes invariably refractory to all hormonally based therapies. Unfortunately, diagnostic tests are nonexistent to distinguish between hormone dependence and independence. Men with a rapid downhill course with widespread systemic metastases should probably receive chemotherapy. Others will have already been treated with all available options of endocrine therapy. Chemotherapy, combination chemo-hormonal therapy, or experimental targeted therapy is chosen at that point. The choice of chemotherapeutic agents is beyond the scope of this chapter and the interested reader is referred to the National Comprehensive Cancer guidelines.[401]

Alternative Approaches to Prostate Cancer Therapy

Monotherapy with anti-androgens, either steroidal or non-steroidal, provides an alternative to surgical or medical castration. Non-steroidal agents, flutamide and bicalutamide, bind to androgen receptors and block the cellular effects of circulating T and DHT on cell proliferation. Interruption of the androgen negative-feedback system results in reflex increments in serum LH, T, and DHT levels. Prior studies with anti-androgens suggested that these agents partially preserve erectile function. However, data suggest that only 20% of all treated men maintain morning erections and sexual activity with anti-androgen therapy. When only men capable of sexual function before treatment are considered, 18% reported a reduction in sexual function score on bicalutamide and 37% after castration.[440] Side effects occur in 4% to 10% of patients and include hot flashes, gynecomastia, hepatotoxicity, nonspecific ophthalmological changes, and diarrhea (particularly for flutamide).[429] Bone density improves in men receiving bicalutamide, most likely secondary to the 146% increase in estradiol and the fact that estradiol is the major mediator of bone density in men.[441]

Randomized trials have compared anti-androgens as monotherapy with medical or surgical castration. Initial studies showed that 50 mg of bicalutamide is not as effective as orchiectomy.[442] Later studies utilized 150 mg of bicalutamide and compared medical or surgical castration with 150 mg of bicalutamide in patients with locally advanced (T1-4, N+, MO) and metastatic disease.[443] Bicalutamide was equivalent to medical or surgical castration for locally advanced but not metastatic disease.[440] Pooled mature data from these studies suggest no survival difference between bicalutamide 150 mg daily and surgical castration. Other available data suggest that bicalutamide is not as effective as castration.[442] Meta-analyses of studies involving 2717 patients comparing monotherapy with various anti-androgens suggested that the hazard ratio for recurrence was greater with anti-androgen monotherapy than with medical or surgical castration but statistical significance was approached but not reached (HR 1.22, 95% CI 0.99, 1.50).[444] Even though anti-androgen monotherapy appears not as effective as castration, some men may choose this therapy in preference to surveillance.[443] The ASCO guidelines include an option for discussion of monotherapy with a nonsteroidal anti-androgen as an alternative to surgical castration or GnRH-A. This recommendation was based upon the data indicating less toxicity with respect to libido and physical capacity but inferior time to progression of disease compared to the LH-RH agonists.[429]

Neo-Adjuvant Hormonal Therapy Prior to Radical Prostatectomy

This approach involves either medical castration with GnRH analogues, steroidal or non-steroidal anti-androgens, or a combination of these agents prior to prostatectomy. Neoadjuvant strategies used for 3 to 6 months prior to radical prostatectomy result in an increase in organ confined cancers and decrease in positive surgical margins.[445] However, no differences in PSA detectable relapse rates have yet been observed in several trials. PFS and OS with neoadjuvant strategies are comparable to other modalities.[446,447] The recent NCCN guidelines strongly discourage this approach.[401]

Complete Androgen Blockade

A more comprehensive strategy for the endocrine treatment of prostate cancer, called complete or maximum androgen blockade (MAB), has been proposed. The rationale rests upon three considerations:

1. The adrenal glands contribute 5% of the total androgen pool.
2. The concentrations of DHT in prostate cancer tissue of patients fall by only 50% to 80% after surgical orchiectomy and DHT levels in that tissue after castration still are higher than in nonandrogen-target tissues.
3. *In vitro* systems demonstrate that some tumor cell clones are hypersensitive to the proliferative effects of androgen.

Based upon these considerations, it was thought that combined inhibition of both testicular and adrenal androgens via MAB might be more effective than inhibition of testicular androgen secretion (TAS) alone. Thirty-six studies have examined the concept of MAB as initial endocrine therapy for advanced disease in randomized trials but the results and conclusions drawn from them conflict. However, three systematic reviews (two with meta-analysis of the literature), one meta-analysis of individualized patient data, one large randomized trial, and one Markov model critically examined this issue.[444,448,449] These studies, involving nearly 90% of men treated worldwide, provided several definitive conclusions regarding this strategy. The benefits of MAB are minimal and range from 1% to 5% improvement in overall survival. The additional cost of MAB as opposed to TAS alone approximates $1,110,000 per quality adjusted life year and the number needed to treat to benefit one patient is estimated to be between 20 and 100.

These meta-analyses provide reasons for discrepancies among prior results. First, MAB regimens using nonsteroidal anti-androgens provide only a 1% to 5% improvement in overall survival. An example of data from one of the meta-analyses is presented in Figure 29.27. This difference is statistically significant $p = 0.005$ but of marginal significance clinically. Second, MAB regimens using the steroidal anti-androgen, cyproterone acetate, produce adverse survival effects compared to TAS. Third, analysis of studies of MAB which pool patients treated either with cyproterone acetate or nonsteroidal anti-androgens demonstrate no survival benefit produced by MAB over TAS. Fourth, men with disease limited to the axial skeleton did not gain more benefit from MAB than those with metastases to the appendicular skeleton. A large prior study had reported this to be the case. Fifth, results were similar when subsets of men treated with surgical or medical castration or with flutamide or nilutamide were examined.

Past controversies about MAB can be explained by fact that some trials used cyproterone acetate, an anti-androgen that shortens survival in these patients. Other trials did not use short-term anti-androgen in the medical castration group and the disease flare may have compromised overall efficacy. Finally, early trials used daily GnRH injections that may not have induced effective medical castration because of the need for compliance with daily injections for 5 years. It should be noted that no properly designed study has compared MAB to sequential androgen blockade (SAB—defined as initial castration followed by anti-androgen upon relapse). In the largest study to date of MAB, use of anti-androgen upon relapse in the placebo group was left to the investigator's discretion and only 50% of men on the placebo arm later took an anti-androgen.[448] For this reason, no information is available whether anti-androgen therapy given at the time of relapse after medical or surgical orchiectomy (i.e., SAB) is as effective as MAB. This is an important issue since flutamide given upon relapse after castration (SAB) causes objective benefit in 23% of patients.

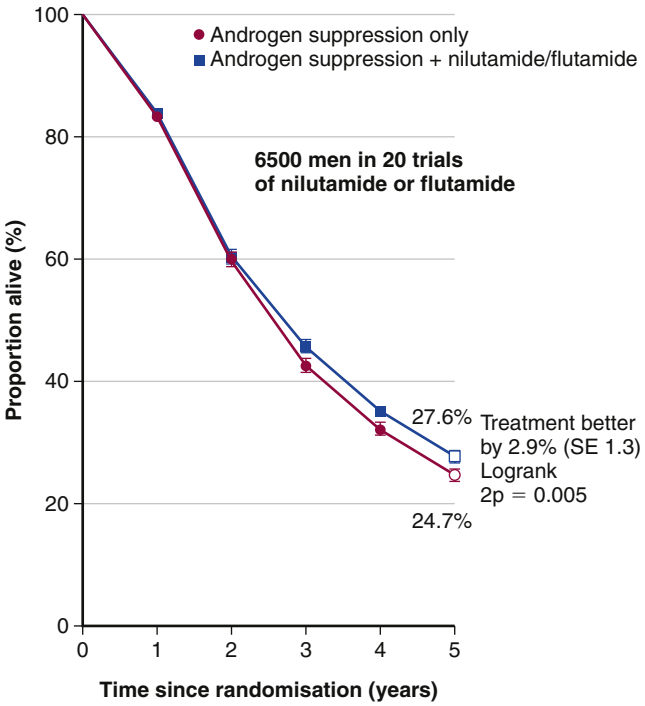


FIGURE 29.27 Meta-analysis of effects of complete androgen blockade versus antiandrogen therapy alone. *(From Santen R. Endocrine responsive cancer. In Kronenberg HM, Melmed S, Polonsky KS, Larsen PR [eds].* Wiliams Textbook of Endocrinology, *11th ed, Philadelphia, WB Saunders, 2008. Copyright National Comprehensive Cancer Network, 2012.)*

Based upon the data presented and additional considerations discussed, ASCO and NCCN guidelines favor the sequential androgen blockade and the use of anti-androgens only after relapse from medical or surgical orchiectomy.[429] However, if GnRH agonists are used to produce a medical orchiectomy, short-term use of an anti-androgen to prevent disease flare is necessary. In patients with metastatic disease, GnRH-A should not be used in men at risk of neurological compromise due to metastasis, ureteral or bladder outlet obstruction due to local encroachment or severe bone pain persisting with narcotic analgesic use.

Intermittent Androgen Deprivation Therapy

Multiple trials are examining the use intermittent ADT in an attempt to reduce the side effects of therapy without altering efficacy.[450,451] The basic tenet of intermittent ADT is to continue ADT until the serum PSA falls to a low level and then stop treatment. No side effects or toxicity are likely to occur during this treatment holiday period. ADT is then started again only when the serum PSA rises to a predetermined level. This cycle is repeated until there is clear evidence of disease progression, either clinically or through continued elevated PSA. Recent data published in abstract form suggest that intermittent androgen deprivation therapy is not as effects as continuous treatment.[452]

Algorithm for Treatment of Prostate Cancer

No standard approach to the treatment of prostate cancer has been universally agreed upon. Specific accepted options for each risk group are included in the NCCN guidelines and detailed as follows:

Initial Treatment of Localized disease

Low Risk

Less than 10-year life expectancy: Active surveillance or radiation therapy.

Greater than 10-year life expectancy: Active surveillance or RT or radical prostatectomy.

Intermediate Risk

Less than 10-year life expectancy: Active surveillance or RT or radical prostatectomy.

Greater than 10-year life expectancy: radical prostatectomy or RT.

High Risk

Androgen ablation (2 to 3 years) plus RT or RT and short term androgen ablation, or radical prostatectomy (if low volume and resectable).

Initial Treatment of Locally Advanced Disease

Androgen ablation for 2 to 3 years or RT and short term androgen ablation.

Initial Treatment of Metastatic Disease

Any T,N1: androgen ablation or RT and androgen ablation.
Any T, Any N, M1: androgen ablation.

Considerations Regarding Initial Treatment of Clinically Localized Prostate Cancer

No consensus exists regarding selection of radical prostatectomy, radiation therapy, or active surveillance. Individual considerations, including age, risk factors for recurrence, overall health of the patient and life expectancy, influence these decisions that are often based on personal choices. In general, the younger and healthier the patient, the more likely is a choice of radical prostatectomy. The older and more debilitated, the more likely is the choice of active surveillance or doing nothing. In men with clinical T3 disease, radiation therapy plus hormonal therapy is generally preferred over radical prostatectomy except in a few T3 cases with low volume disease. Radiotherapy is often selected for patients falling at neither extreme. Adjuvant hormonal therapy used with radiotherapy should be limited to patients with higher risk disease. The likelihood of recurrence with low or intermediate risk disease may be too small to warrant the associated symptoms, bone loss, anemia and other problems associated with androgen deprivation.

Considerations Regarding Initial Therapy in Men with Metastatic Disease at Time of Diagnosis

Men with node positive or metastatic disease at presentation are not treated definitively with radical prostatectomy or irradiation but frequently are offered hormonal therapy prior to development of symptomatic disease.[401] This decision is based upon incomplete data suggesting superior efficacy of early rather than late hormonal therapy in locally advanced disease. Randomized trials with a large number of patients will be required before definitive advice can be given regarding management of patients with PSA relapses.

Which hormonal therapy to use is essentially a matter of choice. Meta-analyses of several trials suggests that medical castration and surgical castration have equal efficacy but individual trials indicate that anti-androgens are less effective.[415,429] In the opinion of the authors, surgical orchiectomy appears preferable for the initial treatment of these patients. The operation is relatively minor; can be performed under local anesthesia if desired, and is cost effective. Rapid and complete cessation of testicular androgen secretion ensues, and patient compliance after surgery is not a factor. Unwarranted cardiovascular or other toxic side effects are unknown. The incidence of impotence and loss of libido is the same as with other available therapies with the exception of the anti-androgens.

In actual practice, nearly half of patients in the United States prefer a form of medical castration for psychological or other reasons. The greater safety of the GnRH-A favor their use over DES and for this reason, DES is no longer available in the U.S. To prevent tumor flare, anti-androgens should be used for at least 2 weeks prior and 2 weeks after initiation of GnRH therapy. Alternatively, the Gn-RH antagonists are not associated with a transient rise in testosterone and can be used without a concomitant anti-androgen. Maximum androgen blockade long term is not recommended based upon the individual studies and meta-analysis reviewed above but should be discussed with the patient.[429] Some patients may choose to continue the anti-androgens long term to gain the slight, 1% to 5% maximal benefit from maximum androgen blockade. An anti-androgen alone could be chosen as an alternative but is not preferred.

At the present time, medical castration with GnRH agonists can be achieved with 3 to 6 monthly injections or yearly implants which are well tolerated and should not be associated with cardiovascular complications. Cost is the limiting factor. When concern regarding erectile dysfunction is a major issue, the persistence of libido and erectile function in a subset of patients treated with anti-androgens as primary therapy may be a decisive factor. Under these circumstances, some men may choose monotherapy with anti-androgens, even though this approach is not as effective as medical or surgical castration. This decision represents a quality of life issue that is an increasingly important consideration in prostate cancer therapy.

Considerations Regarding Disease Recurrence

Upon first recurrence, men treated with radiotherapy or radical prostatectomy are then treated with either medical or surgical orchiectomy. Anti-androgen monotherapy can be offered but is not preferred. Anti-androgen therapy provides a reasonable next option after medical or surgical castration unless previously given as part of a MAB regimen. Those receiving an anti-androgen as part of a MAB regimen are observed for an anti-androgen withdrawal response. Regarding choice of anti-androgen, bicalutamide is preferred to flutamide because of its better side effect profile and potentially superior efficacy. Upon relapse, men are observed for anti-androgen withdrawal response and only treated further when progressive disease is documented.

A variety of therapies are available for men relapsing after anti-androgen withdrawal. One group prefers ketoconazole and hydrocortisone because of its lack of toxicity and observed efficacy (i.e., 60% PSA response rate),[302] but this has now been superseded by abiraterone.[437,438] Each of the other agents listed on Table 29.7 could be used as alternatives or as third line therapies. Further choices of chemotherapy alone or chemotherapy in combination with hormonal agents are a matter of individual preference.

Endometrial Carcinoma

Endometrial cancer is the most common of the gynecological malignancies with 47,130 estimated cases and 8010 deaths from the disease in 2012 in the U.S.[146] The majority occur in postmenopausal women with a small percentage in premenopausal women and up to 14% in women under the age of 45.[453] Endometrial cancer is highly associated with obesity, a condition that is significantly increasing in incidence in the U.S.[454] Endometrial cancer can be categorized into types 1 and 2.[455] Associated with unopposed estrogen excess, type 1 endometrial cancer often has a good prognosis. When well differentiated (grade 1), type 1 cancers commonly expresses the progesterone receptor (PR). In

contrast, type 2 cancers are not associated with estrogen excess, are high grade, and may exhibit aggressive histological subtypes. Treatment considerations require distinction between type 1 and 2 tumors.

The standard of care for endometrial cancer is surgical with total hysterectomy and removal of both fallopian tubes and ovaries via laparotomy, a vaginal approach, laparoscopy, or robotically assisted laparoscopy. In the U.S., the hysterectomy is often associated with staging surgery, including removal of the pelvic and para-aortic lymph nodes (Boxes 29.1 and 29.2, Table 29.8). Lesions are often heterogeneous (Fig. 29.28). With surgical staging and radiation where appropriate, the cure rate for endometrial cancer confined to the corpus (stage I) is ≥80% depending on stage, grade, and histological subtype. Type 1 cancers in general are more likely to present in early stage and to be cured with surgery.

Type 1 endometrial cancer is most often confined to the corpus at the time of diagnosis. Data from the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) program report that 83% of patients had disease confined to the uterus and cervix at the time of diagnosis.[456] Despite the association of this cancer with a good prognosis, 10% to 15% of patients will present with advanced disease. In addition, the rate of recurrence in patients with "good prognosis" early stage disease may be as high as 9%.[457] Adjuvant systemic therapy is often required for those patients with advanced or recurrent disease. Hormonal therapy will likely be instituted at some point in the course of management of recurrent or persistent disease. With respect to type 2 disease, hormonal therapy is less likely to be effective.

Box 29.1 Staging of Endometrial Carcinoma

CORPUS CANCER SURGICAL STAGING, FIGO 2010

Stage 1	Tumor confined to the corpus uteri
Stage 1A	No or less than half myometrial invasion
Stage 1B	Invasion equal to or more than half of the myometrium
Stage 2	Tumor invades the cervical stroma, but does not extend beyond the uterus
Stage 3	Local and/or regional spread of the tumor
Stage 3A	Tumor invades the serosa of the corpus uteri and/or adenxae
Stage 3B	Vaginal and/or parametrial involvement
Stage 3C	Metastases to pelvic and/or para-aortic lymph nodes
Stage 4	Tumor invades bladder and/or bowel mucosa and/or distant metastases
Stage 4A	Tumor invasion of bladder and/or bowel mucosa
Stage 4B	Distant metastases, including intra-abdominal metastases and/or inguinal lymph nodes

Box 29.2 Endometrial Cancer: Histology

Endometrioid
Mucinous
Serous
Clear cell
Squamous
Undifferentiated
Mixed

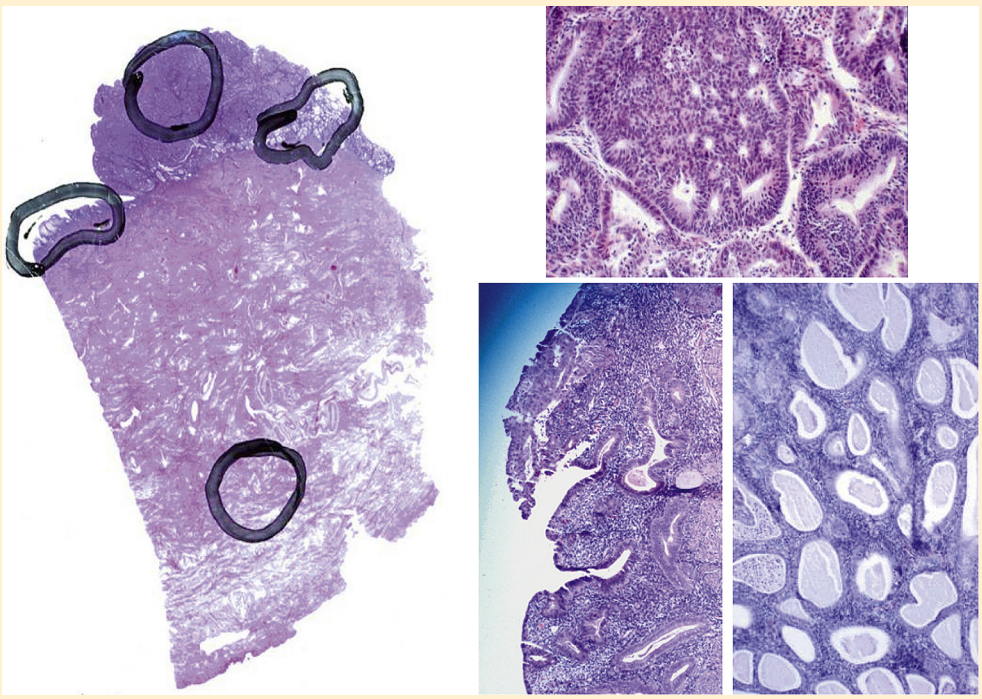


FIGURE 29.28 Example of heterogeneity of endometrial cancer in contiguous areas.

Table 29.8 Criteria for Classification of Endometrial Carcinoma as Type 1 or Type 2

	Type 1	Type 2
Risk factors	Unopposed estrogen	Older age
Grade	Grade 1	High grade
Histology	Endometrioid	Not endometrioid
Stage	I/II	III/IV
Prognosis	Good	Poor
Molecular features	*PTEN* mutations Microsatellite instability	Her-2-neu p53

PTEN, phosphatase and tensin homolog.

This chapter will initially review endometrial cancer with an emphasis on hormone related components and discuss epidemiology, prognosis, and treatment. The features of type 1 and 2 endometrial cancer will be compared and contrasted, and the important implications of this classification highlighted. We will then discuss several key specific issues. The first is primary hormonal therapy with the preservation of the uterus to allow future fertility in premenopausal women. This discussion will also apply to the treatment of complex atypical hyperplasia (CAH), the precursor lesion to type 1 endometrial cancers (also called endometrial epithelial neoplasia). Second, the chapter will address the use of adjuvant hormonal therapy in postmenopausal women presenting with recurrent or persistent advanced endometrial cancer.

Epidemiology

Endometrial cancer is the most common of the gynecological malignancies, the seventh most common cancer in women worldwide, and the fourth most frequent cancer diagnosed in American women. The disease occurs after menopause in over 70% of cases.[458] Endometrial cancer is highly associated with obesity and, in the industrialized world, likely due to lifestyle and dietary habits.

Based on epidemiological, histological, and molecular pathological analyses, two types of endometrial tumors can be distinguished: type 1 and type 2[459] (Table 29-8). Type 1 endometrial cancers are histologically endometrioid, represent up to 80% of endometrial cancers, and are generally associated with CAH, also called Endometrial Intraepithelial Neoplasia (EIN).[460-462] Type 1 tumors are low grade (grade 1 or grade 2), often present in early stage (stage I, confined to the corpus), and are usually curable with surgery alone or surgery plus radiotherapy. Type 1 carcinomas are associated with mutations in the *ras* proto-oncogene and in the *PTEN* tumor suppressor gene, and may show microsatellite instability. Mutations in the *p53* tumor suppressor gene are not usually present. Evidence links endocrine and nutritional lifestyle factors, including obesity, to the risk of type 1 but not type 2 tumors.[463]

Type 2 tumors include three separate histological types: high grade endometrioid (grade 3), serous papillary, or clear cell carcinomas. The suggested precursor lesion for type 2 tumors has been termed endometrial glandular dysplasia.[464] Type 2 tumors may develop from atrophic endometrial tissue in older women.[455,461,465] In contrast to type 1 tumors, these tumors are not generally believed to be hormonally driven and most do not express estrogen (ER) or progesterone receptors (PR). A majority of type 2 tumors have *p53* mutations, but almost never microsatellite instability or *PTEN* mutations.

Table 29.9 Obesity Rates in Women with Endometrial Carcinoma Under the Age of 45

	Duska*	Gitsch†	Solimen‡	Total
Number of patients	92	17	79	188
BMI > 30	44	6	48	98
Obese (%)	48	35	62	52

BMI, body mass index.
*Duska LR, Garrett A, Rueda BR, et al. Endometrial cancer in women 40 years old or younger, *Gynecol Oncol* 83(2):388-393, 2001.
†Gitsch G, Hanzal E, Jensen D, et al. *Obstet Gynecol* 85(4):504-508, 1995.
‡Soliman PT, Oh JC, Schmeler KM, et al. Risk factors for young premenopausal women with endometrial cancer, *Obstet Gynecol* 105(3):575-580, 2005.

Risk Factors for Endometrial Carcinoma

Unopposed Estrogen

Risk factors for type 1 endometrial cancer include any clinical scenario which may result in prolonged estrogen exposure without sufficient progesterone. This "estrogen excess" effect was noted in the U.S. in the late 1970s when it was recognized that the use of unopposed estrogen replacement therapy was associated with a twofold to twelvefold increased risk of uterine cancer.[466-469] The increased risk does not manifest itself for 2 to 3 years and longer use is associated with higher risk. Exogenous estrogen effects are strongest among women who are thin, normotensive, nonsmokers, and nondiabetics.[466,470] Adding a progestogen to the exogenous estrogen as part of so-called "combination" menopausal hormone therapy reduces or eliminates the risk of uterine cancer. Endometrial risk is also increased with the use of tamoxifen as a result of its estrogen agonistic properties in postmenopausal women with breast cancer and an intact uterus.[471,472] Anovulatory menstrual cycles are characterized by lack of luteal phase progesterone secretion resulting in unopposed estrogen effects on the endometrium with an associated increased risk of endometrial neoplasia. A common anovulatory condition associated with endometrial carcinoma is the polycystic ovarian syndrome (usually with associated metabolic syndrome). Insulin resistance may also lead to the development of endometrial cancer via the insulin growth factor receptor.[463]

Obesity

Production of estrogen in extraglandular tissues is catalyzed by the enzyme aromatase which increases linearly as a function of obesity (Table 29.9).[204] Accordingly, plasma estrogen levels increase proportionately with body weight or body mass index. Obesity is thought to increase the risk of endometrial cancer due to the enhancement of stimulation of endometrial tissue by estrogens.[474-476] Increased production of estrogen leads to excess stored estrone in adipose tissue and a reservoir of estrogen available for continuous tissue delivery.[473,477] Excess body weight is also associated with the prevalence of anovulation.

Obesity has been associated with a twofold to fivefold increase in endometrial cancer risk in both pre- and postmenopausal women and has been estimated to account for 40% or more of type 1 endometrial cancer incidence

in affluent societies.[478] While in many studies, risk rose approximately linearly with increasing Body Mass Index (BMI), a few studies showed a threshold effect, with an increase only among obese women with a BMI of 30 kg/m^2 or higher.[478] Epidemiological evidence also suggests a possible protective effect of regular physical activity perhaps by reducing obesity or through other means.[473]

Prolonged Estrogen Exposure

Early age at menarche and late age at menopause are risk factors that may reflect prolonged exposure to estrogen in the presence of anovluatory cycles.[479-481] Other risk factors for endometrial cancer that are associated with increased endogenous estrogen levels include high serum levels of androstenedione, the substrate for aromatase, and lower levels of serum sex-hormone binding globulin (SHBG) which allows increased delivery of estrogen to tissue.[482] Reduction in risk results from alcohol consumption, perhaps due to attenuation of endogenous estrogen, and cigarette smoking. Nulliparity increases the risk of endometrial cancer but the precise mechanisms are unknown.[479,480]

Genetic Factors

While most cases of endometrial cancer are sporadic, genetic factors, such as Lynch syndrome or hereditary nonpolyposis colorectal cancer *(HNPCC)* significantly increase the risk of endometrial cancer.

Environmental and Life-Style Factors

Incidence rates of endometrial cancer are up to 10 times higher in Western, industrialized countries than in Asia or rural Africa. Increases in incidence rates over time after industrial development or migration from low-risk to high-risk areas suggest that endometrial cancer has strong environmental risk factors which are related to the westernization of lifestyle.[463] Life-style factors likely include level of physical activity and obesity.[483,484]

Epidemiological Data Specific to Young Women with Endometrial Cancer

Retrospective reports suggest that between 2% to 14% of women presenting with endometrial cancer will be less than 40 years old.[453,485-493] The majority of young women with endometrial cancer present with menstrual irregularity. In a series from Massachusetts General Hospital (MGH), 29 of 91 women (32%) with endometrial cancer presented with menorrhagia or increasing menorrhagia and 39 of 91 (43%) presented with irregular menses or menometrorrhagia.[453] Similarly, 26 of 32 women in the Crissman series presented with irregular vaginal bleeding.[487]Other studies of young women with endometrial cancer have also reported high rates of irregular bleeding as the presenting complaint.[486,489,492]

Obesity is also a risk factor for endometrial cancer in younger women. The three largest studies regarding BMI are shown in Table 29.9. Data from these studies indicate that obesity rates in younger women range from as low as 38% to as high as 62%.[453] Interestingly, these obesity rates in young women seem to be higher than those of older women with endometrial cancer. In the series from Gallup and Stock, an obesity rate of 43.8% in women younger than 40 years old with endometrial cancer contrasted with 18% in a group of patients treated at the same institution who were older than 40 years old.[488]

All young women with endometrial cancer, however, are not obese. In the series from MGH, 52% of women younger than 40 years old with endometrial cancer were of normal weight (BMI less than 30), and 43% had a BMI of 25 or less.[453] That study reported a trend toward higher stage disease and high-risk histology in the normal weight women, though the differences did not reach statistical significance. Schmeler and colleagues presented a series of women younger than 50 years old and of normal weight seen at the MD Anderson Cancer Center.[494] They suggested that hormonal factors, and in particular polycystic ovarian syndrome (PCOS), may be a risk factor for developing endometrial cancer in those women with normal weight. Retrospective data suggest that normal weight younger women are not at higher risk for poor survival, though the numbers are too small in all studies to reach any conclusion.

Infertility is also associated with endometrial cancer in women under 40 years old, in contrast to their postmenopausal counterparts who are often"fertile" during their premenopausal years. In the series from MGH, 11 patients were diagnosed with endometrial cancer incidentally during infertility evaluation.[453] In Gallup and Stock's study, 44% of women younger than 40 years old with endometrial cancer were classified as "infertile."[488] Schmeler and colleagues reported a 17% prevalence of infertility in women under age 50 with endometrial cancer.[494] The finding of infertility makes sense if we assume that the infertility is a result of anovulation from disorders such as PCOS, which is associated with high levels of circulating unopposed estrogen.

Genetic disorders, particularly Lynch syndrome, are associated with endometrial cancer, usually at a young age. In fact, endometrial cancer is the most common cancer of Lynch syndrome in women and may be the presenting cancer in some patients.[495] Finally, endometrial cancer in a young woman may result from an estrogen producing ovarian tumor such as a granulosa cell tumor.

Complex atypical hyperplasia (CAH) is the precursor lesion for type 1 endometrioid adenocarcinoma. When this diagnosis is made on an endometrial biopsy, there is a high likelihood that a grade I endometrioid adenocarcinoma will coexist and be found on the hysterectomy specimen. Kurman and Norris established retrospectively that a preoperative diagnosis of CAH resulted in a postoperative diagnosis of grade 1 adenocarcinoma on the hysterectomy specimen in 29% of cases.[496] This study has since been confirmed prospectively by the Gynecology Oncology Group (GOG).[497,498] In the GOG's study, all women with a preoperative community diagnosis of CAH underwent hysterectomy within 12 weeks of diagnosis. The rate of carcinoma in the examined uterus was 43%. From the data presented above, it is clear that a diagnosis of CAH on an endometrial biopsy should be treated as if a grade 1 adenocarcinoma were present.

Receptor Mediated Effects

Role of the PR: Evolving data over the past decade have emphasized the complexity of the role of the progesterone receptor (PR) in mediating endometrial cancer in the

corpus. We now have a better understanding of the interactions between the two isoforms of the progesterone receptor: PRA and PRB. The two isoforms can have divergent responses and the ratio between the isoforms are likely to be important.[499-503] Several cofactors and co-repressors can influence PR-mediated action.[504-509]

The presence of a PR in an endometrial tumor does not predict response to progestogens. The study of Arnett-Mansfield and colleagues illustrates the difficulty of utilizing immunohistochemistry and the presence or absence of receptor to predict response.[510] The authors studied PR isoforms in archived endometrial cancer tissue. Ninety-six percent of tumors expressed PR. Only 30% of tumors expressed PRA alone, 42% expressed both isoforms, and 28% expressed PRB alone. PRB-only tumors had low levels of PR and those tumors that expressed both isoforms tended to express predominantly PRA. Based on their data, the authors hypothesized that loss of PRB resulted in the development of endometrial cancer. Other authors support these findings.[502]

Utility of PR Measurements

Most endometrioid adenocarcinomas express PR.[511] The lower grade tumors express PR more frequently, with a decrease in PR expression with increasing tumor grade.[512-514] Tumor heterogeneity (Box 29.2) is an important factor to consider as progesterone therapy may exert responses in some areas of the uterus and not in others. Tumors can have both PR+ and PR− areas.[515-518] Therefore, the presence of PR by immunohistochemistry does not reliably predict response to progesterone therapy. Accordingly, molecular markers of progesterone response that are measurable in paraffin-fixed tissue will be needed in the future to predict response of an individual tumor to progesterone therapy. The significance of PR levels in CAH lesions has not been clarified but it is assumed that they are responsive to progesterone. Retrospective data presented below demonstrate that CAH has a higher response rate to progesterone than well differentiated adenocarcinoma, with resolution rates of 70% to 90% or higher.

Treatment

Primary Treatment of Women Younger Than 40 Years Old for Fertility Preservation

The majority of data supporting primary hormonal therapy of endometrial cancer for the purposes of preserving fertility are derived from small, nonrandomized, retrospective observations of women treated with nonstandardized regimens and are subject to reporting bias. Data from several reviews and individual studies summarize available information.

Ramirez and colleagues published a retrospective literature review of patients treated with progestins[519] and identified 27 studies describing 81 patients treated with hormones for endometrial cancer. Overall, the response to progestin therapy was 76%, with a median time of response of 12 weeks. Documentation regarding pregnancy was available for 20 patients, all of whom were able to conceive at least once following treatment. Gotlieb and colleagues performed a similar and more comprehensive literature review and identified 101 women with a mean age of 29 years treated with hormones for endometrial cancer, with a 71% initial response rate, minimal time to response of 3.6 months, and 56 live births.[520]

Few prospective studies exist for progestin treatment in premenopausal women for the purposes of preserving fertility. A Japanese group reported a multi-center prospective study of patients with EC and CAH younger than 40 years old who were treated with 600 mg of medroxyprogesterone acetate (MPA) for 26 weeks.[521] Forty-five patients were treated, with a complete response (CR) rate of 55% in EC patients and 82% in CAH patients. During the 3-year follow-up period there were 12 pregnancies and 7 live births. Notably, there were also 14 recurrences in 30 patients, demonstrating the high-risk of recurrent disease following treatment cessation.

A recent literature review and meta-analysis of all prospective and retrospective published studies considering young women suggests an overall response rate to progestin therapy of 48% for endometrial cancer and 66% for complex atypical hyperplasia.[522] After complete response, recurrence rates were 23% in patients with CAH and 35% in patients with EC. Forty-one percent of patients with CAH and 35% of patients with EC were able to achieve pregnancy.

These and other studies provide the data to support that the majority of younger women will respond to hormonal therapy with an overall response rate of approximately 70% when EC and CAH are combined. The response rates for EC are lower than for CAH. The presence of ER and PR does not necessarily imply response to hormonal therapy and the recurrence risk is not insignificant, with a range of 8% to 66%.[523]

These studies provide the basis for agreement among most experts that only those women with grade 1 tumors lacking myometrial invasion (as assessed radiologically) may be considered for hormonal treatment for the purposes of fertility preservation (Box 29.3 gives an example of a treatment algorithm for this clinical scenario). Higher grade tumors or invasive tumors should be treated with curative surgery, as progression of disease and death from disease is a real phenomenon that has been reported in the literature.[524]

There is no standardized drug, delivery system, or dosing schedule for treating women with primary endometrial cancer for the purpose of preserving fertility with hormones. Most gynecological oncologists choose megestrol acetate (MA) as a first choice, but doses and schedules are not standardized. Doses as low as 40 mg daily and as high as 160 mg four times daily have been reported. Oral medroxyprogesterone acetate (MPA), depo-medroxyprogesterone acetate, and combinations of tamoxifen and progesterone have also been suggested. While some authors suggest using cyclic therapy to induce a monthly withdrawal bleed, most advocate continuous treatment which ultimately results in an atrophic endometrium. Since progestin may be poorly tolerated by many women, with breast tenderness and weight gain being frequent complaints, it is probably best to use the lowest dose that will also be successful in reversing the neoplastic endometrium. Notably this lowest dose probably varies from woman to woman and likely its success is dependent on patient's BMI and individual tumor.

Many women with endometrial cancer treated with progestins will require ART to achieve pregnancy. Since ART generates very high serum estradiol levels (which thereby put the patient at risk for recurrence if pregnancy is not achieved), many community *in vitro* fertility (IVF) programs may feel uncomfortable managing these patients.

Box 29.3 Treatment Algorithm for Primary Hormonal Treatment of Premenopausal Women with Endometrial Carcinoma Who Wish to Preserve Fertility

- Confirm that the tumor is endometrioid and grade 1 by pathological review. If diagnosis was made by endometrial biopsy, perform dilatation and curettage (D&C) to ensure complete sampling of the endometrial cavity.
- Obtain a careful medical history and perform physical examination with particular attention to family history. A family history that suggests Lynch syndrome should result in genetic counseling and possible testing as patient is at increased risk for colon and ovarian cancer. Attention should also be paid to medical history that might complicate future pregnancies (obesity, diabetes, hypertension).
- MRI and/or ultrasound should be performed to rule out adnexal metastases and evaluate for myometrial invasion.

If the tumor is well sampled and grade 1 with no evidence of extrauterine disease or myometrial invasion, the patient should undergo:

- Informed counseling, preferably with a gynecological oncologist.
- Treatment with progesterone, either continuous or cyclic.
- Resampling in 3 months to assess response.
- If resolution of disease: patient should be encouraged to achieve pregnancy quickly. Many of these patients will require artificial reproductive technology (ART).
- If incomplete resolution of disease: patient may be retreated with another medication regimen or a higher dose of the same formulation and resample in 3 months.

Once childbearing is complete or if treatment fails, the patient should be counseled for definitive surgical therapy consisting of TAH–BSO with lymphadenectomy.

Moreover, many of these women are in the older range of reproductive age and therefore have lower success rates for IVF, perhaps requiring multiple attempts at ovulation induction to achieve pregnancy. These risks must be considered in the overall counseling of patients when they are contemplating hormonal management for preservation of fertility.

Pathological Assessment of Progestin Therapy

The GOG is trying to understand the molecular basis of progestin response in several prospective ongoing studies. In GOG study 211, postmenopausal women undergoing hysterectomy for a known diagnosis of endometrioid adenocarcinoma were treated with a single intramuscular (IM) dose of depo-medroxyprogesterone acetate 400 mg 21 to 24 days prior to hysterectomy. As described in a preliminary abstract,[525] 60 patients had initial biopsies and hysterectomy specimens available for study. Only one complete response was seen, but 37 tumors had partial response, more frequently in tumors of lower grade. While 90% of tumors were initially ER and PR positive, it was noted that both ER and PR were significantly down-regulated following progesterone treatment.

Alternative Methods of Treatment

Several authors have suggested the use of a progesterone intrauterine device (IUD) as a means of treating endometrial cancer with high doses of progesterone without systemic side effects. In the study from Montz and colleagues, women with clinical stage I, grade 1 endometrial cancer underwent hysteroscopy and curettage followed by placement of a progesterone IUD and resampling every 3 months.[526] Seven of 11 patients demonstrated complete response at 6 months and 6 of 8 at 12 months. Dhar and colleagues performed a similar study using a levonorgestrel containing IUD.[527] Four women with grade 1 adenocarcinoma that expressed PR were treated with IUD; only one patient had a complete response within 6 months. However, this study did not exclude myometrial invasion prior to the treatment with the IUD. In both studies, the majority of patients were postmenopausal and underwent hormonal treatment because it was felt that they were poor surgical candidates; thus, it is impossible to know whether a similar treatment regimen in premenopausal women would have similar outcome. Moreover, two women were reported to possibly have developed adenocarcinoma in the uterine isthmus while using a levonorgestrel IUD, suggesting that either the uterine cavity does not receive a uniform dose of progestin, or that the cancer is not uniformly receptive to progesterone treatment.

Follow-Up of Therapy

Appropriate follow-up has not been not standardized with respect to frequency of endometrial resampling, duration needed before a complete response may occur, or duration of required follow-up to rule out recurrence. It is clear, however, that responses may not be seen at the first 3-month resampling, and that the recurrence risk is high. The authors' opinion is that patients be resampled 3 months after beginning hormonal therapy. If an incomplete response is documented, a further 3-month trial of treatment, perhaps with increased dose or different medication, may be appropriate. If pregnancy is not desired in premenopausal women, a "maintenance" hormonal treatment must be utilized to prevent recurrence once regression of the lesion has been documented histologically. This maintenance therapy might consist of the birth control pill, monthly withdrawal bleeds with progesterone, or continuous progestin therapy, either by mouth, intramuscular, or by intrauterine device. The subgroup of premenopausal women desiring pregnancy require a modified approach. Once complete remission is established, pregnancy should be aggressively pursued, with artificial reproductive technology (ART) if required.

Management of Recurrent or Persistent Endometrial Cancer

Data Upon Which to Base Conclusions

In most clinical trials of hormonal therapy, the histological type and grade of endometrial cancer have not been specified and interpretation is confounded by these factors. Because type 2 cancers are more likely to present in late stages, and to persist or recur, there may be a majority of this type in any clinical trial of this nature. (Sub-types of endometrial cancer are listed in Table 29.8.)

Progestational Agents

Use of these agents to successfully treat patients with advanced or recurrent cancer has a long history. The activity of progestins in this setting was reported in the 1950s by Kelley and Baker.[528] They and others demonstrated that

30% to 40% of patients responded to progestin therapy, with response durations ranging from 23 to 29 months.[529] More recent studies, using standard response criteria as well as new imaging procedures, have indicated that response rates are more likely to be in the 10% to 15% range.[530,531] Nevertheless there has been enthusiasm for progestin (hormonal) therapy in this disease setting for years, especially given the historically low response to standard chemotherapy agents and the relatively low rate of side effects of hormonal therapy. Early reports regarding progestin treatment of advanced or recurrent endometrial cancer considered intravenous progesterone delivery.[532] Subsequently, after it was demonstrated that serum concentrations of medroxyprogesterone acetate (MPA) are similar whether given by parenteral or oral routes, studies began to focus on oral progestins.[533]

The Gynecology Oncology Group (GOG) has completed multiple studies of hormonal treatment of advanced or recurrent endometrial cancer (Tables 29.10 and 29.11). While different forms of progestin have been used most

Table 29.10 Gynecology Oncology Group (GOG) Studies of "Active" Hormonal Agents in Advanced or Recurrent Endometrial Cancer

Study	Drug	Number of Evaluable Patients	Response Rate
Thigpen*	Megestrol acetate 200 mg QD Megestrol acetate 1000 mg QD	145 154	CR 17% PR 8% ORR 25% CR 9% PR 10% ORR 15%
Whitney†	Tamoxifen citrate 20 mg PO BID *plus* Medroxyprogesterone acetate 100 mg PO BID on alternating weeks	58	CR 10% PR 23% ORR 33%
Lentz‡	Megestrol acetate 800 mg PO daily in divided doses	58	CR 11% PR 13% SD 22%
Fiorica‖	Megestrol acetate 80 mg PO BID for 3 weeks Tamoxifen citrate 20 mg PO BID for 3 weeks	56	CR 22% PR 5% ORR 27%

*Thigpen JT, Brady MF, Alvarez RD, et al. Oral medroxyprogesterone acetate in the treatment of advanced or recurrent endometrial carcinoma: a dose-response study by the Gynecologic Oncology Group, *J Clin Oncol* 17(6):1736-1744, 1999.
†Whitney CW, Brunetto VL, Zaino RJ, et al. Gynecologic Oncology Group study. Phase II study of medroxyprogesterone acetate plus tamoxifen in advanced endometrial carcinoma: a Gynecologic Oncology Group study, *Gynecol Oncol* 92(1):4-9, 2004.
‡Lentz SS, Brady MF, Major FJ, et al. High-dose megestrol acetate in advanced or recurrent endometrial carcinoma: a Gynecologic Oncology Group study, *J Clin Oncol* 14(2):357-361, 1996.
‖Fiorica JV, Brunetto VL, Hanjani P, et al. Gynecologic Oncology Group Study. Phase II trial of alternating courses of megestrol acetate and tamoxifen in advanced endometrial carcinoma: a Gynecologic Oncology Group study, *Gynecol Oncol* 92(1):10-14, 2004.

Table 29.11 Gynecology Oncology Group (GOG) Studies of "Nonactive" Hormonal Agents in Advanced or Recurrent Endometrial Cancer

Study	Drug	Number of Evaluable Patients	Response Rate
Podratz*	Tamoxifen 20 mg PO BID	68	CR 4% PR 6% ORR 10%
Asbury†	Lupron 3.6 mg SQ monthly	40	CR 5% PR 7% ORR 11%
Rose‡	Anastrozole 1 mg PO QD	23	No responders 2 short-term SD
Covens, 2003‖	Danazol 100 mg PO QID	22	No responders SD 27%
Covens, 2011¶	Fulvestrant 250 mg IM monthly	53	No responders in ER negative patients ORR 16% in ER positive patients

*Podratz KC, O'Brien PC, Malkasian GD Jr, et al. Effects of progestational agents in treatment of endometrial carcinoma, *Obstet Gynecol* 66(1):106-110, 1985.
†Asbury RF, Brunetto VL, Lee RB, et al. Gynecologic Oncology Group Goserelin acetate as treatment for recurrent endometrial carcinoma: a Gynecologic Oncology Group study, *Am J Clin Oncol* 25(6):557-560, 2002.
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‖Covens A, Brunetto VL, Markman M, et al. Gynecologic Oncology Group. Phase II trial of danazol in advanced, recurrent, or persistent endometrial cancer: a Gynecologic Oncology Group study, *Gynecol Oncol* 89(3):470-474, 2003.
¶Covens AL, Filiaci V, Gersell D, et al. Phase II study of fulvestrant in recurrent/metastatic endometrial carcinoma: a Gynecologic Oncology Group study, *Gynecol Oncol* 120(2):185-188, 2011.

frequently, the GOG has also studied combination therapy with tamoxifen, the aromatase inhibitor anastrozole, gonadotropin releasing hormone analogues, among others. Unfortunately many of these studies have demonstrated these drugs to have activity that does not merit further study.

Tables 29.10 and 29.11 outline selected GOG studies that have been published using hormonal therapy in advanced or recurrent endometrial cancer. Table 29.10 shows those studies that were felt to be positive studies that merited further study. The details of select studies will be discussed below.

GOG protocol 48 was one of the first large studies of progestin in the modern clinical era.[534] In this study, with 331 women with measurable disease, the response rate to 150 mg MPA daily was 18%, and the duration of response was short. Subsequently, GOG protocol 81 was undertaken to determine if higher doses of progestin would result in higher response rates. This prospective trial accrued 324 women between 1985 and 1989[535] and randomized subjects to either 200 or 1000 mg per day of MPA orally. Determination of the presence of estrogen or progesterone receptor was not required for study entry, but the data were collected when available. Interestingly, the overall response rates (complete plus partial; 25% and 16% for low-dose and high-dose regimens, respectively) favored the low-dose regimen. The crude relative odds of responding to the high-dose regimen was 0.56. When the data were controlled for pretreatment prognostic factors, including tumor grade and stage, age of patient, and performance status, the response rate still favored the low-dose regimen.

Not surprisingly, the response to progestin treatment was highly associated with tumor grade. Patients with grade 1 cancers experienced tumor regression more frequently in response to treatment than patients with high grade tumors with response rates of 37% (22 of 59 patients), 23% (26 of 113), and 9% (12 of 127) for those with grade 1, 2, or 3, respectively. A statistically significant trend demonstrated greater response rates with increased differentiation of the tumor. Similarly, tumors with a positive hormone receptor status responded more frequently than negative tumors with response rates of 37% (17 of 46) for patients who were progesterone receptor-positive and 8% (7 of 86 patients) for those negative. On average, the overall survival and progression-free survival were longer for those assigned to the low-dose regimen than for those assigned to the high-dose regimen. After the initial performance status, progesterone-receptor level, tumor grade, and age were adjusted for, the risk of death, was 31% greater for those on the high-dose regimen than for those on the low-dose regimen. Similarly, the risk of first progression or death was 35% greater for those on the high-dose regimen. The oral progestin therapy was well tolerated, with thrombophlebitis, the most frequent adverse effect, occurring in only 5% of patients.

GOG 121 expanded on the results of GOG 81, utilizing a different formulation of progestin.[536] In this phase II study, patients were treated with megestrol acetate (MA) at a dose of 800 mg daily in divided doses. While the ORR was only 24%, it is notable that the clinical trial population was heterogeneous: 47% of the patients had high grade histology (grade 3) and only 50% of patients had endometrioid histology. Thus less than half of the patients had endometrial cancers that would be considered to be type 1 and likely to respond to hormonal therapy. When only the patients with grades 1 to 2 disease were considered (which eliminates all of the high risk histologies), the response rate was 37%. Toxicities of MA were minimal, with weight gain being the most common side effect.

A potential consequence of progestin treatment is the decrease in PR concentration, resulting in a relatively short treatment response.[537] Tamoxifen citrate has been shown to increase PR in animal models.[538] The aim of GOG protocols 119 and 153 was to utilize alternating treatment with tamoxifen and MA to see if the tamoxifen would counterbalance the MA down-regulation of PR.[539,540] In GOG 153, patients were treated with MA 80 mg BID for 3 weeks followed by tamoxifen citrate 20 mg BID for 3 weeks. This alternating sequence was continued at 3-week intervals until disease progression or adverse events. There were 21.4% CRs and 5.4% PRs for an ORR of 27%. The response rate was highest at 38% for those tumors that were grade 1. Weight gain was the most common adverse event and thromboembolic events the most serious. These data, while not directly comparing results with megestrol–acetate or MPA, would not appear superior to use of progestogens alone.

The GOG also evaluated the combination of daily tamoxifen and intermittent weekly MPA in GOG 119. Thirty-three percent of patients responded (6 CR and 13 PR). A scientific corollary study was performed as part of GOG 119 to determine the relationship of ER and PR to clinical outcome.[541] Pretreatment tumor tissue from metastatic or recurrent endometrial cancer lesions was obtained from all patients on study. Immunohistochemistry was performed for ER and PR on paraffin fixed tissue as well as the PR isoforms PRA and PRB on fresh frozen tissue. As expected, ER and PR significantly decreased with increasing tumor grade. ER prior to hormonal therapy was significantly related to clinical response to daily tamoxifen and intermittent MPA. ER expression was highly correlated with the expression of PRA, but not PRB. Except for ER, there was no correlation between receptor expression and clinical response.

Other cooperative groups have performed hormonal studies in the advanced or recurrent population with similar results.[542,543] Combinations of hormones as well as hormones plus chemotherapy have been considered.[544]

Hormonal Treatment with New Biological Agents

Low response rates to hormonal agents in patients with advanced or recurrent endometrial cancer have prompted investigators to consider novel targeted agents in this population. One example is the drug LY353381 (arzoxifene), a third generation selective estrogen modulator (SERM). While first generation SERMs such as tamoxifen have mixed estrogen agonist and antagonist activity, second and third generation SERMs have more selective estrogen antagonism. As a third generation SERM, arzoxifene is a potent estrogen antagonist. A recent phase II study of arzoxifene in 34 patients revealed a 31% response rate; all responses occurred in patients whose tumors expressed PR.[545] Toxicity was minimal, with hot flashes the most common side effect. Further study of this drug and others like it are ongoing.

Conclusions

A subgroup of endometrial cancers are hormonally driven and will respond to hormone based therapy, but our current

understanding of the molecular aspects of response are limited. There is a significant need for incorporation of translational studies in clinical trials in this disease in order to obtain a better understanding of the mechanism of action of hormones and to identify markers for response. Since hormonal treatment has fewer side effects than chemotherapy, identifying those patients who will respond to hormonal treatment in the setting of advanced or recurrent disease may significantly impact quality of life.

Finally, obesity remains a significant and increasing clinical problem in the U.S. and other developed countries, and we expect to see rising rates of endometrial cancer across all age groups in this setting. Education about avoiding obesity and increasing physical activity is expected to significantly impact the rate of endometrial cancer development, particularly in young women for whom fertility preservation may be paramount.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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Table S29.7 Secondary Hormonal Therapies for Recurrent Disease

Modality	Responders	Total Patients	Percent Responding	Clinical Setting	Reference
Flutamide	23	100	23%	First relapse after medical or surgical orchiectomy	(1)
Prednisone	21	101	21%	First relapse after medical or surgical orchiectomy	(2)
Flutamide withdrawal	29	138	21%	Relapse after combined androgen blockade or flutamide	(3; 4)
Aminoglutethimide + hydrocortisone	14	29	49%	After anti-androgen withdrawal	(5)
Hydrocortisone	16	82	20%	After anti-androgen withdrawal	(6)
Ketoconazole Hydrocortisone	43	72	60%	After anti-androgen withdrawal	(7)
Megestrol l acetate	17	119	14%	After anti-androgen withdrawal	(8)
DES	71	243	29%	After anti-androgen withdrawal	(9)
Abiraterone	797	231	29% (PSA response); improved survival 14.8 months vs placebo 10.9 months ($P < 0.001$)	After anti-androgen withdrawal and docetaxel	(8)

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CHAPTER 30

Medical Approaches to Ovarian Stimulation for Infertility

Bart C.J.M. Fauser

Concepts of Ovarian Stimulation

Ovarian stimulation is a central component of many infertility therapies. At the outset of this chapter, it is important to emphasize that two different concepts of ovarian stimulation exist. These approaches differ in both the starting point (i.e., the type of patients) and end points (i.e., the aim of the medical intervention).

Ovulation Induction

In the strict sense of the term, ovulation induction refers to the triggering of ovulation, that is, the rupture of the preovulatory follicle and release of the oocyte. In the clinical context however, this term refers to the type of ovarian stimulation for anovulatory women aimed at restoring normal fertility by generating normo-ovulatory cycles (i.e., to mimic physiology and induce single dominant follicle selection and ovulation). Ovulation induction represents one of the most common interventions for the treatment of infertility.[1] Anovulation represents one of the few states of absolute infertility, but excellent cumulative pregnancy rates can be achieved if normal menstrual cyclicity is restored.

After the exclusion of intrinsic ovarian abnormalities (e.g., premature ovarian failure), follicle development can be stimulated by various pharmacological compounds, and normo-ovulatory cycles can usually be obtained. This can be achieved with appropriate monitoring of ovarian response and in the hands of skillful clinicians. Because of various more subtle inherent ovarian abnormalities in most of these women, especially in patients suffering from polycystic ovary syndrome (PCOS), the risks of multiple pregnancy and ovarian hyperstimulation syndrome (OHSS) are considerable. However, the occurrence of these complications can be reduced to an acceptable level, especially with low-dose gonadotropin protocols.[2] The therapeutic window for an acceptable ovarian response is small, with a major individual (and to some extent cycle-to-cycle) variability in response. Approaches for gonadotropin ovulation induction include slowly and prudently surpassing the individual follicle-stimulating hormone (FSH) threshold for ongoing follicle development, as will be discussed later in this chapter.

Many other approaches for ovulation induction are available. These approaches include interfering with negative estrogen feedback anti-estrogens or aromatase inhibitors, the use of insulin-sensitizing agents, and laparoscopic surgical methods.

Ovarian Hyperstimulation

This treatment modality has become an integral part of assisted reproductive technologies (ART). The aim of ART is to bring more male and female gametes closer together and thereby increase the chances of pregnancy. The goal of ovarian hyperstimulation per se is to induce ongoing development of multiple dominant follicles and to mature many oocytes in order to improve chances for conception either *in vivo* (empirical ovarian hyperstimulation with or without intrauterine insemination, IUI) or *in vitro* with *in vitro* fertilization (IVF). This approach of interfering with physiological mechanisms underlying single dominant follicle selection is usually applied in normo-ovulatory women. Although ovarian hyperstimulation can also be performed in anovulatory women, this approach should be clearly differentiated from ovulation induction. The physiological concepts that underlie current approaches to ovulation induction and ovarian hyperstimulation are described later in this chapter.

Concepts of Follicle Development Regulation Relevant to Ovarian Stimulation

Initiation of growth of primordial follicles, also referred to as primary recruitment, occurs continuously and in a random fashion and development from the primordial up to the preovulatory stage takes several months.[3,4] The great majority of primordial follicles which enter this development phase undergo atresia prior to reaching the antral follicle stage. The regulation of early follicle development and atresia and the degree to which early stages of follicle development are influenced by FSH remain unclear, but evidence suggests that the transforming growth factor β superfamily and factors regulating apoptosis (i.e., programmed cell death) are involved.[5,6] Only at more advanced stages of development do follicles become responsive to FSH and obtain the capacity to convert the theca cell–derived substrate androstenedione (AD) to estradiol (E_2) by the induction of the aromatase enzyme.

Owing to demise of the corpus luteum during the late luteal phase of the menstrual cycle, E_2, inhibin A,

and progesterone levels fall. This results in an increased frequency of pulsatile gonadotropin-releasing hormone (GnRH) secretion inducing rising FSH levels at the end of the luteal phase.[7,8] Although each growing follicle may initially have an equal potential to reach full maturation, only those follicles that happen to be at a more advanced stage of maturation during this intercycle rise in FSH (levels surpassing the so-called *threshold* for ovarian stimulation) gain gonadotropin dependence and continue to grow[2] (Fig. 30.1). This process is referred to as cyclic, gonadotropin-dependent recruitment as opposed to the recently mentioned initial, gonadotropin-independent recruitment of primordial follicles.[4] Based on indirect observations it is believed that the cohort size of healthy early antral follicles recruited during the luteofollicular transition is around 10 per ovary.[9,10] During the subsequent follicular phase, FSH levels plateau during initial days[11,12] and are gradually suppressed thereafter by ovarian inhibin B[13] and E_2[14] negative feedback. A rise in inhibin B occurs just after the intercycle rise in FSH. It may therefore be proposed that inhibin B limits the duration of the FSH rise. Decremental follicular phase FSH levels (effectively restricting the time when FSH levels remain above the threshold, referred to as the FSH *window*) (Fig. 30.1) appear to be crucial for selection of a single dominant follicle from the recruited cohort.[11] Only one follicle escapes from atresia by increased sensitivity for stimulation by FSH and luteinizing hormone (LH).[2] The important concept of increased sensitivity of the dominant follicle for FSH has been confirmed by human studies showing developing follicles to exhibit a variable tolerance for GnRH antagonists-induced gonadotropin withdrawal.[15,16] On the other hand, early stages of follicle development being independent from gonadotropins is confirmed in hypophysectomized women presenting with preovulatory graafian follicles within 2 weeks after the initiation of ovarian stimulation with exogenous gonadotropins.[17]

A central role has also been demonstrated for LH in monofollicular selection and dominance in the normal ovulatory cycle.[18-20] Although granulosa cells from early antral follicles respond only to FSH, those from mature follicles also contain LH receptors and therefore become responsive to both FSH and LH. The maturing dominant follicle may become less dependent on FSH because of the ability to respond to LH. It is suggested that the leading follicle continues its development owing to LH responsiveness, whereas smaller follicles enter atresia because of insufficient support by decreasing FSH concentrations during the late follicle phase. The dominant follicle can be distinguished by ultrasound from other cohort follicles by a size greater than 10 mm diameter.[10] The concept of both endocrine and autocrine up-regulation is supported by several other observations that characterize the dominant follicle, including the *in vitro* induction of aromatase enzyme activity,[21] ovarian morphology,[22] and endocrine changes in follicle fluid[23] and serum. These observations all show that enhanced E_2 biosynthesis is closely linked to preovulatory follicle development.

These concepts of follicular development and selection have come to underlie contemporary approaches to therapeutic ovulation induction in women suffering from anovulatory infertility. Moreover, our increasing understanding of the processes underlying monofollicular selection has enabled the development of new approaches to ovarian hyperstimulation for assisted reproduction treatments.

Preparations Used for Ovarian Stimulation

Evidence of the endocrine pituitary-gonadal axis arose early in the 20th century, when it was observed that lesions of the anterior pituitary resulted in atrophy of the genitals. The first convincing evidence supporting the existence of two separate gonadotropins (initially referred to as Prolan A and Prolan B) was provided by Fevold and Hisaw in 1931, and both LH and FSH were subsequently isolated and purified. In 1928, Aschheim and Zondek described the capacity of urine from pregnant women to stimulate gonadal function. The concept of stimulating ovarian function by the exogenous administration of gonadotropin preparations has

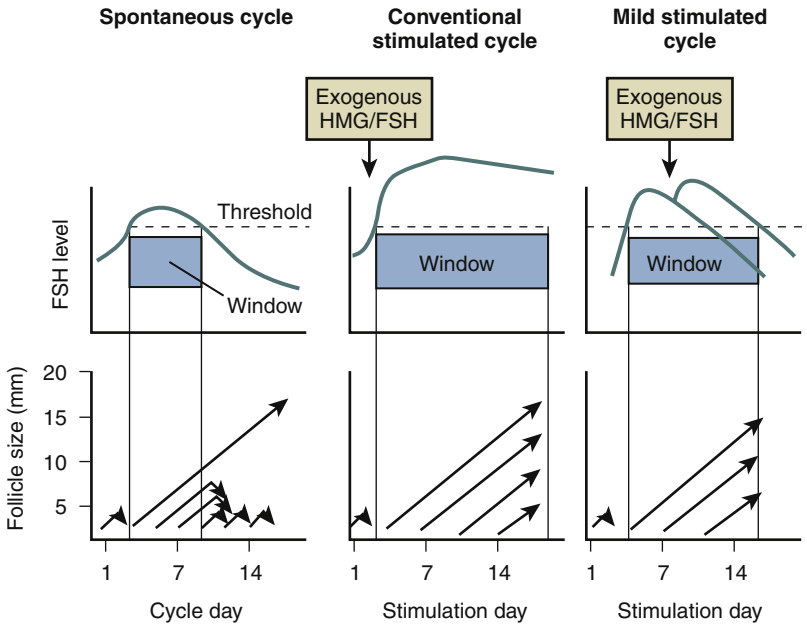


FIGURE 30.1 The follicle-stimulating hormone (FSH) threshold and window concept for monofollicular selection *(left panel)*, as conventionally applied to achieve multifollicular development *(middle panel)*. Each *arrow* represents a developing follicle. The *right panel* represents the concept of extending the FSH window by administering exogenous FSH in the midfollicular phase to maintain FSH levels above the threshold allowing multifollicular development. HMG, human menopausal gonadotropin. *(Adapted from Macklon NS, Stouffer RL, Giudice LC, Fauser BC. The science behind 25 years of ovarian stimulation for in vitro fertilization.* Endocr Rev *27[2]:170–207, 2006.)*

intrigued investigators for many decades. As early as 1938, Davis and Koff had already described the ability of purified pregnant mare serum to induce ovulation in humans by intravenous administration. However, these initial attempts had to be stopped due to species differences and resulting antibody formation impacting on efficacy and safety. Not until 1958 did Gemzell describe the first successful use for ovulation induction of gonadotropin preparations derived from human pituitaries. Shortly thereafter, Lunenfeld reported the clinical use of gonadotropin extracts from urine of postmenopausal women (for an historical overview, see Gruhn and Kazer[24] and Lunenfeld[25]).

A second important development allowing for ovarian stimulation on a large scale was a fine example of medical serendipity. The first estrogen antagonist tested in cancer patients was found to induce ovulation.

Clomiphene Citrate

In the late 1950s the first nonsteroidal estrogen antagonist (MER-25) was tested in patients to assess the efficacy of the compound in women with cystic mastitis, breast cancer, endometrial hyperplasia, or endometriosis. Some of these women with endometrial hyperplasia were of reproductive age and suffering from long-standing amenorrhea due to the Stein-Leventhal syndrome. To the great surprise of the investigators, the initiation of the medication in these women was followed by the recommencement of menstrual cycles.[26] Shortly thereafter, the ovulation-inducing capacity of the next generation of closely related anti-estrogens (MRL/41; clomiphene citrate, CC) (Fig. 30.2) was recognized.[27] More than half a century later, CC is still the most applied drug for infertility therapies worldwide, accounting for around two thirds of all prescriptions.

Clomiphene citrate is a racemic mixture of two stereoisomers. The enclomiphene isomer has a relatively short half-life, whereas the zuclomiphene isomer has an extended clearance. The two isomers demonstrate different patterns of agonistic and antagonistic activity *in vitro*.[28] Stimulation of ovarian function is elicited by raised pituitary FSH secretion due to blockage of E_2 steroid feedback by CC. Overall a 50% to 60% increase of serum FSH levels above baseline has been described.[29] The exact nature of the mechanism of action of CC is still uncertain.[30,31] Induced changes in other systems, like insulin-like growth factor (IGF) may partly explain the capacity of CC to stimulate the ovary.[30] However, anti-estrogenic effects at the uterine level (cervical mucus production and endometrial receptivity) are believed to underlie the observed discrepancy between achieved ovulation and pregnancy rates. The impact of a concomitant rise in LH on ovarian response to CC is also uncertain. CC for ovulation induction is considered to be relatively safe because steroid negative feedback remains intact. The oral route of administration and low costs represent additional advantages of this preparation. CC was originally developed for clinical use by the Merrel company in 1956, and it is still considered to represent the first-line treatment strategy in most anovulatory infertility. In addition, this compound was a central component in the early days of IVF[32,33] and is still often applied for the empirical treatment of unexplained infertility, alone or in combination with IVF.

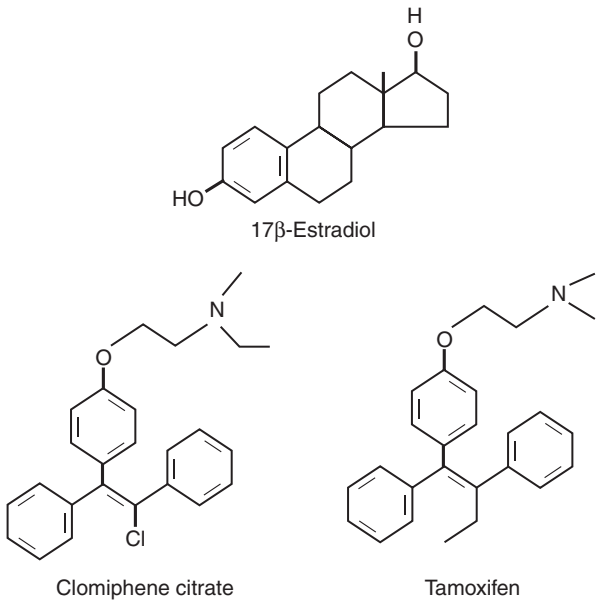


FIGURE 30.2 Structure of 17β-estradiol and the anti-estrogenic triphenylethylene derivates clomiphene citrate and tamoxifen.

Gonadotropin Preparations

Clinical experiments in the late 1950s demonstrated that extracts derived from the human pituitary could be used to stimulate gonadal function.[34] Subsequently, experiments involving the extraction of both the gonadotropic hormones LH and FSH from urine of postmenopausal women led to the development of human menopausal gonadotropin (hMG) preparations. From the early 1960s these preparations were used for the stimulation of gonadal function in the human.[35] It soon became clear that hMG was a very potent compound. Its ability to directly stimulate the ovaries was accompanied by the inherent risks of ovarian hyperstimulation. Initial use in the treatment of anovulation was associated with high rates of multiple pregnancy and OHSS. The potential for dangerous complications induced the need for monitoring of ovarian response and dose adjustment. More recently introduced low-dose protocols applied in conjunction with intense ovarian response monitoring have substantially contributed to improved treatment outcomes.

Initial attempts in the 1970s by Edwards and Steptoe to enable the conception of a baby through IVF also involved hMG stimulation protocols. Because of a lack of pregnancies (presumed due to abnormal luteal function) it was decided to switch to natural cycle IVF. It was an unstimulated cycle that led to the conception of the first IVF baby, Louise Brown, who was born on July 25, 1978.[36] Subsequent IVF pregnancies were reported from Australia to occur after ovarian stimulation with CC.[32] The more widespread use of hMG for successful IVF was developed thereafter in the United States.[37] For over 2 decades, gonadotropin preparations have also been extensively applied for ovarian stimulation in ovulatory women for empirical treatment of unexplained subfertility. Here the aim is to increase monthly fecundity rates by increasing the number of oocytes available for fertilization *in vivo* (with or without the additional use of IUI). These trends, and the rapid expansion in the use of IVF treatment, underlie the enormous increase in worldwide demand and sales for gonadotropin preparations.

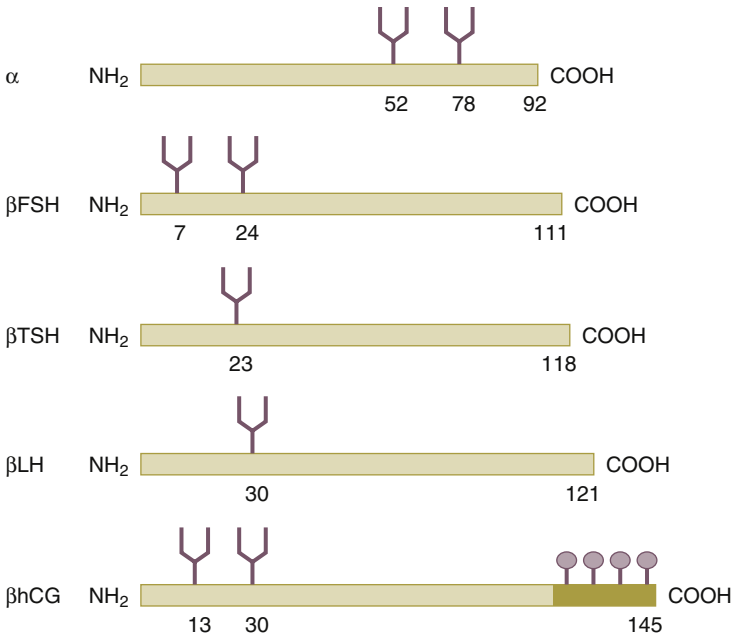


FIGURE 30.3 Structure of the common α subunit and hormone-specific β subunit of the human glycoprotein hormones: follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and human chorionic gonadotropin (hCG).

The early extraction techniques were very crude, requiring around 30 L of urine to manufacture enough hMG needed for a single treatment cycle. The FSH to LH bioactivity ratio of these early preparations was 1:1. As purity improved, it was necessary to add human chorionic gonadotropin (hCG) in order to maintain this ratio of bioactivity.[38] These initial preparations were very impure with many contaminating proteins; only less than 5% of the proteins present were bioactive. Bioactivity of gonadotropin preparations continues to be assessed by the crude *in vivo* rat ovarian weight gain Steehlman and Pohley assay. This rather anachronistic technique has the disadvantage of allowing considerable batch-to-batch inconsistency in bioactivity. However, improved protein purification technology allowed for the production of hMG with reduced amounts of contaminating nonactive proteins and eventually the development of purified urinary FSH (uFSH) preparations by using monoclonal antibodies since the late 1980s.[39] The currently available pure products allow for less hypersensitivity reactions, and less painful subcutaneous administration. Because of the worldwide increased need for gonadotropin preparations, demands for postmenopausal urine increased tremendously and adequate supplies could no longer be guaranteed. In addition, concern regarding the limited batch-to-batch consistency along with possibilities of urine contaminants emerged.[39]

Through recombinant DNA technology and the transfection of human genes encoding for the common α subunit and hormone-specific β subunit of the glycoprotein hormone (Fig. 30.3) into Chinese hamster ovary cell lines,[40] the large-scale *in vitro* production of human recombinant FSH (recFSH) has been realized.[41] The first pregnancies using this novel preparation in ovulation induction[42] and in IVF[43] were reported in 1992. Since then, numerous large-scale, multicenter studies have been undertaken, demonstrating their efficacy and safety. The recombinant products offer improved purity, consistency, and large-scale availability. Because of its purity, recFSH can now be administered by protein weight rather than bioactivity, and so-called "filled-by-mass" preparations[44] are now available for clinical use. During recent years, recLH and rechCG have also been introduced for clinical application.[29] Finally, a long-acting recFSH agonist (a man-made chimeric hormone generated by the fusion of the carboxy-terminal peptide of hCG to the FSH β chain) has recently been introduced into the clinic after efficacy and safety had been established in large sample size trials where IVF clinics from all over the world participated.[45-47]

GnRH Analogs

In 1971, the small decapeptide gonadotropin-releasing hormone (GnRH) was isolated and its structure elucidated by Schally and Guillemin (Fig. 30.4). Some years later, both investigators jointly received the Nobel prize for this discovery. Amino acid substitutions have revealed the significance of specific regions for its stability, receptor binding, and activation of the gonadotrope cells. This decapeptide is secreted by the hypothalamus into the portal circulation in an intermittent fashion, stimulating the pituitary gonadotropes to synthesize and secrete LH and FSH. Early studies demonstrated that pituitary down-regulation could be induced by the continued administration of GnRH.[48] Clinically safe GnRH agonists were developed relatively easily by replacing one or two amino acids. An increased potency could be achieved by replacing glycine for D-amino acids at position 6 and by replacing Gly-NH_2 at position 10 by ethylamide.[49] Such simple structural changes render these compounds more hydrophobic and more resistant to enzymatic degradation. The administration of GnRH agonists induces an initial stimulation of gonadotropin release for 2 to 3 weeks (the so-called "flare effect") followed by a down-regulation (or desensitization) due to the clustering and internalization of pituitary GnRH receptors.

GnRH agonists have been used clinically since 1981 to induce a "chemical castration" for steroid-dependent disease states such as fibroids and endometriosis in females and prostate cancer in males. The first paper concerning its use in IVF for the prevention of a premature LH rise also

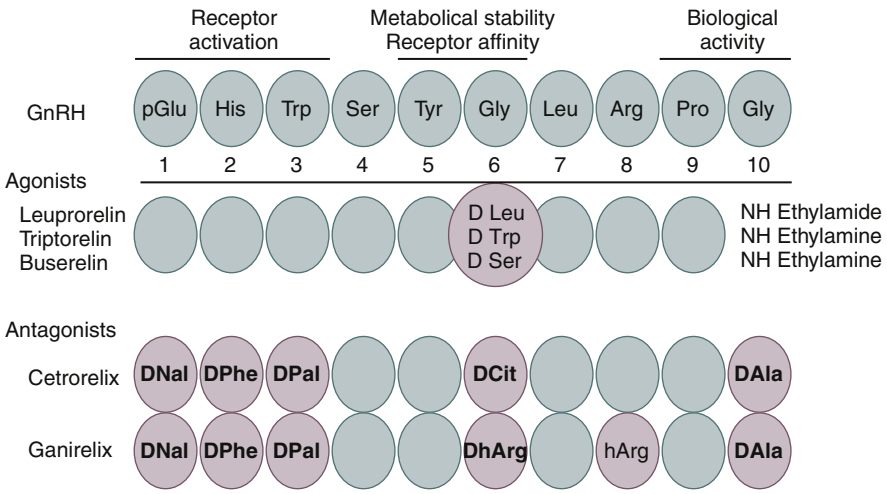


FIGURE 30.4 Structure of the native decapeptide gonadotropin-releasing hormone (GnRH), as well as the modified, commercially available GnRH agonists and antagonists. Arg, arginine; D, dextro; DAla, D-alanine; DCit, D-citroline; DhArg, D-homoarginine; DNal, D-naphthylalanine; DPal, D-pyridylalanine; DPhe, D-phenylalanine; Gly, glycine; hArg, homoarginine; His, histidine; Leu, leucine; pGlu, pyroglutamate; Pro, proline; Ser, serine; Trp, tryptophan; Try, tyrosine.

appeared in the early 1980s.[50] Shortly thereafter, the use of GnRH agonists such as buserelin, triptorelin, or leuprorelin to down-regulate the pituitary prior to administration of gonadotropins (a strategy that became known as the "long protocol") became the standard of care. The recent clinical introduction of GnRH antagonists may ultimately lead to a new standard of care in IVF practice.

It has taken almost 3 decades to develop GnRH antagonists with acceptable safety and pharmacokinetic characteristics. The first-generation antagonists were developed by replacing amino acids histidine at position 2 and tryptophan at position 3, but these compounds suffered from low potency. In second-generation compounds, the activity was increased by incorporating a D-amino acid at position 6. However, the widespread clinical application of these compounds was hampered by frequent anaphylactic responses due to histamine release. By introducing further replacements at position 10, third-generation compounds were developed.[51,52] Subsequently both the compounds ganirelix and cetrotide were shown to be safe and efficacious in IVF. These third-generation GnRH antagonists were registered in 2001 for use in IVF. The immediate suppression and recovery of pituitary function renders these compounds appropriate for short-term use in IVF. Clinical uptake of GnRH antagonists in IVF has been rather slow. Recent meta-analysis has confirmed the use of antagonist cotreatment to be effective and safe[53,54] It is estimated that at present around the world almost 50% of all IVF is with GnRH antagonist co-treatment. Further extended use of GnRH antagonist in steroid-dependent disease states such as endometriosis or myomas will depend on the development of depot or slow release formulations.

Outcomes of Ovarian Stimulation

Ovulation Induction

Amenorrheic women with anovulation exhibit virtually no chance of spontaneous conception and ovulation induction may restore normal fertility. However, the aim of mimicking normo-ovulatory cycles cannot always be achieved, and therefore, the chances of complications such as multiple pregnancy or OHSS should be taken seriously, especially in PCOS patients. Oligomenorrheic women may or may not have incidental spontaneous ovulations, and therefore, spontaneous pregnancies may occur. For obvious reasons, fertility specialists see only oligomenorrheic women who have failed to conceive, and these patients will usually respond well to ovulation induction. The balance between success and complications resulting from ovulation induction is dependent on many factors, including patient characteristics, gonadotropin preparations and dose regimens used, the intensity of monitoring ovarian response to stimulation, and willingness to cancel the cycle in case of hyper-response. An alternative option under those circumstances would be to convert ovulation induction to IVF. Cumulative live birth rates of ovulation induction have been reported to be around 75% to 80%,[55,56] with a coinciding incidence of multiple pregnancies of more than 10% and of OHSS of less than 2%.

OHSS is a potentially life-threatening complication characterized by ovarian enlargement, high serum sex steroids, and extravascular fluid accumulation, primarily in the peritoneal cavity. In severe cases, hypotension, increased coagulability, reduced renal perfusion, and oliguria may occur. Deranged liver function tests, venous and arterial thrombosis, renal failure, and adult respiratory distress syndrome can ensue, and fatalities have been reported.[57] Moderate to critical OHSS is very rare with CC but constitutes an important complication of gonadotropin use.[58] The incidences of mild, moderate, and severe OHSS following gonadotropin ovulation induction have been reported to be 20%, 6% to 7%, and 1% to 2%, respectively.[29] In addition to PCOS, risk factors for the development of OHSS include young age and low body weight.[58] The risk is further increased when adjuvant GnRH agonist treatment is employed.[59]

The contribution of ovulation induction treatment to the number of triplet and higher order pregnancies is considerable.[59,60] It has been calculated that 40% of higher order multiple births in the United States could be attributed to the use of ovulation-inducing drugs without assisted reproduction.[61]

Ovarian Stimulation

As previously outlined, the aim of ovarian hyperstimulation alone or in combination with assisted reproductive techniques is to bring an increased number of gametes (oocytes and sperm) together in order to augment pregnancy

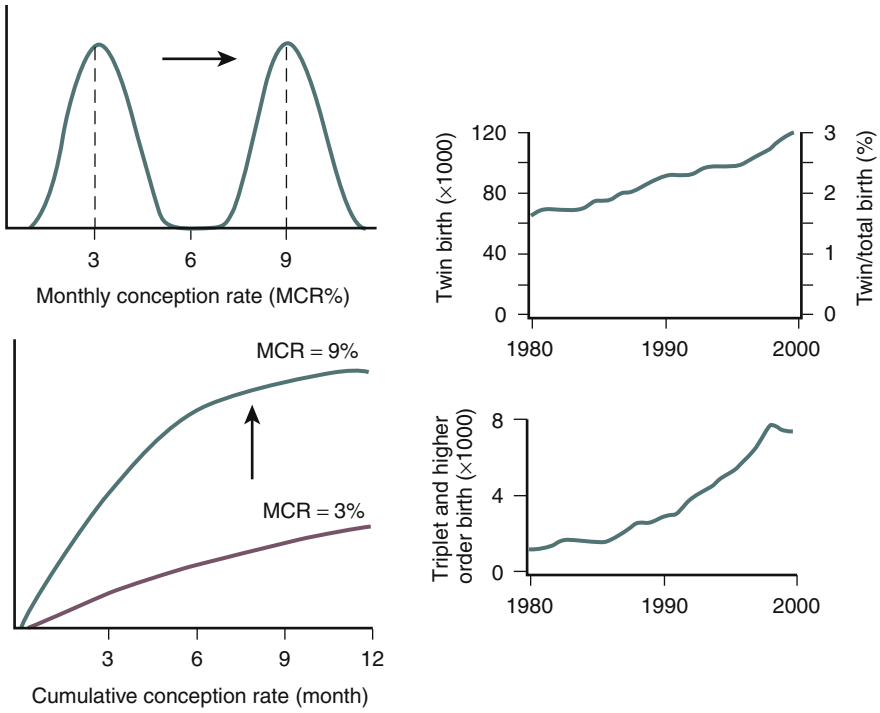


FIGURE 30.5 Monthly conception rate in unexplained infertility increasing from 3% to 9% per cycle due to ovarian stimulation and resulting increase in cumulative conception rates over a 12-month period *(left)*; increased occurrence of multiple pregnancies (twin, triplet, and higher order) between 1980 and 2000 associated with ovarian stimulation *(right)*. *(Left from Stovall DW, Guzick DS. Current management of unexplained infertility.* Curr Opin Obstet Gynaecol *5:228–233,1993; right from Rowland Hogue CJ. Successful assisted reproduction technology: the beauty of one.* Obstet Gynecol *100:1017, 2002; Jones HW. Multiple births: how are we doing?* Fertil Steril *79:17–21, 2003.)*

chances. Ovarian hyperstimulation alone may give rise to a two- to fourfold increase in pregnancy rates. The associated risk of OHSS and the occurrence of twin and higher order multiple births is dependent on the magnitude of ovarian stimulation, the intensity of ovarian response monitoring, and the criteria applied for cycle cancellation should too many follicles develop. The overall incidence of severe ovarian OHSS associated with ovarian hyperstimulation is less than 5%.[62]

Initial studies suggested that a threefold increase in monthly probability of pregnancy can be achieved with empirical ovarian hyperstimulation in the treatment of unexplained infertility[63] (Fig. 30.5). Subsequently, a large multicenter study showed that ovarian hyperstimulation with gonadotropins and IUI both exhibit an independent additive effect on pregnancy chances. Moreover, overall cumulative pregnancy rates with this combined therapy were reported to be 33% within three cycles, but at the price of an unacceptably high multiple pregnancy rate of 20% for twins and 10% for higher-order multiple pregnancy.[64] It has been proposed that a similar cumulative pregnancy rate could be achieved by expectant management over a 6-month period, obviously with much lower chances of multiple pregnancy. [65] In an analysis from one large European fertility center of 1878, pregnancies obtained from IUI cycles stimulated with gonadotropins, 16% were twins and 6% were triplets or higher order.[66] Less intense ovarian stimulation may reduce the incidence of higher order multiple pregnancies, but probably at the expense of a reduction in overall conception rate. On the basis of a 2-year experience in a large U.S. infertility clinic involving 3347 consecutive ovarian stimulation cycles (ovulation induction and ovarian hyperstimulation combined) in approximately 1500 women, a 30% pregnancy rate was described. Twenty percent of these pregnancies were twins, along with 5% triplets and 5% quadruplets or higher order.[67] The most worrying conclusion of this analysis was that the number of large antral follicles or serum E_2 levels during the late follicular phase had only limited value in predicting higher order multiple gestations. The true rate of multiple pregnancies arising from ovarian stimulation with or without IUI remains uncertain, however,[68] as few national registers record the outcome of ovarian stimulation. The most recent summary of the European IVF monitoring consortium involving 33 countries, over 1000 clinics and almost 500,000 IVF/ICSI cycles reported for the year 2007 a multiple delivery rate of 22.3% (including 1% triplets). For approximately 170,000 IUI cycles performed in Europe, twin deliveries were reported in 11.7% of cycles whereas the triplet rate was 0.5%.[69]

Some years ago it was estimated that ovarian stimulation with or without IUI is responsible for around 30% of multiple births (Fig. 30.6). It is easier to influence chances for multiple gestations after IVF, because the occurrence is primarily dependent on the number of embryos transferred. Therefore, ovarian hyperstimulation for IVF is merely the factor allowing for the generation of multiples, but not the sole determining factor like in IUI. Unsurprisingly, the incidence of twin pregnancies following IVF without stimulation[70] or with hyperstimulation combined with single embryo transfer is close to normal.[71,72] Over the years, the number of embryos transferred in IVF has decreased, but larger numbers continue to be transferred in the United States compared to Europe. On the basis of a large nationwide data set from the United Kingdom, it was reported quite some years ago that the number of embryos transferred could be reduced from three to two without a concomitant drop in overall pregnancy chances.[73] The policy of two embryo transfer was adopted by many major European IVF centers during the 1990s. Subsequently it was demonstrated that in young women in whom two high-quality embryos are transferred, the chances of a twin pregnancy are actually higher than for a singleton pregnancy.[74,75] An increasing number of centers, especially

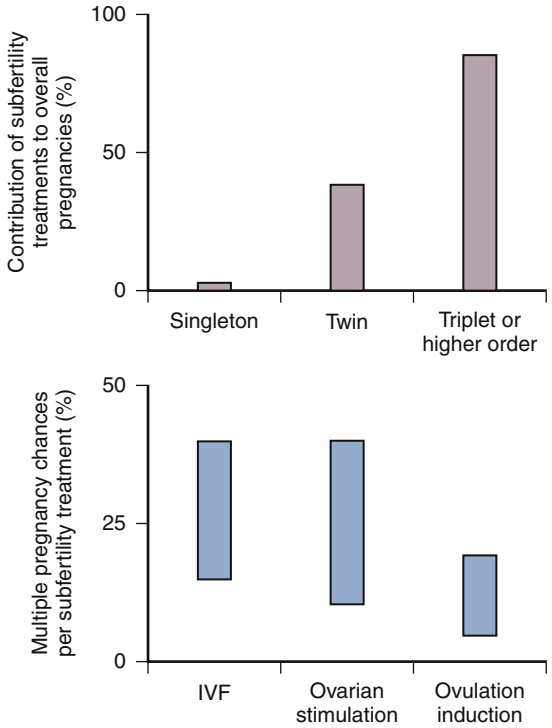
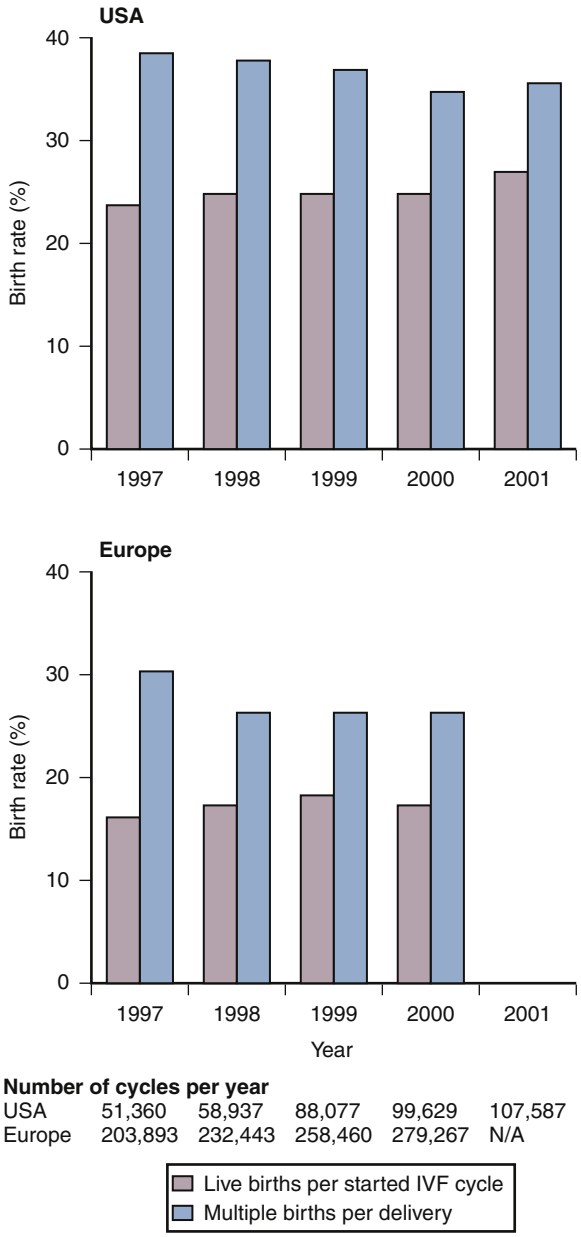


FIGURE 30.6 Contribution of subfertility treatments to overall pregnancies *(upper)* and reported frequency of multiple pregnancy in relation to *in vitro* fertilization (IVF), ovarian stimulation, and ovulation induction *(lower). (From Fauser BC, Devroey P, Macklon NS. Multiple birth resulting from ovarian stimulation for subfertility treatment.* Lancet *365[9473]:1807–1816, 2005.)*



Number of cycles per year

USA	51,360	58,937	88,077	99,629	107,587
Europe	203,893	232,443	258,460	279,267	N/A

Live births per started IVF cycle
Multiple births per delivery

FIGURE 30.7 Rates of live births per started *in vitro* fertilization (IVF) cycle and multiple births. N/A, not available. *(From Fauser BC, Devroey P, Macklon NS. Multiple birth resulting from ovarian stimulation for subfertility treatment.* Lancet *365[9473]:1807–1816, 2005.)*

in Northern in Europe, have adopted a single embryo transfer policy in selected women. Overall good clinical results have been reported, escpecially in the context of improved results of cryopreserved surplus embryos. Provided that the embryology lab performance is good, single embryo transfer policy can be introduced in the clinic without any decrease in overall pregnancy chances per oocyte harvest (hence the cumulative fresh plus cryo embryo transfer).[76-79]

The number of embryos transferred in the United States is substantially higher, with current American Society for Reproductive Medicine (ASRM) practice committee guidelines proposing more focus on wider society benefits of single embryo transfer.[80,81] In general, overall IVF results in Europe are slightly lower compared to the United States, but with an overall reduced incidence of multiple and premature birth (Fig. 30.7).

Given the risks associated with ovarian stimulation, couples should be well counseled regarding their spontaneous chances for pregnancy prior to commencing therapy (Table 30.1). These chances are often underestimated,[82] both by the doctor and by the patient and the price to be paid for interventions (the increased incidence of multiple pregnancies) may frequently be underemphasized. How should the increased chances for pregnancy on the one hand be balanced with the high complication rates inevitably associated with ovarian hyperstimulation? Until recently, fertility specialists tended to lean heavily in the direction of increasing pregnancy rates at all costs. In doing so, an iatrogenic epidemic of multigestation came about, with major health, psychosocial, and financial consequences.

Higher order multiple pregnancies have a major adverse impact on perinatal morbidity and mortality rates. Mortality rate is increased fourfold to sevenfold in twins and up to twentyfold in triplets.[60] Children born from multiple pregnancies have more chances for perinatal complications and subsequent health problems, chiefly associated with prematurity and low birth weight.[61] Chances for cerebral palsy are increased almost fiftyfold in children from triplet pregnancies.[83] Even the second child from a twin pregnancy delivered at term presents with a significant increased risk for death due to complications of vaginal delivery.[84] Besides the medical and emotional burden, the financial costs associated with multiple pregnancies should be taken into account by policy makers. Obstetric and neonatal costs are increased

Table 30.1 Hypothetical Model of Cumulative Spontaneous Pregnancy Rates in Five Categories, According to Duration of Subfertility

Category	MFR (%)	Cumulative Pregnancy Rate After (%)			
		6 Mo	12 Mo	24 Mo	60 Mo
Superfertile	60	100	—	—	—
Normally fertile	20	74	93	100	—
Moderately subfertile	5	26	46	71	95
Severly subfertile	1	6	11	21	45
Infertile	0	0	0	0	0

MFR, monthly fecundity rate.
From Evers JLH. Female subfertility. *Lancet* 360:151–159, 2002.

five- to sevenfold in higher order multiples, and by the age of 8 costs for low-birth-weight children are increased eightfold.[61] Finally, possibilities of more subtle health risks which may be revealed only later in life should also be taken seriously.

Perhaps one strategy that may help improve the situation would be to agree to a new way of defining success from infertility therapy. The appropriate outcome measure should be shifted from pregnancy rate per treatment cycle toward live birth or preferably, healthy singleton child per started course of treatment.[85]

Ultimately, however, the risk to benefit ratio of ovulation induction and ovarian hyperstimulation is determined by the practice of the clinician. In the following sections we provide an overview of the medical approaches applied in contemporary practice.

Induction of Ovulation in Anovulatory Women

Principles of Ovulation Induction

The aim of induction of ovulation in anovulatory women is to stimulate a single follicle to develop up to the preovulatory stage and subsequently ovulate. As stated before, this therapeutic goal should be clearly distinguished from two other forms of ovarian stimulation. First, ovulatory women with unexplained infertility may undergo a mild form of ovarian hyperstimulation aimed at producing two or three follicles and an increased chance of fertilization in a given cycle. This treatment, which is frequently combined with IUI, is discussed later in the chapter. Second, ovarian hyperstimulation may be applied in ovulatory women undergoing IVF treatment, where multifollicular development is required to produce multiple oocytes. In contrast to these two therapeutic approaches, which produce a superphysiological situation, ovulation induction aims to mimic the normal physiological monofollicular ovulatory cycle. Ovulation induction is characterized therefore by tighter therapeutic margins and a need for careful monitoring and skilled management if success without complications is to be achieved. Ovarian surgical techniques such as laparoscopic drilling offer an alternative to medical therapies in this context. Again, the aim of this treatment paradigm is to institute monofollicular ovulatory cycles.

Anovulatory disorders account for around 25% of causes of infertility.[86] This proportion may increase with the rising prevalence of obesity. Anovulation is usually manifested as the absence (amenorrhea) or infrequent occurrence (oligomenorrhoea) of menstrual periods. Although oligomenorrhoea may be associated with occasional ovulation, the chance of a woman conceiving within a year of unprotected intercourse is clearly diminished unless therapeutic steps are taken. Many medical approaches have been developed to achieve the goal of inducing the monthly development of a single dominant follicle and subsequent ovulation. In recent years, increased understanding of the pathophysiology of ovarian dysfunction has enabled the development of clinical strategies which aim to mimic the endocrine control of normo-ovulatory cycles. Achieving this within the narrow therapeutic margins of stimulating single rather than multiple follicular developments remains a challenge to clinicians.

The second European Society of Human Reproduction and Embryology (ESHRE) and ASRM sponsored PCOS consensus workshop acknowledged that much attention should be paid to the condition of the woman (in terms of food intake, lifestyle, smoking habits, etc.) before embarking on any form of ovulation induction.[87]

Classification of Anovulation

Ovarian dysfunction can be readily classified in everyday clinical practice on the basis of the assessment of serum gonadotropin and estrogen levels in peripheral blood. This concise approach, currently known as the World Health Organization (WHO) classification of anovulation, was developed by Insler and colleagues.[88] Amenorrhea may coincide with either low or normal E_2, whereas oligomenorrhea is associated with normal estrogens only. Low estrogens combined with low gonadotropin levels suggest a central origin of the disease at the hypothalamic-pituitary level.[89] This cause of anovulation occurs in less than 10% of infertile women and is termed WHO class 1. Low estrogens in combination with high gonadotropins suggest defective ovarian function per se, usually on the basis of premature ovarian failure (POF) (currently referred to as primary ovarian insufficiency [POI]) or ovarian dysgenesis. This cause of anovulation, termed WHO class 3, occurs in around 5% of infertile women and around 1% to 2% of the general female population. Anti-müllerian hormone (AMH) may help to identify women with POI with some residual ovarian activity.[90]

The majority (80% to 90%) of anovulatory women present with estrogen and FSH levels within normal limits. LH levels may be increased in these women. Polycystic ovary syndrome (PCOS), exhibiting FSH and E_2 concentrations within the normal range, represents the great majority of these women. Recently, new criteria for the diagnosis of PCOS have been supported by the ASRM and ESHRE. The so-called Rotterdam consensus criteria are broader than the NIH (National Institutes of Health) criteria primarily because polycystic ovaries are now included. The incidence of PCOS as defined by the Rotterdam criteria is therefore higher.[91]

An additional cause of anovulation with an endocrine etiology is hyperprolactinemia which may present with normal or reduced gonadotropin and E_2 concentrations. This

may be considered as a variant of WHO class 1 anovulation because high serum prolactin levels suppress GnRH release by the hypothalamus by altering opioid receptor stimulation. Hyperprolactinemia may also present with normal gonadotropin and E_2 concentrations, and may then be considered as a variant of WHO class 2. The pathophysiology and treatment of hyperprolactinemia are discussed in detail in Chapter 3.

Preparations for Treating Anovulation

Anti-Estrogens

Background

The most widely used anti-estrogen for treating anovulation is CC, the development and pharmacology of which are addressed in the introduction to this chapter. In terms of its relative efficacy, safety, cost, and ease of use, it remains some 40 years after its introduction into clinical practice the most important therapeutic agent in use. The principal indication for CC is the treatment of anovulatory infertility in women with an intact hypophyseal-pituitary-ovarian axis. In this role it remains the first-line therapy. Given orally in the early to midfollicular phase, it causes a 50% rise in the endogenous serum FSH level,[92] thus stimulating follicle growth. This rise in FSH is accompanied by a similar rise in serum LH levels. Limitation of the duration of administration to 5 days is aimed at allowing FSH levels to fall in the late follicular phase and the mechanisms for monofollicular development and ovulation to operate. However, elevated gonadotropin levels may persist into the late follicular phase in some women.[93] The long half-life zuclomiphene isomer (which exhibits predominant estrogen agonist activity) has been shown to persist and accumulate across consecutive cycles of treatment.[94] However, the resulting concentrations are well below those demonstrated to have any effects *in vitro* and are unlikely to be of clinical significance.

Preparations and Regimens

The conventional starting dose of CC is 50 mg/day, starting from day 2 until day 5 of the menstrual cycle, for 5 consecutive days. In normogonadotropic amenorrheic women, treatment can be initiated following a progesterone-induced withdrawal bleeding. Whether CC is commenced on cycle day 1 or 5 does not appear to affect outcomes.[95] Should 50 mg/day fail to elicit follicle growth, the dose should be increased to 100 mg/day in the subsequent cycle, followed by 150 mg/day, which is usually considered to be the maximum dose beyond which alternative treatments are indicated. The LH surge occurs between 5 and 12 days following the last day of CC administration. Intercourse is therefore advised for a week from the fifth day after the last day of CC administration. Some advocate hCG administration as a surrogate for the LH surge to trigger ovulation and to time intercourse. However, recent studies showed no improvement in outcomes, despite the increased monitoring required to time hCG administration.[96,97]

Clinical Outcome

Between 60% and 85% of anovulatory women will become ovulatory with CC, and 30% to 40% will become pregnant.[87] In a meta-analysis based on four placebo-controlled studies in oligomenorrheic patients, the odds ratio with CC was 6.8 for ovulation and 4.2 for pregnancy.[98]

Why some women with WHO class 2 anovulation do not respond to CC is not fully understood. Altered individual requirements for FSH at the ovarian level, the local intraovarian effect of autocrine or paracrine factors, and variations in FSH receptor expression or FSH receptor polymorphisms may contribute. A number of studies have pointed to being overweight as a factor.[99] In a multivariate analysis of factors found to predict outcome of CC ovulation indication, the free androgen index (FAI), body mass index (BMI), presence of amenorrhea (as opposed to oligomenorrhea), and ovarian volume were found to be independent predictors of ovulation.[99] The possibility of using clinical data to individualize treatment and optimize outcomes is discussed later in this chapter.

The occurrence of ovulation can be identified by the use of temperature charts and midluteal urinary pregnanediol or serum progesterone measurements.[87] Although results of large trials indicate that monitoring by ultrasound is not mandatory to ensure good outcomes,[87] the practice in many centers is to monitor the first cycle to allow adjustment of dose where necessary. The cumulative pregnancy rate in ovulatory women with CC in 6 to 12 months of treatment is around 70%,[99] with conception rates per cycle around 22%.[87]

Why do some women who become ovulatory with CC not conceive? Reasons include patient selection, the regimen used, and the presence of other causes of subfertility. The anti-estrogenic effects of CC on the reproductive tract have been particularly implicated. Negative effects on tubal transport, quantity and quality of cervical mucus,[29] and the endometrium[100] have all been reported.

Miscarriage rates of 13% to 25% are reported. Although these numbers appear high, they are similar to the spontaneous miscarriage rate[101] and those observed in infertile women undergoing IVF. In general, it does not appear that the miscarriage rate is significantly increased in anovulatory women treated with CC.

Side Effects and Complications

Apart from hot flushes, which may occur in up to 10% of women taking CC, side effects are rare. Nausea, vomiting, mild skin reactions, breast tenderness, dizziness, and reversible hair loss have been reported, but less than 2% of women are affected. The mydriatic action of CC may cause reversible blurred vision in a similar number.[26]

The multiple pregnancy rate is less than 10%, and OHSS is rare.[87] The putative increased risk of ovarian cancer reported to be associated with the use of CC for more than 12 months[102] has led CC to be licensed for just 6 months of use in some countries.

Tamoxifen is, like CC, a nonsteroidal selective estrogen receptor modulator (SERM). In contrast to CC, tamoxifen contains only the zu-isomer and appears to be less anti-estrogenic at the uterine level. The possible advantages of tamoxifen over CC include an agonistic effect at the endometrium. Many uncontrolled studies in the area of ovulation induction have suggested that tamoxifen may be a safe and efficacious alternative to CC. A meta-analysis of four randomized controlled studies revealed tamoxifen to be as effective as CC in inducing ovulation. However, despite the

theoretical benefits, no significant improvement in pregnancy rates was observed compared with CC.[103] Clinicians should therefore base their choice of treatment on familiarity with the given regimen.

Insulin-Sensitizing Agents

Background

The role of insulin-resistance in the pathogenesis of ovarian dysfunction in many PCOS patients has led to the introduction of insulin-sensitizing agents as adjuvant or sole treatment regimens for the induction of ovulation. The most extensively studied insulin-sensitizing drug in the treatment of anovulation is metformin. Metformin (dimethylbiguanide) is an orally administered drug used to lower blood glucose concentrations in patients with non-insulin-dependent diabetes mellitus (NIDDM).[104] It is antihyperglycemic in action, and increases sensitivity to insulin by inhibiting hepatic glucose production and by increasing glucose uptake and utilization in muscle. These actions result in reduced insulin resistance, lower insulin secretion, and reduced serum insulin levels.

Many papers have been published in recent years advocating the clinical usefulness of this compound for ovulation induction. The absence of well-designed and properly powered studies did not dampen enthusiasm for metformin in this context, and it has been widely introduced into clinical practice. Recently, however, two large, placebo-controlled randomized studies comparing metformin to CC and metformin as adjunctive therapy to CC have shown no benefit of metformin.[105,106]

Preparations and Regimens

The first studies reporting the use of metformin as an ovulation induction agent suggested that metformin improved insulin sensitivity; lowered LH and total and free testosterone concentrations; and increased FSH and sex hormone–binding globulin levels.[107,108] This, and subsequent uncontrolled studies, indicated that correction of hyperinsulinemia has a beneficial effect in anovulatory women, by increasing menstrual cyclicity, improving spontaneous ovulation, and thus promoting fertility.[109,110] It is recommended that metformin be commenced at 500 mg/day orally, rising to 500 mg three times a day over 7 to 10 days.[109] Depending on response, this may be increased to 1000 mg twice a day. The optimal duration of treatment remains unclear. However, most studies reporting a beneficial effect from metformin have shown this within 2 to 4 months.[111,112]

Clinical Outcome

The majority of studies on the outcome following metformin therapy are small and uncontrolled or simply case series.[113] Most of the available data on restoration of menses following metformin therapy are on predominantly obese hyperinsulinemic women with PCOS. Similarly, studies of the ability of metformin to induce ovulation have been primarily carried out in obese women. In a meta-analysis of 15 studies involving 543 participants with PCOS, metformin was found to be effective in achieving ovulation with odds ratios of 3.88 (CI 2.25 to 6.69) for metformin versus placebo and 4.41 (CI 2.37 to 8.22) for metformin and clomiphene versus clomiphene alone.[114] However, a recent large multicenter

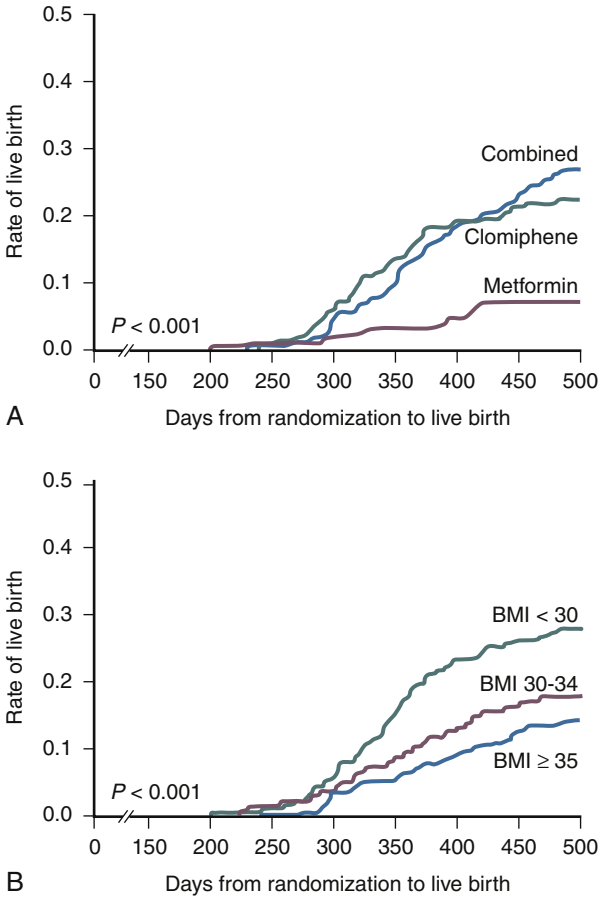


FIGURE 30.8 Kaplan-Meier curves for live births following ovulation induction with clomiphene, metformin, or both treatments combined **(A)** and body mass index (BMI) **(B)**. *(Adapted from Legro RS, Barnhart HX, Schlaff WD, et al. Clomiphene, metformin, or both for infertility in the polycystic ovary syndrome.* N Engl J Med *356:551–566, 2007.)*

study has clarified the role of metformin as an alternative first-line ovulation induction agent in women with PCOS.[106] In this study, 626 women with PCOS were randomized to receive 50 to 150 mg CC plus placebo from cycle day 3 to 7500 to 2000 mg daily doses of extended release metformin plus placebo, or a combination of metformin and CC. Treatment was continued for up to 6 months. Obesity was not an exclusion criterion. The results of this study are summarized in Figure 30.8. The primary end point of live birth rate was 22.5% after treatment with CC, compared with a significantly lower rate of just 7.2% following metformin treatment. Combination therapy with both metformin and CC yielded a live birth rate of 26.8%, which did not differ significantly from that achieved with CC treatment alone. The relatively poor performance of metformin in terms of live birth rates was partly explained by a low conception rate, which was just 21.7% following metformin, compared with 39.5% in the CC group. In terms of ovulation rates alone, the combination of metformin and CC was superior to either individual therapy. However, in a study of 228 women randomized to receive CC plus metformin or CC plus placebo, no significant difference in ovulation rates was observed (64% versus 72%, respectively).[105] A significantly larger proportion of women in the metformin group discontinued treatment because of side effects (16% versus 5%).

Box 30.1 Maternal and Perinatal Risks Associated with Polycystic Ovary Sydrome

MATERNAL
- Gestational diabetes*
- Pregnancy-induced hypertension*
- Preeclampsia
- Delivery by cesarean section

NEONATAL
- Admission to a neonatal intensive care unit
- Perinatal mortality
- Premature deliveries

*Outcome confirmed by subgroup analysis of higher validity studies.
From Boomsma CM, Eijkemans MJ, Hughes EG, et al. A meta-analysis of pregnancy outcomes in women with polycystic ovary syndrome. *Hum Reprod update* 12:673–683, 2006.

It has been suggested that metformin may reduce the rate of miscarriage compared with CC-derived pregnancies. However, in the study of Legro and colleagues, the rate of first trimester loss did not differ significantly between the treatment groups, although the study was not powered to detect this.[106]

Metformin therapy has also been proposed to aid weight loss in obese women with PCOS. Many studies have now examined the effect of metformin on BMI, and the evidence is conflicting. However, the majority of observational studies addressing weight loss with metformin have revealed a reduction in the BMI of 1% to 4.3%.[111] More recently, a double-blinded randomized trial compared metformin 850 mg twice daily treatment with placebo in 143 PCOS women with a BMI greater than 30. After 6 months' treatment no significant difference in weight loss or menstrual frequency was observed. In contrast, lifestyle modification was to improve cycle regularity by improving weight loss.

Attention has turned in recent years to the possible benefits and safety of metformin administration during pregnancy. PCOS pregnancies demonstrate a greater incidence of perinatal and maternal complications such as gestational diabetes, preeclampsia, and premature delivery[115] (Box 30.1). A number of studies have appeared suggesting a role for metformin to ameliorate these complications. However, most of these studies are not randomized or suffer from small numbers and surrogate outcomes. Although metformin crosses the placenta, there is no clear evidence of toxicity when taken during pregnancy. Larger well-designed studies are still required to address the possible therapeutic benefits of metformin during pregnancy in women with PCOS.

In summary, although some individual studies continue to suggest a benefit of metformin pretreatment[116] a recent Cochrane analysis involving 40 trials failed to establish improved live birth rates of metformin cotreatment.[117]

Side Effects and Complications

Metformin has been used for many years for the treatment of diabetic patients and appears to be safe for long-term use, with few side effects reported. Rarely, lactic acidosis may occur[114] if hepatic or renal disease is present, and these patients should be excluded before commencing therapy. The main side effects of metformin are nausea and diarrhea, which may occur in 10% to 25% of patients and contribute to the weight loss effects observed with metformin. If these symptoms persist despite lowering the dose, alternative therapy should be given. For this reason, metformin should be started at a low dose that gradually rises (see earlier discussion).

Gonadotropins

Background

Women with WHO class 2 anovulation who fail to ovulate or conceive following ovulation induction with antiestrogens can be successfully treated with exogenous gonadotropins. Exogenous gonadotropins have been widely used for the treatment of anovulatory infertile women since 1958.[2,25] Improvements in purification techniques led to increasing relative amounts of the active ingredients and the first urine-derived preparation containing only FSH (uFSH) became available in 1983. The development and application of production techniques based on immunoaffinity chromatography with monoclonal antibodies enabled the production of highly purified uFSH. In the 1980s, recombinant DNA technology led to the development and, later, the clinical introduction of human recombinant FSH (recFSH). This advance promised not only unlimited availability, but improved purity and batch-to-batch consistency compared to urinary derived products.

The development of recombinant gonadotropins also provided the opportunity to elucidate more clearly the physiology of ovarian E_2 synthesis. During further follicular development, LH has a synergistic action with FSH. Theca cells are stimulated by LH to convert cholesterol into androstenedione (AD) and testosterone (T) by cytochrome P450 side chain cleavage oxidases and 3β-hydroxysteroid dehydrogenase. Aromatase activity in the granulosa cells is induced by FSH and converts AD and T into estrone and E_2. The involvement of two cell types (granulosa and theca cells) and two hormones (LH and FSH) to produce estrogens from cholesterol has led to the "two cell, two gonadotropin" concept. In addition to stimulating aromatase activity, FSH also induces LH receptors and further increases FSH receptor formation on granulosa cells while stimulating DNA and protein synthesis by the cell.[118] Clinical observations in the treatment of anovulatory women have supported this concept.

In the treatment of WHO class 1 (hypogonadotropic hypogonadal) anovulation, women with intact pituitary function can be treated with pulsatile GnRH therapy to restore the periodic release of FSH and LH. The treatment of hypogonadotropic women with FSH alone leads to follicular development but not pregnancy.[17] Exogenous LH is therefore required to treat this form of anovulatory infertility. Until recently, hMG was the only source of exogenous LH for this group of patients. Now recLH or rechCG offers the possibility for a more sophisticated approach to treatment.

Recent studies have demonstrated the safety and appropriate dose required to affect follicle development and subsequent pregnancy. It has been established that baseline levels of at least 0.5 to 1 IU LH should be sufficient to provide maximal stimulation to thecal cells.[119] In a study of hypogonadotropic women undergoing treatment with

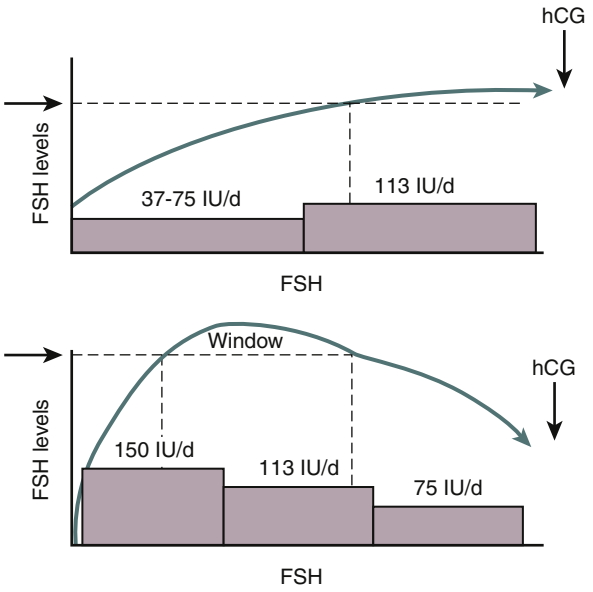


FIGURE 30.9 Schematic representation of serum follicle-stimulating hormone (FSH) levels and daily dose of exogenous FSH during low-dose step-up or step-down regimens for ovulation induction.

recFSH and recLH, a dose of 75 IU per day of recLH was observed to result in follicular development and pregnancy. However, further increases in LH levels above the threshold level needed to gain a response did not appear to induce a greater degree of ovarian stimulation.[120]

Preparations and Regimens

In addition to urinary derived FSH products, recFSH has been clinically available since 1996 in the form of follitropin alpha and follitropin beta. More recently, a long-acting recFSH (corifollitropin alpha), a recLH, and a rechCG have been added to the clinical arsenal for ovarian stimulation.

In order to achieve development of a single dominant follicle with exogenous gonadotropins, specific treatment and monitoring protocols are needed. The two most frequently encountered in the literature and in clinical practice are the low-dose step-up and, more recently, step-down protocols (Fig. 30.9). The initially described standard step-up protocol had a starting dose of FSH 150 IU/day.[121] However, this regimen was associated with a high complication rate. Multiple pregnancy rates of up to 36% were reported, and ovarian hyperstimulation occurred in up to 14% of treatment cycles.[2] As a result, this protocol has been largely abandoned.

The concept of the FSH threshold proposed by Brown[122] postulated that FSH concentrations must exceed a certain level before follicular development will proceed (Fig. 30.1). Once this level is reached, normal follicular growth requires only a minor further increase above this threshold. Exposure to excessive FSH serum concentrations may lead to excessive follicular development. This concept formed the theoretical basis for low-dose step-up regimens for ovulation induction. A low-dose, step-up protocol designed to allow the FSH threshold to be reached gradually has now become the most widely used regimen, reducing the risk of excessive stimulation and development of multiple preovulatory follicles. The recommended initial starting dose of FSH is 37.5 to 50 IU/day.[87] The dose is increased by 50% if after 14 days, no response is observed on ultrasonography (and serum estradiol monitoring). The detection of an ovarian response is an indication to continue the current dose until hCG can be given to trigger ovulation. If equal daily doses of FSH are given from the beginning of the follicular phase, steady-state serum FSH concentrations are reached after 5 to 7 days.[123] During step-up regimens, elevated FSH serum concentrations may occur during the late follicular phase, which may, in a similar manner, interfere with selection of a single dominant follicle. Previous suppositions that steroid negative feedback remained intact during low-dose step-up regimens have not been substantiated by scientific data.

In contrast to the concept of the FSH threshold on which the low-dose step-up protocol is based, the concept of the FSH "window" stresses the significance of the duration of FSH elevation above the threshold level, rather than the magnitude of elevation of FSH for single dominant selection.[124] This concept was substantiated by the demonstration that elevating FSH levels high above the threshold level for a short period of time in the early follicular phase does not increase the number of dominant follicles.[125] Conversely, when the physiological decrease of FSH in a normal cycle is prevented by administration of FSH in the late follicular phase, the augmented sensitivity for FSH allows several follicles to gain dominance[126] (Fig. 30.10). As demonstrated previously in the monkey model, when the negative feedback effect of estradiol on gonadotropin production is suppressed by administration of anti-estrogens, selection of the preovulatory follicle is overridden.[127] Further studies regarding the process of selection of the dominant follicle in the normal cycle have indicated that throughout the cycle up to 10 nondominant follicles (measuring between 2 and 10 mm in diameter) can be visualized by transvaginal ultrasound. The dominant follicle itself can be identified once it has reached a diameter beyond 9 mm.[10] Endocrine studies have confirmed that E_2 levels in the serum[11] and follicle fluid[23] begin to rise only after a dominant follicle is present. The above-mentioned initial research findings provided the theoretical basis for developing and monitoring a step-down regimen of ovulation induction.

It was subsequently demonstrated that the late follicular phase FSH profile during a step-down regimen closely resembled serum FSH levels in the spontaneous cycle.[128] Moreover, a median daily fall of 5% to 10% in serum FSH levels was observed in women treated with the step-down regimen, and in those treated with a low-dose step-up regimen a reduction was observed in just 39% of treated women.[129] In the majority of women, the FSH levels remained stable in the late follicular phase.

With the aim of rapidly achieving the FSH threshold for stimulating follicle development, step-down regimens normally begin therapy with 150 IU/day, started shortly after a spontaneous or progesterone-induced bleed. This dose is continued until a dominant follicle (≥10 mm) is observed. The dose is then decreased to 112.5 IU/day followed by a further decrease to 75 IU/day 3 days later, which is continued until hCG is administered to induce ovulation.[130] Should no ovarian response be observed after 3 to 5 days, the FSH starting dose should be continued.

For some patients an initial dose of 150 IU/day is too high, reflecting major individual differences in the FSH

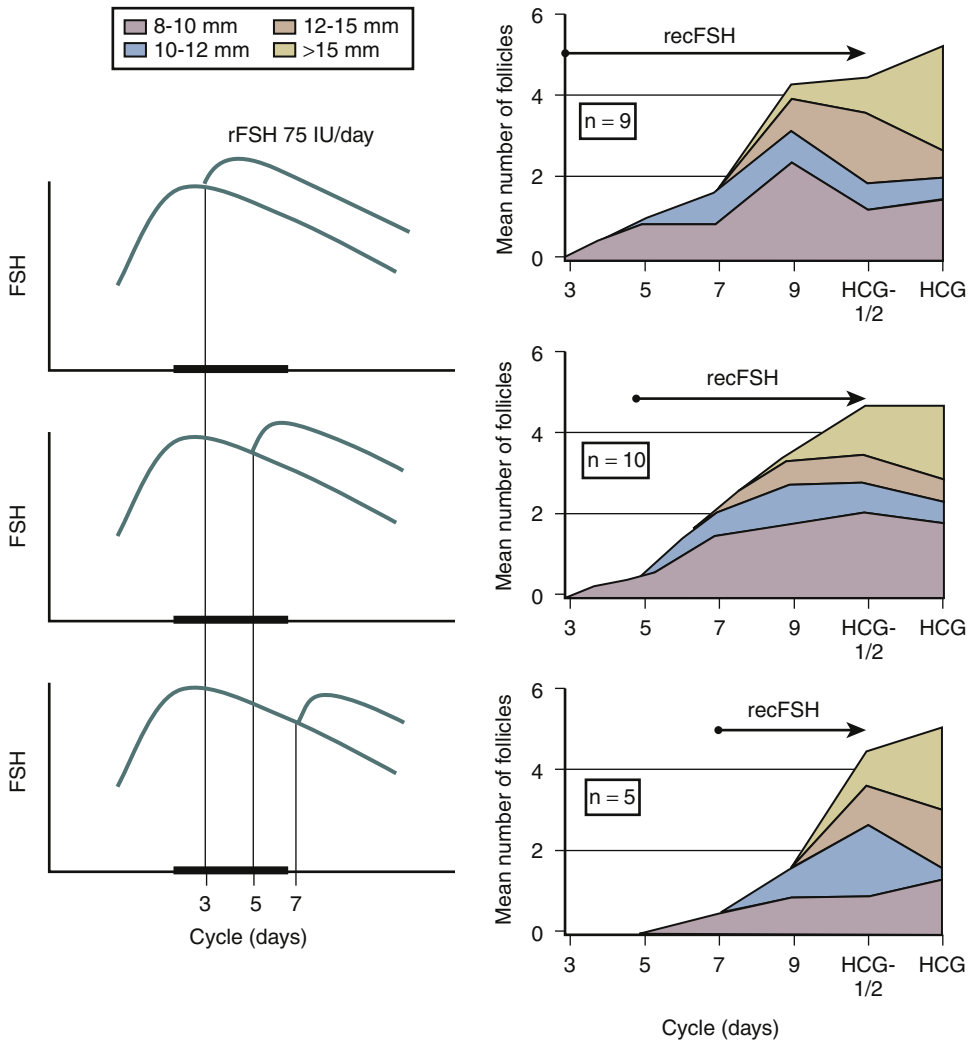


FIGURE 30.10 Observations substantiating the follicle-stimulating hormone (FSH) window concept in the human. Multiple dominant follicle growth can be observed in normo-ovulatory women receiving daily low doses of exogenous FSH, starting on cycle day 3, 5, or 7. *(From Hohmann FP, Laven JS, de Jong FH, et al. Low dose exogenous FSH initiated during the early, mid or late follicular phase can induce multiple dominant follicle development.* Hum Reprod *16:846–854, 2001.)*

threshold. The appropriate starting dose may be determined by using the low-dose step-up regimen for the first treatment cycle in order to assess the individual FSH response dose.[131] Patients who demonstrate good follicular growth with a fixed regimen of 75 IU/day, (the "good responders" who might have been at risk of OHSS with the normal starting dose of the step-down regimen) can thus be identified. Conversely, those who do not respond with ongoing follicle growth to the initial dose should have the daily dosage increased. The second cycle may then be initiated as a step-down regimen with a starting dose 37.5 IU above the effective dose in the preceding low-dose step-up cycle.

Experience to date has indicated that the major drawback of the step-down regimen is the risk that the initial starting dose is too high for some patients. In an effort to overcome this problem, sequential low-dose step-up and step-down regimens have been proposed.[132] Starting with a step-up regimen, the FSH dose is reduced when the leading follicle has reached 14 mm diameter. Comparisons with a group treated with a low-dose step-up regimen showed the incidence of monofollicular cycles to be similar. This approach requires further evaluation and the problem of the large individual variability in the dose of exogenous FSH required for monofollicular development remains to be properly addressed.

Clinical Outcomes

In what remains one of the largest series describing outcomes using the low-dose step-up regimen, 225 women with PCOS, with ovulation and pregnancy rates of 72% and 45%, respectively, were reported.[133] Studies focusing on further reducing the starting dose have reported the feasibility of commencing with 50 IU or 37.5 IU.

In a randomized trial comparing outcomes following the low-dose step-up versus step-down protocol, the clinical benefits of a more physiological means of stimulating follicle development were reflected in an incidence of monofollicular cycles of 88% compared to 56% observed in women treated with the step-up regimen, presumably reducing the risk of multiple pregnancy and hyperstimulation[129] (Table 30.2). Potential health and economic benefits were also apparent because those treated with the step-down regimen required a mean duration of treatment of just 9 days, as opposed to 18 days in women treated with the low-dose step-up regimen.

A multicenter randomized study comparing the step-up versus step-down protocol using recFSH reported a shorter duration of stimulation when the step-down protocol was used.[134] The cumulative rate of clinical gestations did not differ between the two groups, but in contrast to the findings

Table 30.2 Comparison of Ovarian Response and Clinical Outcome Following the Low-Dose Step-Up and the Step-Down Regimens for Gonadotropin Induction of Ovulation

	Low-Dose Step-Up			Step-Down	
Response and Outcome	Hamilton (1991)	Hull (1991)	Christin-Maitre (2003)	Christin-Maitre (2003)	van Santbrink (1995)
Number of patients	100	144	44	39	82
Number of cycles	401	459	85	72	234
Duration treatment (days)	14	NR	15	10	11
Ampules per cycle	19	NR	13	13	14
Ovulation rate (%)	72	74	70	61	91
Monofollicular cycles					
% of ovulatory cycles	73	NR	77	35	62
% of all started cycles	55	NR	68	32	56
Pregnancy rate (%)					
Per started cycle	11	11	19	16	16
Per ovulatory cycle	16	15	NR	NR	17
Cumulative pregnancy rate (%)	55	NR	37	31	47
Multiple pregnancy rate (%)	4	11	12	25	8
Ongoing singleton pregnancy rate (%)	7	10	NR	NR	12
Ovarian hyperstimulation syndrome rate (%)	1	NR	2	11	2

NR, not recorded.

of an earlier single-center study, the step-up protocol was associated with a higher rate of monofollicular development and a lower rate of ovarian hyperstimulation (Table 30.2). These differences may reflect the necessity for increased skill and care in monitoring step-down stimulation cycles, which is easier to ensure in a single-center setting. Largely for this reason, low-dose step-up protocols remain the most widely used approach.

The degree to which the type of FSH compound employed may influence outcomes in ovulation induction continues to be subject of some controversy. Two meta-analyses comparing the effectiveness of daily uFSH to daily hMG for inducing ovulation in women with PCOS who had not responded to CC demonstrated no difference in pregnancy rate per treatment cycle. However, the women given FSH were less likely to have moderately severe or severe ovarian hyperstimulation syndrome.[59] The total dose of recFSH needed and duration of treatment was less, and the complication rates were similar. In a later meta-analysis of randomized controlled trials comparing recFSH with uFSH for ovulation induction in women with CC-resistant PCOS, no significant differences were demonstrated for the ovulation rate (OR 1.19, 95% CI 0.78 to 1.8). Furthermore, the odds ratios for pregnancy rate (0.95, 95% CI 0.64 to 1.41), miscarriage rate (1.26, 95% CI 0.59 to 2.7), multiple pregnancy rate (0.44, 95% CI 0.16 to 1.21), and ovarian hyperstimulation syndrome (1.55, 95% CI 0.5 to 4.84) showed no significant difference between recFSH and uFSH.[135] In terms of cost-effectiveness, a recent randomized study showed that a lower total dose of recFSH than highly purified urinary FSH was required to achieve the same outcomes. This translated into a 9.4% cost reduction in favor of recFSH.[136]

The success in early clinical studies of pure FSH preparations, increasingly devoid of LH, has served to enhance the impression that excess LH is detrimental to oocyte development and chances of pregnancy following therapeutic intervention. However, a number of recent clinical studies, together with an increasing understanding of the function played by LH in oocyte maturation, have begun to redefine the role of LH as a therapeutic agent in anovulatory fertility. In normogonadotropic anovulation, endogenous LH does not normally require supplementation. Indeed, the focus on LH in this group of patients has been primarily directed at reducing the potential detrimental effects associated with excessive LH.[137] More recently, however, the demonstration of the importance of late follicular LH in optimizing dominant follicle development oocyte quality has reopened the debate as to the role of LH in ovulation induction.[19] Supplementation of LH activity may offer advantages in some patients by hastening large follicle development and therefore shortening the duration of treatment.[138] Moreover, the work of Zeleznik and co-workers[18] referred to a potential therapeutic role for LH in effecting monofollicular stimulation as part of a sequential ovarian stimulation protocol following initiation with recFSH. This concept has been supported in a study in which anovulatory women with a hyper-response to recFSH were randomized to continue treatment with the addition of either placebo or recLH.[139] In those in whom LH was administered, a trend toward fewer preovulatory follicles was observed. As the availability of recombinant gonadotropins leads to increasing knowledge of the processes of follicular development and selection, further refinements in the efficacy and safety of ovulation induction are likely.

Adverse Effects and Complications

The complications of ovulation induction with gonadotropins are primarily related to excessive ovarian stimulation. Although the aim of therapy is monofollicular growth, multiple follicular developments may occur, causing symptoms of OHSS. Moreover, the development of multiple follicles raises the real risk of multiple pregnancies. In order to increase the chance of therapeutic success and reduce the risks of complications, careful monitoring of treatment is required. Ovarian response to gonadotropin therapy is monitored using ultrasound to measure follicular diameter. The scans, usually performed every 2 or 4 days, should be focused on identifying follicles of intermediate size; hCG (5000 to 10,000 IU subcutaneously or intramuscularly) is

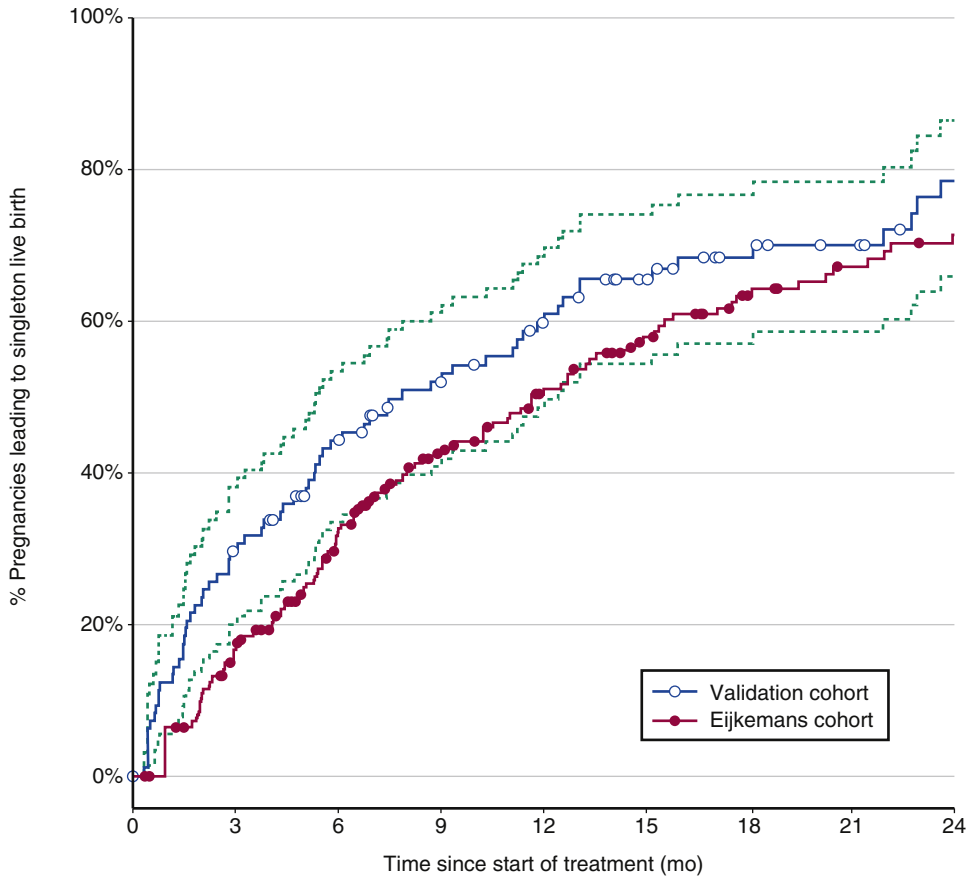


FIGURE 30.11 Cumulative pregnancy rates leading to single live-birth following ovulation induction in PCOS. *(Adapted from Veltman-Verhulst SM, Fauser BC, Eijkemans MJ: High singelton live birth rate confirmed after ovulation induction in women with anovulatory polycystic ovary syndrome: validation of a prediction model for clinical practice,* Fertil Steril *98, 761–768, 2012.)*

given on the day that at least one follicle measures more than 18 mm. If more than three follicles larger than 15 mm are present, stimulation should be stopped, hCG withheld, and use of a barrier contraceptive advised in order to prevent multiple pregnancies and OHSS. Measurements of serum E_2 may also be useful.[130] Ovarian stimulation with gonadotropins has not been shown to be associated with long-term risks. Urinary derived FSH is associated with a theoretical risk of transmission of prion proteins. However, the risk of infection is considered to be minimal and not in itself a reason to prescribe recFSH over uFSH.[140]

As confirmed recently, high cumulative singleton live birth rates of almost 80% can be achieved using CC as first line and low dose FSH as second line ovulation induction treatment in PCOS, with 14% multiple pregnancy rates[55,56] (Fig 30.11). PCOS women with a poor prognosis for ovulation induction, in whom alternative treatment strategies such as IVF may be considered, can best be identified by age, duration of infertility, and body weight.

Pulsatile GnRH

In the normo-ovulatory woman, the pattern of GnRH pulse stimulation alters with the phase of the menstrual cycle, effecting differential gonadotropin synthesis and secretion.[141] During the luteal-follicular transition, pulses occur every 90 to 120 minutes. This slow pulse frequency, in the presence of low E_2 and inhibin A levels, favors FSH production. In the midfollicular or late follicular phase, GnRH frequency increases, favoring LH secretion.[142] In the luteal phase, the production of progesterone increases hypothalamic opioid activity, thus slowing GnRH pulse secretion. This again favors FSH secretion in the luteofollicular transition.

The application of pulsatile GnRH therapy has been demonstrated to be an effective, reliable, and safe alternative to gonadotropin therapy for treating this form of anovulation.[143] Due to the intact ovarian-pituitary feedback system during pulsatile GnRH treatment, the resulting serum FSH and LH concentrations remain within the normal range and the chances of multifollicular development and ovarian hyperstimulation are therefore low. Little ovarian response monitoring is therefore needed during treatment.

The intravenous route appears superior to the subcutaneous route.[144] In order to mimic the normal pulsatile release of GnRH, a pulse interval of 60 to 90 minutes is used with a dose of 2.5 to 10 μg per pulse. The lower dose should be used initially in order to minimize the likelihood of multiple pregnancies.[145] The dose should then be increased to the minimum dose required to induce ovulation. Pulsatile GnRH administration may be continued throughout the luteal phase until menses or a positive pregnancy test. Alternatively, it may be discontinued after ovulation, and the corpus luteum supported by hCG.[49]

Clinical Outcomes

Pulsatile GnRH administration is primarily indicated for women with hypogonadotropic hypogonadal anovulation (WHO type 1) who have normal pituitary function.[146] In these patients, cumulative pregnancy rates of 83% to 95% after six cycles have been reported, with multiple

pregnancies accounting for 3% to 8% of pregnancies.[145,147] Lower ovulation and pregnancy rates have been observed in women with WHO type 2 anovulation, including PCOS.[148] This may be because anovulation in PCOS in part reflects the effects of a persistant, rapid frequency of GnRH stimulation of the pituitary, causing increased LH and T levels.[142] In a recent meta-analysis of four trials comparing pulsatile GnRH with gonadotropins for ovulation induction in women with PCOS, the small size and short follow-up of the studies meant that the authors were unable to draw conclusions on their relative effectiveness.[149]

Regular menstruation occurring approximately every 4 weeks indicates that the woman is having ovulatory cycles. Ultrasonography and measurements of serum progesterone are not usually needed for monitoring therapy. Local complications such as phlebitis may occasionally be encountered when intravenous administration is used. To avoid this, pulsatile GnRH can be administered subcutaneously. This route is certainly simpler than the intravenous approach. However, pharmakinetic studies comparing the two routes have shown that the plasma GnRH profiles are damped after subcutaneous administration, and that bioavailability is reduced.[150] However, the increased convenience offered by the subcutaneous route has led to this approach being favored by many. Smaller devices and more patient friendly delivery systems continue to be developed.

Aromatase Inhibitors

In recent years the use of aromatase inhibitors to mimic the actions of CC has been proposed.[151] Rather than antagonizing estrogen feedback activity at the hypothalamic-pituitary axis, this approach aims at reducing the amount of estogens being synthesized. Aromatase inhibitors block the conversion of AD and T to estriol (E_3) and estradiol (E_2), respectively.[152] This increases gonadotropin secretion, resulting in stimulation of ovarian follicles.[151] Aromatase inhibitors have been in clinical use for more than 20 years, primarily in the treatment of postmenopausal patients with advanced breast cancer. The more recently developed third generation of aromatase inhibitors are characterized by their potency in inhibiting the aromatase enzyme without significantly inhibiting inhibition of other steroidogenesis enzymes. One of the third generation compounds, letrozole, has been the focus of study as a potential therapeutic agent for inducing ovulation.

Clinical Outcomes

When given in the early follicular phase, letrozole reduces estrogenic feedback at the pituitary-hypothalamic axis, causing a subsequent increase in gonadotropin secretion. This was shown in monkeys to stimulate follicle development.[153] Subsequent small clinical studies employing a dose of 2.5 mg/day from day 3 to day 7 of the menstrual cycle have suggested that it may be an effective ovulatory agent in CC-resistant women.[151] A local effect at the ovary to increase sensitivity to FSH by blocking the conversions of androgens to estrogens has also been proposed, because accumulating intraovarian androgens may increase FSH receptor gene expression.[154]

Although the concept of applying aromatase inhibitors as an alternative to CC or as adjuvant therapy to CC or gonadotropins seems attractive and preliminary data on pregnancy outcome were encouraging[155,156] (adequately powered comparative controlled randomized studies are limited), a recent systematic review and meta analysis, involving 13 randomzed controlled trials, concluded that aromatase inhibitors should not be recommended as first line ovulation induction treatment in the absence of good quality evidence of efficacy.[157]

Adverse Effects and Complications

Although letrozole has a half-life that allows rapid disappearance following cessation of treatment in the midfollicular phase, the possible effects of this drug on ensuing pregnancy remain to be clarified. The performance of further clinical studies has been inhibited because an association was reported between letrozole and fetal toxicity. However, a recent analysis of outcomes in 911 newborns following conceived by CC or letrozole showed no difference in the overall rates of major and minor congenital malformations.[158] In the absence of sufficiently powered, randomized controlled trials establishing efficacy and safety, the routine clinical use of aromatase inhibitors is not advocated.[87]

Opioid Antagonists

Background

Endogenous opioid peptides have been shown to play an important role in regulating the pulsatile secretion of gonadotropins by inhibiting the hypothalamic pulse generator that directs GnRH secretion.[159] Infusion of the opiate receptor antagonist naloxone was shown to increase serum LH levels when administered during the late follicular and luteal phase of the cycle.[160]

Clinical Outcomes

Several groups have used naltrexone, an orally active opioid receptor antagonist, to treat ovulatory disorders, with varying degrees of success. The earlier observation that gonadal steroids enhance opioid modulation of gonadotropin secretion was postulated to explain the inability of two groups to demonstrate an increase in gonadotropin secretion or resumption of ovulation in women with WHO class 1 anovulation.[161] However, others have observed restoration of the menstrual cycle.[162] In an uncontrolled study, 19 of 22 women with CC-resistant anovulation were observed to become ovulatory under naltrexone treatment (sometimes in combination with CC), with 12 conceiving.[163] Treatment with 25 mg twice daily was commenced on the first day of a spontaneous or progesterone-induced cycle and continued until a positive pregnancy test occurred or, if no response was observed, for 21 days of treatment. Others have employed doses of up to 100 mg/day.[164] Conclusions as to the efficacy, optimal regimen, and safety of opiate antagonists for inducing ovulation cannot yet be made, however. No randomized controlled studies demonstrating their value for this condition have yet been published, and opiate anatagonists remain at best second-line, alternative therapy.

Dopamine Agonists

These agents are primarily used in the treatment of anovulation secondary to hyperprolactinemia. The treatment of hyperprolactinemia and the agents available for treatment is covered in detail elsewhere (see Chapter 3).

Adjunctive Therapies

Dexamethasone

Glucocorticoids have been proposed as a useful adjuvant to both CC and gonadotropin ovulation induction in women with PCOS with a therapeutic rationale based on reducing ovarian androgen levels, improving ovulatory function, and reducing resistance to ovulation induction agents.[166] Although the source of high androgen secretion in anovulatory women with PCOS is primarily ovarian, 50% to 70% also demonstrate excessive adrenal androgen levels.

In order to normalize (without suppressing) adrenal steroid production, daily oral doses of dexamethasone (0.25 to 0.5 mg) or prednisone (5 to 10 mg) have been employed in a continuous regimen. Although widely used, the value of adjuvant corticosteroid administration with CC or gonadotropins for ovulation induction remains questionable. In a study of women with PCOS, the chance of ovulation after glucocorticoid suppression of adrenal androgens was not predicted by either basal DHEAS (dehydroepiandrosterone sulfate) levels or suppressed levels, and limited effects on ovulation were observed.[165] A randomized controlled study in 80 women with CC resistance and normal serum DHEAS levels showed significantly higher ovulation and pregnancy rates when 2 mg/day dexamethasone was added from cycle day 2 to 12 to CC 100 mg.[166]

While major complications from the adjuvant use of low-dose glucocorticoids are rare, weight gain is a common problem. Other reported side effects include glucose intolerance and osteoporosis. Given possible side effects, their use should remain as a second-line therapy subject to further research.

Gonadotropin-Releasing Hormone Agonists

Adjuvant GnRH agonist treatment has also been proposed to improve outcomes and reduce complications of ovulation induction. Early uncontrolled studies indicated that the concomitant use of GnRH agonist with ovarian stimulation regimens in women with PCOS was safe and improved treatment outcome.[167] Further studies indicated that premature luteinization could be prevented by employing GnRH agonists, but no clear difference in pregnancy rates was demonstrated.[168] Although, a meta-analysis of five prospective studies[169] suggested that improved pregnancy rates could be achieved at similar ovulation rates when GnRH agonists were also employed, a later systematic review concluded that GnRH agonist as an adjunct to FSH and hMG does not improve pregnancy and OHSS rates, and should therefore not be recommended as a standard treatment for this patient group.[170]

Conflicting data on the effects on ovulation and pregnancy rates, combined with reports of severe OHSS with adjuvent GnRH agonist therapy and the additional burden for the patient of prolonged treatment cycles, mean that adjuvant GnRH agonists remain a second-line therapy in conjunction with FSH stimulation.

The availability of GnRH antagonists provides new opportunities to modify ovulation induction regimens. Particular attributes of GnRH antagonists that might be of value in this context include their competitive binding properties, immediate suppression of the pituitary without a flare-up effect, and rapid resumption of gonadal function on discontinuation. However, few studies have appeared which further explore its role in this clinical context.

Additional Treatable Factors Influencing the Balance of Efficacy and Risks

Obesity

Among women with WHO class 2 anovulation, obesity may be present in up to 50%. In addition to enhancing the features of insulin resistance mentioned earlier, overweight (BMI greater than 32) is also associated with reproductive dysfunction, despite regular menstrual cycles.[171] In recent years, considerable attention has been given to the role of lifestyle factors and management in improving outcomes in obese anovulatory women. Even a small (2% to 5%) reduction in weight has been shown to improve metabolical indices including insulin resistance.[172] In addition, weight loss can lead to a rise in sex hormone–binding globulin (SHBG) concentrations, a decrease in FAI and T levels, and improvement in cyclicity.[173-175] A relatively modest reduction in weight has been shown to increase the frequency of ovulation in obese anovulatory women to more than 70%.[176] Energy restriction acting to temporarily improve insulin senstivity may be important,[175] because improvements in endocrine and clinical parameters occurred maximally during the period of energy restriction. During subsequent weight maintenance, many benefits were reversed.[175]

The evidence for the benefits of weight loss, combined with recent data confirming BMI to be a major factor influencing outcome of ovulation induction,[87] make the treatment of obesity an important adjuvant treatment that should precede ovulation induction.[87] Given the baseline risks of ovulation induction, and the possible risks of obesity for subsequent pregnancy and general health, weight loss in cases of obesity should be considered as a prerequisite to medical ovulation induction treatment.[177-180]

Tobacco Smoking

Epidemiological data provide strong evidence for a causal association between cigarette smoking and other lifestyle factors and decreased fertility. For a recent review of the impact of smoking and other lifestyle factors on fertility treatment outcomes, see Homan and colleagues.[181] Dose-dependent effects of smoking have been reported in relation to the duration to conception.[182] Moreover, there is evidence of increased risk of early pregnancy loss in smokers[183] and a reduced mean age at menopause.[184] Although properly designed studies of the effect of smoking on outcomes of ovulation induction are scarce, data from studies in assisted conception point to detrimental effects on ovarian function and oocyte quality, which are likely to be applicable to the situation concerning ovulation induction.[185] In any discussion of infertility therapy, the clinician should emphasize the risks of smoking for outcome of treatment. Indeed, preconceptional care and lifestyle advice should be an integral part of the modern fertility clinic.

Ovarian Stimulation in the Empirical Treatment of Unexplained Infertility

Principles of Ovarian Stimulation

The aim of ovarian stimulation is to intervene in the mechanisms regulating single dominant follicle selection in order to mature multiple follicles and obtain multiple oocytes for

fertilization *in vivo* (either after timed intercourse or IUI) or *in vitro* (IVF). Ovarian stimulation is usually performed in normo-ovulatory infertile women in order to increase chances for pregnancy. However, the development of multiple follicles inherently also increases the undesired risk of (higher order) multiple pregnancies and OHSS. In IVF, OHSS risks are reduced because of the puncture of all visible large follicles to retrieve the oocytes, and incidences of multiple pregnancies can be controlled by limiting the number of embryos transferred.

Obviously, oligovulatory and anovulatory women may also qualify for either IUI or IVF after failed ovulation induction. Hyperstimulation may also be performed in these women, aiming at multiple follicle development. It should again be emphasized that this condition of hyperstimulation in these patients is distinctly different from ovulation induction in which the aim is to mimic physiology and stimulate ongoing growth and ovulation of a single dominant follicle. However, these patients are usually difficult to manage because of an unpredictable major individual variability in response and a tendency to hyper-respond to stimulation protocols.[186]

Although daily administration of ovary stimulating agents allows for dose adjustments based on individual ovarian response monitoring, the clinical evidence for the efficacy of such an approach is scant. A hyper-response may be counteracted by a dose decrease or the complete cessation of exogenous gonadotropins for some days (the latter strategy is referred to as "coasting").[187] An excessive number of follicles for ovulation induction or hyperstimulation for IUI may be reduced by follicle puncture[188] or cycle cancellation. When, in contrast, low ovarian response to standard stimulation is observed, recent evidence indicates that a gonadotropin dose increase does not result in improved outcome.[189,190] This is not surprising if the pathophysiological background of low response is taken into consideration. Low response to ovarian hyperstimulation may be the first sign of advanced ovarian aging.[191] Women with a previous low response to hyperstimulation have been shown to enter menopause at an earlier age.[192]

During the normal menstrual cycle the midcycle LH surge represents the trigger for inducing final oocyte maturation, the rupture of the follicle and release of the oocyte, and finally luteinization of granulosa and theca cells allowing for the formation of the corpus luteum. As mentioned before, the synchrony of endocrine events inducing the LH surge is disrupted in ovarian hyperstimulation. Therefore, the endogenous LH surge is replaced by an exogenous hCG bolus injection, timed by the visualization of large graafian follicles upon ultrasound. Finally, these follicular phase interventions result in luteal phase abnormalities[193] requiring luteal phase supplementation by either hCG or exogenous progestins.

Therapeutic Approaches

Unexplained infertility is usually diagnosed by exclusion, when standard infertility investigation shows no abnormalities. However, no agreement exists with regard to the preferred extent of standard investigation as well as the interpretation and prognostic value of many of these tests. Usually, ovulation is assessed by a midluteal phase serum progesterone assay, tubal patency is established by hysterosalpingogram, and male factor infertility excluded by semen analysis. Again, the interpretation of any of these tests is not without difficulty and many clinicians perform additional tests to further explore possible causes of infertility.[62] Hence, the term *unexplained infertility* is notoriously ambiguous and may mean anything in between undiagnosed infertility and normal fertility in which a pregnancy did not occur merely by chance. This may especially be the case in young women who have been attempting to conceive for a relatively short time.[65]

It should be realized that many biologically relevant processes important for obtaining a pregnancy—such as oocyte chromosomal constitution, subtle sperm abnormalities, *in vivo* conception, embryo transport and nidation, and finally endometrial receptivity—cannot be studied accurately as yet. It is to be expected that with the advancement of our understanding of these processes, the percentage of couples diagnosed with unexplained infertility, and therefore potential need for empirical ovarian hyperstimulation, will decrease.

When a couple presents with unexplained infertility, it is extremely important to assess chances of spontaneous pregnancy before commencing on any kind of empirical therapy. As mentioned before, ovarian hyperstimulation (with or without additional interventions such as IUI) may enhance pregnancy chances per cycle, but at the cost of patient stress and discomfort, chances for side effects such as multiple gestation and OHSS, and high costs[194,195] (Fig. 30.5). Similar cumulative pregnancy rates may be achieved with expectant management for 6 to 12 months.[65] Expectant management may represent the most favorable approach in many young women with a short duration of infertility.

Results are frequently reported from combined interventions such as ovarian hyperstimulation and IUI. These studies are often uncontrolled, and few are sufficiently powered to differentiate between the independent effects of hyperstimulation and IUI and the potential additive effects of combining both interventions. In recent years, the picture has become clearer. Although the absolute treatment effect appears relatively limited, given the low cost and ease of administration, CC can be recommended as first choice medication for the treatment of unexplained infertility. In terms of pure efficacy, however, a meta-analysis of five trials indicated that gonadotropins may be superior to CC as ovarian stimulation agents for the treatment of unexplained infertility.[196] Treatment with CC was associated with significantly reduced odds ratios of pregnancy per woman compared to gonadotropins (OR 0.41; 95% CI 0.17 to 0.8). As far as complications are concerned, no significant differences could be found for miscarriage (OR 0.61; 95% CI 0.09 to 4) or multiple birth (OR 1.1; 95% CI 0.2 to 7). The incidence of OHSS or cycle cancellation rates could not be assessed.

For unexplained infertility, the combination of IUI with ovarian hyperstimulation potentially bypasses several possible barriers to fertility, including minor sperm abnormalities, sperm-cervical mucus interactions, timing of sperm delivery problems, and a possible beneficial effect of ovarian stimulation on endometrial receptivity. The most important benefit is likely to be the stimulation of multiple follicles. Although a meta-analysis by Hughes[197] has addressed questions relating to the benefits of FSH and IUI alone compared with combined therapy, less than a third of the studies included in the analysis make use of treated control subjects.

Moreover, the conclusions that both FSH and IUI improve fecundity are derived from regression analysis and are open to discussion.[198] Other studies have indicated that ovarian hyperstimulation with both CC and gonadotropins improve the fecundity rate compared to IUI alone.[68] However, a study comparing intracervical insemination alone with FSH in combination with IUI showed a statistically higher pregnancy rate with the latter treatment combination.[64] The number needed to treat was 31 cycles. This implies that it would take 31 cycles of treatment before there would be one more singleton live birth with FSH and IUI than with intracervical insemination alone.[199] The number needed to treat of FSH in combination with IUI in order to obtain an extra pregnancy above that obtained with IUI alone is even greater.[199] When the costs of multiple pregnancies arising from multiple follicle development are taken into account, the cost effectiveness of FSH and IUI combined therapy for this indication may be limited. Cost-effectiveness analyses have led to the conclusion that IUI with or without hyperstimulation should precede IVF.[200] In clinical practice the benefits of ovarian hyperstimulation in combination with IUI need to be weighed against the additional discomfort and costs of monitoring applied, often unsuccessfully,[67] to avoid multiple pregnancy.[201] Clearly, more studies are needed to elucidate the optimal approach to treating unexplained infertility, and the role ovarian hyperstimulation should play. Although it is increasingly recognized that treatment success should be defined in terms of cumulative multiple cycles, at present cumulative live birth rates remain poorly reported, and comparisons with expectant management after multiple cycles have not been made.

Preparations for Ovarian Stimulation

Clomiphene Citrate

Preparations and Regimens

Daily doses of 50 to 100 mg are given usually from days 5 until 9[28] and ovulation is triggered by exogenous hCG. Little ovarian response monitoring is required, and luteal support is probably not necessary.

Clinical Outcome

A retrospective analysis of 45 published reports conclude that the adjusted pregnancy rate per initiated cycle is 5.6% for CC alone, versus 8.3% for CC plus IUI compared to an estimated pregnancy rate from expectant management of 1.3%.[201] A meta-analysis on the basis of six randomized trials[202] concluded that CC administration was superior to no treatment, with an odds ratio for clinical pregnancies of 2.4 (95% CI 1.2 to 4.6) per patient and 2.5 (1.4 to 4.6) per cycle. As stated before, an earlier meta-analysis[197] indicated an independent significant improvement in pregnancy rates for clomiphene, exogenous FSH, and IUI. Most recent analysis suggests no benefit of CC in unexplained infertility.[202]

Adverse Effects and Complications

Adverse effects include hot flushes, mood swings, headache, and visual disturbances. The principal complication remains multiple pregnancy, which occurs in around 10% of pregnancies, and a slightly increased chance for OHSS. Long-term use of CC (more than 12 months) may be associated with a slight increase in the risk of ovarian epithelial cancer.[102]

Gonadotropins

Preparations and Regimens

Usually exogenous gonadotropin administration is started around cycle day 3 to 5 at daily doses of 75 to 225 IU for several days in fixed dose regimens. Thereafter, doses may be adjusted on the basis of ovarian response monitoring by ultrasound and/or E_2 assays. The therapeutic window for gonadotropins achieving the desired goal (two to three preovulatory follicles) is rather small, and a considerable proportion of treatment cycles are canceled because of hyper-response (and the related increased chance of higher order multiple pregnancy) or because they fail to achieve multiple dominant follicle development. The need for cancellation is highly dependent on the stimulation protocol applied and the rigidity of cancellation criteria applied. This in turn depends on whether higher order multiple pregnancies are considered an acceptable side effect of treatment, or whether this should be seen as a failure of treatment to be prevented at any price. Moreover, premature luteinization during ovarian hyperstimulation for IUI may occur more frequently than generally assumed. This may have a detrimental impact on treatment outcome. Recent studies of GnRH antagonist cotreatment during gonadotropin hyperstimulation have demonstrated a reduced incidence of a premature LH rise but no significant improvement in pregnancy rates.[203] However, this approach renders ovarian stimulation protocols more complicated and expensive, increasing the frequency of hospital visits required for monitoring.

Clinical Outcome

A meta-analysis based on 5214 cycles reported in 22 trials concluded an odds ratio for pregnancies associated with FSH compared to expectant management of 2.35 (95% CI 1.9 to 2.9).[197] A retrospective analysis based on 45 previous papers concluded a significantly increased pregnancy rate occurred after either hMG alone (7.7%) or hMG plus IUI (17.1%).[201] A subsequent large multicenter study[64] confirmed that ovarian hyperstimulation with gonadotropins and IUI both exhibit an independent additive effect on pregnancy chances. The applied treatment regimen for ovarian hyperstimulation (150 IU/day FSH from cycle day 3 to 7) resulted in high frequency of conception. Overall cumulative pregnancy rates when this was combined with IUI therapy were reported to be 33% within three cycles, but at the price of an unacceptable high multiple pregnancy rate of 20% twins and 10% higher-order multiple pregnancy.[64] Women undergoing combined hyperstimulation and IUI were 1.7 times more likely to achieve a pregnancy in a given cycle compared to those receiving IUI alone. However, only 53% of these pregnancies resulted in a live birth with a substantial number of triplet and quadruplet births, despite the fact that fetal reduction has been applied in some of these women. Indeed, 30% of occurring pregnancies were multiples, including 9% triplets and quadruplets. No information was provided regarding perinatal mortality and morbidity rates.

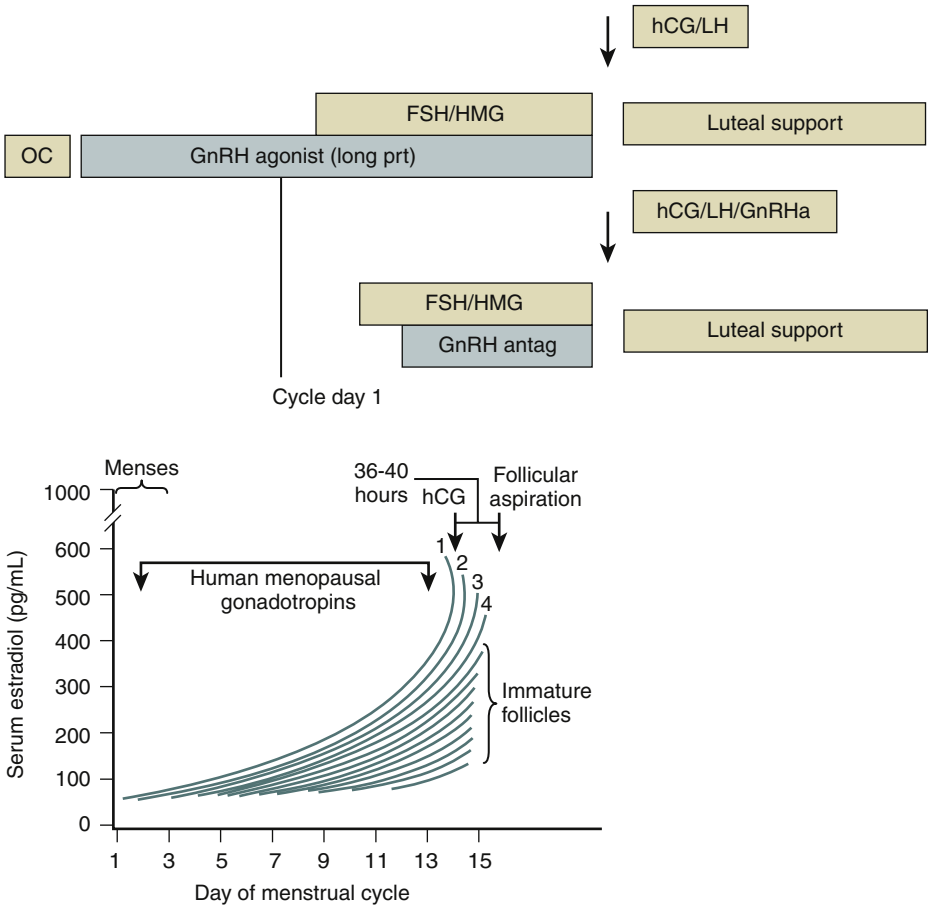


FIGURE 30.12 Schematic representation of complex medication regimens involved in ovarian hyperstimulation for *in vitro* fertilization *(top)*, and the heterogeneous cohort of recruited and selected follicles *(bottom)*. Antag, antagonist; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; HMG, human menopausal gonadotropin; LH, luteinizing hormone; OC, oral contraceptives; prt, protocol. *(Graph from Oehninger S, Hodgen GD. Introduction of ovulation for assisted reproduction programmes.* Baillieres Clin Obstet Gynecol *4:451–573, 1990.)*

Adverse Effects and Complications

Those effects relating to gonadotropins in general are discussed earlier. In the context of ovarian stimulation for the treatment of unexplained infertility, we again stress the risk of multiple pregnancy associated with the use of these drugs. The ability of careful monitoring to allow prevention of this complication is limited even in highly skilled hands,[67] and the decision to employ gonadotropins in the context of treating ovulatory women for unexplained infertility should be preceded by an open and informed discussion with the couple over the risks of treatment and the limitations of monitoring. It is clear that an individual approach is required when addressing these issues, and that there is a need to individualize treatment in order to ensure optimal outcomes.

Ovarian Stimulation for in Vitro *Fertilization*

Therapeutic Approaches

The general aim of ovarian stimulation in this clinical context is to induce the development of multiple dominant follicles in order to be able to retrieve many oocytes to allow for inefficiencies in subsequent fertilization *in vitro*, embryo culture, and embryo selection for transfer and implantation (Fig. 30.12).[29] Hence, multiple embryos can be transferred in the great majority of patients and often spare embryos can be cryopreserved to allow for subsequent chances of pregnancy without the need for repeated ovarian stimulation and oocyte retrieval.[28] The paradigm of so-called "controlled" ovarian stimulation by high doses of exogenous gonadotropins and GnRH agonist long protocol cotreatment for IVF has constituted the gold standard for clinicians throughout the world since the early 1990s. It appears that large numbers of developing follicles is still considered a useful surrogate marker of successful IVF, whereas its significance in relation to the chance of achieving a pregnancy resulting in a healthy baby born is in doubt.[72,203] The ovarian stimulation protocols required to produce a large number of follicles have become extremely complex and costly over the years,[28,52] creating considerable side effects, risks of complications, and the need for intense monitoring of ovarian response.[204] The total number of retrieved oocytes required to achieve a single live birth is somewhere around 25, suggesting a very substantially oocyte wastage.[205] Physicians appear to be in control of ovarian stimulation, owing to their ability to adjust the gonadotropin doses or the type of preparation on the basis of ovarian response monitoring. However, the major individual variability in response is out of the doctor's control and is an extremely important determining factor for both success and complications of IVF treatment.[206] A good ovarian response to standard stimulation indicates normal ovarian function and a good prognosis for successful IVF. A low ovarian response suggests ovarian aging and is therefore associated with poor IVF outcome. A low response can to some extent be predicted by chronological age and endocrine and ultrasound aging parameters assessed before the initiation of treatment, as

will be discussed later.[192,207] However, the widely applied approach to increase gonadotropin doses administered in case of insufficient ovarian response has very little scientific foundation. The occurrence of a severe hyper-response comes as a surprise in most cases.[58,197] However, recent research indicates that the initial AMH level may represent a useful tool to identify women and risk for an exaggerated response to ovarian stimulation.[208] Severe OHSS is induced by hCG and is therefore often associated with pregnancy. This can be prevented from happening by refraining from embryo transfer in the cycle at risk and cryopreserving all available embryos for transfer in another cycle. Current approaches also include the triggering of an endogenous LH surge inducing final oocyte maturation by a bolus dose of GnRH agonist.[209] For obvious reasons, such an approach can only be used in stimulation protocols employing GnRH antagonist cotreatment.

Slowly, ovarian stimulation protocols have shifted from the use of hMG to uFSH to recFSH.[210] In recent years, several groups have focused on the potential significance of late follicular phase LH levels for clinical IVF outcome. Indeed, it has been shown that dominant follicle development can be stimulated exclusively by LH rather than FSH, opening new possibilities for therapeutic interventions,[19] as discussed in more detail later.

Despite the fact that the first child born after IVF was conceived in a spontaneous menstrual cycle, natural cycle IVF received little attention. The major focus has been the improvement of complex ovarian stimulation regimens. Natural cycle IVF offers major advantages such as negligible complications (arising from multiple pregnancy or OHSS), reduced patient discomfort, and a low cost. The efficacy of natural cycle IVF is hampered, however, by high cancellation rates due to premature ovulation or luteinization. A systematic review of 20 selected studies involving a total of 1800 cycles showed a 7.2% overall pregnancy rate per started cycle, and 16% per embryo transfer.[70] Cumulative pregnancy and live birth rates over four cycles of 42% and 32%, respectively, have been reported.[211] Despite the relatively high failure rate, the approach of natural cycle may still be cost effective. In one study, it was calculated that natural cycle IVF could be offered at 23% of the cost of a stimulated cycle.[212]

More recently a modified natural cycle[213] has been proposed, in which GnRH antagonists are instituted to prevent premature ovulation, and low-dose exogenous gonadotropin co-treatment is given as add-back to prevent a GnRH antagonist induced involution of follicle development. Using this approach, which (like natural cycle IVF) aims to achieve monofollicular development, cumulative pregnancy rates of 44% have been reported over nine cycles of treatment.[213]

Preparations

Clomiphene Citrate

Background

After the first baby born following IVF in a natural cycle,[39] four normal IVF pregnancies were reported following ovarian stimulation with CC.[35] In subsequent years, many groups reported IVF results following CC, with or without gonadotropin cotreatment.[214] Combined CC and hMG regimens were considered the standard of care before GnRH agonist cotreatment to induce pituitary down-regulation came into use. (For a comprehensive historical overview, see reference 198.) The advantages of these combined regimens included reduced requirements for hMG and higher luteal phase progesterone levels alleviating the need for luteal phase supplementation.[32] Recent studies have reported clinical outcomes of combined regimens applying CC, gonadotropins, and GnRH antagonist.[32]

CC usually induces the development of at least two follicles, which may sometimes elicit a premature LH rise. By virtue of the fact that CC is therapeutically active through interference with estrogen feedback, this compound cannot be combined with GnRH agonist cotreatment for prevention of a premature LH surge. Moreover, undesired antiestrogenic effects of CC at the level of the endometrium have been implicated by some in the observed discrepancy between relatively low embryo implantation rates coinciding with successful ovarian hyperstimulation.

Preparations and Regimens

Clomiphene citrate administration is usually initiated on cycle day 2, 3, or 5, and given daily for 5 subsequent days with doses varying between 100 and 150 mg/day. In most applied regimens, exogenous gonadotropin medication (150 IU/day) is initiated after cessation of CC. It seems that CC alone induces a limited but dose-dependent increase in the number of developing follicles. However, the addition of gonadotropins elicits a more intense ovarian response. Sufficiently powered randomized comparative trials to support one approach over the other are lacking.

Clinical Outcome

Reported outcome is variable in the literature, but in general pregnancy rates appear higher compared to natural cycle IVF, but lower compared to conventional gonadotropin and GnRH agonist protocols. Again, most studies are uncontrolled but an extensive summary of almost 40,000 cycles reported in the literature suggests an overall pregnancy rate per embryo transfer of 20.5%.[215]

Adverse Effects and Complications

Because of the relatively mild stimulation, the incidence of side effects or complications of CC treatment for IVF is low, as discussed earlier. Overall side effects are CC dose related and are completely reversible once medication is stopped.

Gonadotropins

Background

Gonadotropin preparations have been used for ovarian stimulation since the early days of IVF and were originally developed in the United States.[214] The daily administration of these preparations is usually efficacious in the induction and maintenance of growth of multiple dominant follicles, allowing for the retrieval of many oocytes for IVF. Preparations initially used were hMG (containing both LH and FSH bioactivity), followed by purified uFSH and more recently recFSH. No general consensus exists with regard to starting day and doses of gonadotropins. In conclusion, based on seven randomized controlled trials (RCTs) involving a

total of 2563 cycles, although higher gonadotropin doses may result in the retrieval of 1 or 2 more oocytes, improved clinical outcomes in terms of pregnancy rates could not be demonstrated.[216]

A chimeric FSH agonist (so-called recFSH-CTP), generated by the fusion of the carboxy-terminal peptide (CTP) of hCG (responsible for its prolonged metabolical clearance compared to LH) with the FSH-β chain has recently been introduced in the IVF clinic.[45] Corifollitropin alpha can be administered with a 7 day injection free interval and has been shown to be safe with high efficacy.[217]

The type, duration, and dosing of GnRH analog cotreatment to suppress endogenous pituitary gonadotropin release (as will be discussed later in the chapter) may also affect the preferred gonadotropin preparation. Classical principles teach us that both LH and FSH are required for adequate ovarian estrogen biosynthesis and follicle development. Theca cell–derived androgen production (which is under LH control) is mandatory as a substrate for the conversion to estrogens by FSH-induced aromatase activity of granulosa cells.[29] A number of studies have indicated that excessively suppressed late follicular phase LH concentrations may be detrimental for clinical IVF outcome.[218,219] Under these circumstances the use of urinary preparations containing both LH and FSH activity or the addition of recLH or rechCG next to exogenous FSH may be useful.[29] It is uncertain as yet, however, for which patients this approach may be beneficial. Recent meta-analyses failed to show clinically relevant differences in relation to late follicular phase LH concentrations,[220] or when cycles with or without the addition of exogenous LH are compared.[221]

Recently the concept that exogenous LH is capable of selectively stimulating the development of the more mature dominant follicles has been developed. A shift from FSH to LH preparations during stimulation may therefore be useful in order to stimulate a more homogeneous cohort of follicles for IVF.[18,19,222]

Preparations and Regimens

To allow for the clinical introduction of recombinant FSH, large-scale, multicenter, comparative trials in IVF were published from 1995 onward.[223] It should be noted, however, that these studies, including several hundreds of women, were sponsored by pharmaceutical companies. The results should therefore be interpreted with an appropriate degree of caution. For instance, it was arbitrarily chosen for all initial studies that recFSH would only be compared with uFSH and not hMG, although the latter preparation was still considered to be the gold standard by the majority of clinicians. Several independent comparative trials have been published since then, but sample size of these single-center studies was usually insufficient to allow for the detection of relatively small differences. An early meta-analysis[224] as well as health economics studies[225,226] indicate a slightly improved outcome for recFSH compared to uFSH. However, recently published multicenter, company-sponsored trials reported similar clinical outcomes comparing uFSH versus recFSH, or hMG versus recFSH[227,228] which was confirmed in the most recent meta-analysis.[229]

Many different regimens are applied with little if any proof of their efficacy and safety. Different starting days and doses are applied worldwide along with incremental or decremental doses. In case of imminent OHSS resulting from the development of too many follicles, the possibility of complete cessation of gonadotropin administration (coasting) has been advocated by several investigators.[187] Studies of the efficacy of this approach thus far undertaken have been limited and inconclusive. Adequate doses for gonadotropin preparations may also vary, depending on whether GnRH agonist or antagonist co-treatment is used.[230] Major individual differences in body weight may also determine response.[231] Because endogenous gonadotropins are suppressed by GnRH antagonists for a limited period of time (as will be discussed later), less exogenous FSH is required. The ideal day of initiation of gonadotropin therapy is another variable that has been poorly characterized so far, and may also vary dependent on GnRH agonist or antagonist co-treatment. It is surprising to conclude that very few of the previously mentioned questions with regard to applied dose regimens can be answered on the basis of scientific evidence by properly designed studies.

Usually starting doses vary between 100 and 300 IU/day and doses are often altered depending on the observed individual ovarian response. A typical daily starting dose would currently be 150 to 225 IU in Europe and 225 to 300 IU in the United States. Only few randomized studies regarding dose regimens can be found in the literature. A single-center RCT from Rotterdam showed that a doubling of the hMG dose in low responders after a 225 IU/day dose for 5 days is not efficacious compared to continued similar doses.[232] Moreover, an RCT in which higher versus standard dose of FSH was administered to expected poor responders showed no difference in pregnancy rates.[233] All together a meta analysis[216] encompassing all RCTs comparing different FSH doses failed to show a difference in favor of high-dose regimens where pregnancy chances are concerned (Fig 30.13), indicating that the widely applied practice of a gonadotropin dose increase in case of low response is not efficacious.

The approach of starting exogenous FSH early during the luteal phase of the preceding cycle recognizes the physiological principle of early recruitment of a cohort of follicles for the next cycle.[2] However, this protocol did not result in improved ovarian response in women with a low oocyte yield during previous IVF attempts.[234]

The perceived need to allow programming of oocyte retrieval led to a number of studies addressing the role of oral contraceptives (OCs) for this indication. Fixed schedule protocols were developed by a number of groups in which OCs were administered in advance of ovarian stimulation and planned follicle aspiration. Despite their apparent efficacy, ease of administration, and fewer side effects, subsequent randomized studies comparing OCs to GnRH agonists as a means of preventing premature luteinization showed the superiority of the latter and because of this, OCs are no longer widely used for this indication. To facilitate the planning of the initiation of exogenous gonadotropins in a GnRH antagonist cycle, independent of the menstrual period, OC pretreatment has been evaluated in a number of small studies and a recent meta-analysis.[235] Although there is evidence that OC pretreatment may aid in the scheduling of IVF cycles when GnRH anatagonists are used, at present there is no evidence that they improve live birth rates.

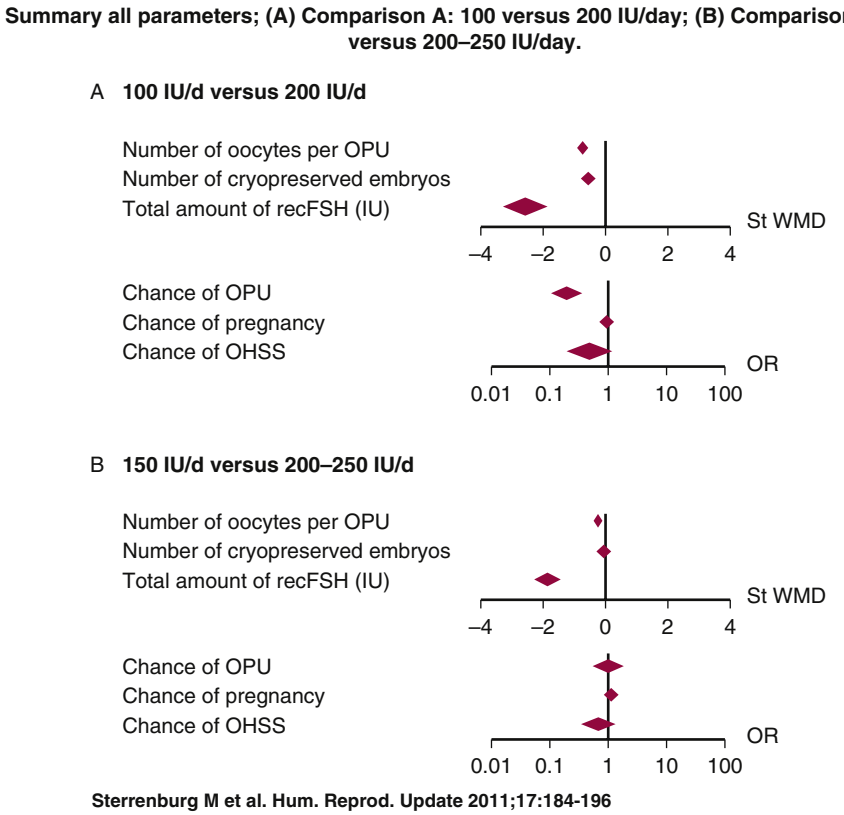


FIGURE 30.13 Summary of clinical outcomes from a meta-analysis comparing different FSH doses for ovarian stimulation for IVF. *(Graph from Sterrenburg et al. Clinical outcomes in relation to the daily dose of recombinant follicle-stimulating hormone for ovarian stimulation in* in vitro *fertilization in presumed normal responders younger than 39 years: a meta-analysis.* Human Reprod Update *17:184, 2011.)*

Gonadotropin-Releasing Hormone Agonist Cotreatment

During initial studies with hMG stimulation of multiple follicle development for IVF it became apparent that a premature LH peak occurred in around 20% to 25% of cycles, due to positive feedback activity by high serum E_2 levels during the midfollicular phase of the stimulation cycle.[32] This advanced exposure to high LH resulted in premature luteinization of follicles and either cycle cancellation due to follicle maturation arrest or severely compromised IVF outcome. The clinical development of GnRH agonists in the early 1980s[60] allowed for the complete suppression of pituitary gonadotropin release during ovarian stimulation protocols for IVF.[32] Induced pituitary down-regulation indeed resulted in significantly reduced cancellation rates and improved overall IVF outcome.[236,237] Moreover, the approach of GnRH agonist cotreatment did facilitate scheduling of IVF and timing of oocyte retrieval. Frequently used preparations include buserelin, triptorelin, nafarelin, and leuprorelin. To some degree, the extent and duration of pituitary suppression are dose related, but surprisingly few dose finding studies have been performed. In addition, randomized studies comparing different GnRH agonists are scarce.

Due to the intrinsic agonist activity of the compound, pituitary down-regulation is preceded by an initial stimulatory phase (referred to as the "flare" effect) which lasts for around 2 weeks. In this long protocol, GnRH agonist treatment therefore usually commences in the luteal phase in the preceding cycle and is continued until hCG administration. Stimulation with gonadotropins is started when pituitary and ovarian quiesence has been achieved. Moreover, it is uncertain whether ovarian response to exogenous stimulation is affected by GnRH agonist cotreatment.[238] Some women suffer from serious hypoestrogenic side effects, such as mood changes, sweating, and flushes. Alternative approaches include the short (and sometimes ultrashort) protocols in which the initial flare effect of GnRH agonist treatment is used to stimulate the ovaries. Attempts to discontinue GnRH agonist administration during the ovarian stimulation phase[239,240] have not shown beneficial effects. Reported clinical results of these alternative clinical protocols remain variable, and the GnRH agonist long protocol has remained the standard of care for over a decade.[32]

Gonadotropin-Releasing Hormone Antagonist Cotreatment

Two third-generation GnRH antagonists (cetrorelix and ganirelix) became available for large-scale clinical studies around 1995. Previous generations of the antagonist suffered from problems with pharmaceutical formulation and related bioavailability along with the local or systemic induction of histamine release. The potential advantage of a GnRH antagonist is that pituitary gonadotropin secretion is suppressed immediately after initiation of therapy. Therefore the co-treatment with GnRH antagonist can be restricted to the time in the cycle at risk for a premature rise in LH (i.e., the midfollicular to late follicular phase of the cycle).[32]

Both single, high-dose and multiple, low-dose GnRH antagonist regimens have been described. Multiple, daily dose regimens are most widely used at present. Initial dose finding studies suggested that a daily injection of 0.25 mg represents the minimal effective dose to suppress a

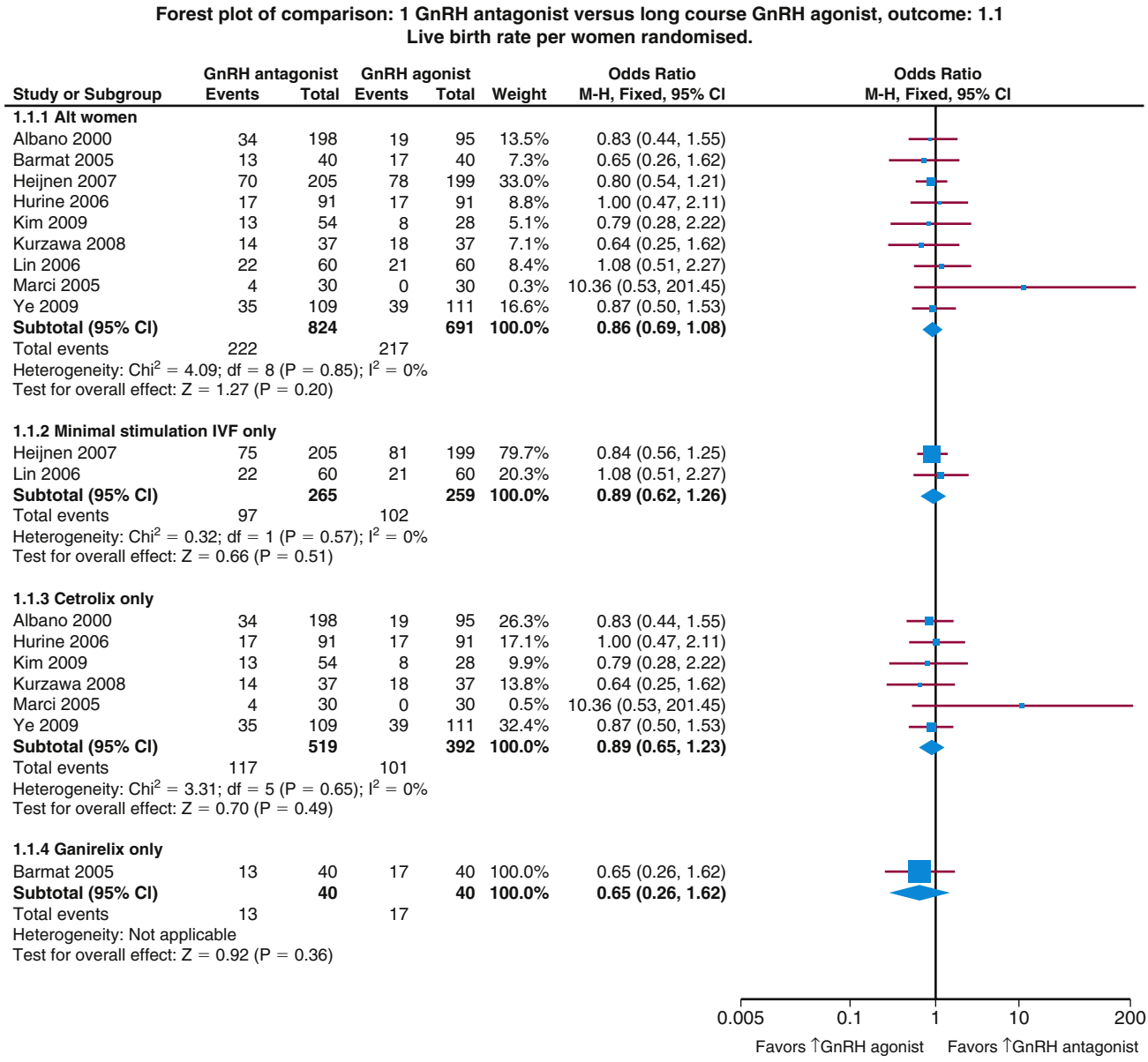


FIGURE 30.14 Forest plot of live-birth rate per women randomized comparing GnRH antagonist versus GnRH agonist long protocol cotreatment for IVF. *(Adapted from Al Inani, Gonadotrophin-releasing hormone antagonists for assisted reproductive technology.* Cochrane database *11; [5]:2011, CD001750.)*

premature LH rise in most patients. In all phase 3 comparative trials of the daily GnRH antagonist co-treatment regimen, it was initiated on cycle day 6. However, in principle, GnRH antagonists need only be given when there is follicular development and rising E_2 levels which might give rise to a premature elevation in pituitary LH release due to positive feedback mechanisms. However, a meta-analysis of four studies comparing fixed with flexible regimens showed a trend toward lower pregnancy rates following the flexible protocol (OR 0.7, 95% CI 0.47 to 1.05).[241] The first meta-analysis published comparing outcomes following cotreatment with GnRH antagonist versus GnRH agonist[230] based on five multicenter RCTs concluded that the GnRH antagonist is as efficient as GnRH agonist in preventing a premature LH surge in IVF (OR 1.76, 95% CI 0.75 to 4.16). However, a small but significant reduction in pregnancies was observed per started cycle (OR 0.79, 95% CI 0.63 to 0.99). Since then, protocols have been refined, and a recent meta-analysis of later studies has shown no difference in live birth rates[242] (Fig. 30.14).

Concerns have been raised regarding the possibility of direct effects of GnRH antagonists on the embryo. However, no adverse effects were observed on the freeze–thaw embryos of GnRH antagonist cycles.[243] Possible detrimental effects of GnRH antagonists at the endometrial level or on follicle development could not be confirmed.[32] Moreover, recent studies have indicated that gonadotropin regimens do not need to be adjusted when GnRH antagonists are commenced.[244,245] Furthermore, exogenous LH is probably not required next to FSH.[221,246]

Despite improving outcomes, the debate regarding the advantages and disadvantages compared with GnRH agonists continues (Box 30.2).[247] The most recent meta-analysis comparing GnRH agonist versus antagonist cotreatment,

Box 30.2 Advantages and Disadvantages for the Use of GnRH Antagonists in IVF

ADVANTAGES

- Prevention of premature LH increase is easier and takes less time.
- GnRH antagonists are not associated with an acute stimulation of gonadotropins and steroid hormones.
- The initial stimulation by GnRH agonists can induce cyst formation, which is avoided with GnRH antagonists.
- No hot flushes are observed with GnRH antagonists.
- Inadvertent administration of the GnRH analog in early pregnancy can be avoided as GnRH antagonist is administered in the midfollicular phase.
- Requirements for exogenous gonadotropins are reduced, rendering ovarian stimulation less costly.
- Duration of ovarian stimulation protocols is shortened, improving patient discomfort.

DISADVANTAGES

- GnRH antagonist cotreatment represents a novel approach and more knowledge is necessary for its optimization.
- GnRH antagonists offer less flexibility regarding cycle programming as compared with the long GnRH agonist protocol.
- Reduced ability to gain an orderly daily volume of oocyte retrievals compared with GnRH agonist, although this can be improved by using the oral contraceptive pill.

GnRH, gonadotropin-releasing hormone; IVF, *in vitro* fertilization; LH, luteinizing hormone.

Adapted from Tarlatzis B, Fauser BCJM, Kolibianakis EM, et al. GnRH antagonists in ovarian stimulation for IVF. *Hum Reprod Update* 12:333–340, 2006.

demonstrated comparable live birth rates and reduced OHSS rates.[248] In light of a reduced duration of treatment, less injections needed, similar success rates and reduced complications, GnRH antagonist should be considered first line treatment.

Approaches for Induction of Final Oocyte Maturation

In the natural normo-ovulatory cycle, rupture of the dominant follicle and release of the oocyte are triggered by the midcycle surge of LH. This sudden enhancement of pituitary synthesis and release of LH (and FSH) is elicited by high late-follicular phase E_2 levels in combination with slightly elevated progesterone levels.[249] In stimulated cycles for IVF, estrogen levels are prematurely elevated, which may induce unpredictable but advanced LH rises. As mentioned before, GnRH agonist cotreatment is required in order to prevent this from happening. Consequently, exogenous hCG should be used during the late follicular phase under these circumstances to replace the endogenous LH surge. This approach has been considered the standard of care for the induction of final stages of oocyte maturation before oocyte retrieval along with corpus luteum formation in IVF.[32] Exogenous hCG is also implicated in sustained luteotropic activity[239] due to its prolonged circulating half-life.[249] Unfortunately, hCG is therefore also believed to contribute to chances of developing OHSS.[187]

Initial studies during ovarian hyperstimulation for IVF (before the widespread use of GnRH agonist cotreatment) showed that an endogenous LH surge could be induced reliably by the administration of GnRH or a bolus injection of GnRH agonist.[250] The induction of an endogenous LH (and FSH) surge is more physiological compared to exogenous hCG because of the much shorter half-life.[251,252] Moreover, luteal phase steroid concentrations seem closer to the physiological range,[253] which may improve endometrial receptivity.[254] As the follicular phase cotreatment with GnRH agonist has been the standard of care for over a decade, alternative approaches for the induction of oocyte maturation has received little attention. However, the suppressive effect of follicular phase GnRH antagonist administration can be reversed immediately by administering native GnRH or GnRH agonist.[255] Indeed, a randomized trial confirmed that the triggering of final stages of oocyte maturation can be induced effectively by a single bolus injection of GnRH agonist even after the follicular phase co-treatment with a GnRH antagonist. This was demonstrated by the observed gonadotropin surge and quality and fertilization rate of recovered oocytes. The concept of GnRH agonist triggering as a valid alternative to hCG is appealing due to the virtual elimination of OHSS.[255] However, the preferred luteal phase supplementation under those circumstances is not yet well defined.

Recombinant LH and recombinant hCG have recently become available for clinical use. An early large randomized trial comparing 250 μg rechCG versus 5000 IU uhCG for the induction of oocyte maturation in a total of 190 women undergoing IVF showed that the number of mature oocytes retrieved and luteal phase serum concentrations of progesterone and hCG concentrations were significantly higher.[256] Considering the short half-life of recLH two injections with a 1- to 3-day interval may be considered.

The introduction of GnRH antagonists into clinical practice now makes it possible to employ a bolus injection of GnRH agonist to induce an endogenous LH surge. Although previously shown to be effective in achieving this,[253] randomized studies comparing this approach to hCG administration showed lower implantation and ongoing pregnancy rates.[257] Recent data indicate that standard luteal support regimens may be insufficient in this setting, and improved results may be achieved when this is addressed.[258,259]

Luteal Phase Supplementation

Since the early days of IVF it has been described that the luteal phase of stimulated IVF cycles is abnormal. In fact, it was already stated in the first extended report on IVF by Edwards and Steptoe[39] that "the luteal phase of virtually all patients was shortened considerably after treatment with gonadotropins" and it was suggested that high follicular phase estrogen levels due to ovarian hyperstimulation might be involved. Initial studies in the United States in 1983 concerning hMG-stimulated IVF cycles also confirmed the occurrence of an abnormal luteal phase in IVF cycles with characteristic features of elevated progesterone levels along with a significantly reduced luteal phase length[260] (Fig. 30.15).

As mentioned earlier, GnRH agonist cotreatment became the standard of care for the prevention of a premature rise in LH. Typically, GnRH agonist treatment is initiated in the luteal phase of the preceding cycle and continued until the late follicular phase. It became apparent, however, that

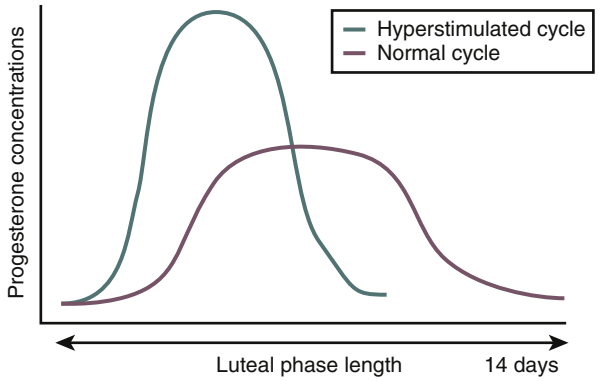


FIGURE 30.15 Schematic representation of changes in luteal phase length and endocrine profile induced by ovarian hyperstimulation for *in vitro* fertilization. *(From Jones HW]. What happened? Where are we?* Hum Reprod *11[Suppl 1]:7–21, 1996.)*

prolonged pituitary recovery from down-regulation during the luteal phase[261] resulted in lack of support of the corpus luteum by endogenous LH and advanced luteolysis.[262] It was observed shortly thereafter that the corpus luteum can be rescued by the administration of hCG,[32] and this treatment modality became the standard of care for luteal support during the late 1980s. Outcome was better compared to progesterone supplementation, but 5% of hCG-supplemented patients developed OHSS. Because of this association between hCG and OHSS,[187] luteal phase hCG support has been largely replaced over the years by luteal phase progesterone supplementation.[263] A recent meta-analysis of luteal support in stimulated IVF cycles involving a total of 69 studies and 16,327 women concluded supplementation by synthetic progesterone represents the current method of choice, with no improved outcomes using either estrogen or hCG supplementation.[264,265]

Attempts to secure pituitary recovery during the luteal phase by the early follicular phase cessation of GnRH agonist cotreatment all failed, because it takes at least 2 to 3 weeks for LH secretion to recover.[32] Because of the rapid recovery of pituitary gonadotropin release after discontinuation of GnRH antagonist it has been speculated that luteal phase supplementation may not be required following the late follicular phase administration of antagonist.[266] Preliminary observations related to ovarian stimulation and GnRH antagonist cotreatment for IUI seem to favor this contention.[267] However, various studies in IVF applying GnRH antagonist cotreatment have now clearly shown that luteolysis is also initiated prematurely resulting in a significant reduction in the length of the luteal phase along with greatly compromised chances for pregnancy.[268-270] More detailed studies could confirm that early luteal and midluteal phase LH levels remained suppressed following the follicular phase administration of GnRH antagonist.[270,271]

Moreover, luteolysis is advanced in the nonsupplemented luteal phase after either hCG or GnRH agonist triggering of oocyte maturation.[270] Collectively, this indicates that high early luteal phase steroid production is primarily responsible for advanced luteolysis, due to massive negative feedback resulting in greatly suppressed LH secretion.[203] Mild stimulation regimens resulting in lower serum steroid levels have therefore been advocated as a means of benefiting the luteal phase.[264]

Clinical Outcome of IVF

The most recent report concerning IVF in Europe performed in the year 2008 involves 532,000 cycles (from over a thousand clinics in 36 countries) with a clinical pregnancy rate per aspiration of 28.5% (including 21% twins and 1% triplets).[272] The most recent U.S. data involve almost 150,000 cycles in 2009 with live birth rates per fresh embryo transfer using nondonor oocytes of around 30% with a total multiple pregnancy rate of 31%.[273]

Adverse Effects and Complications

Complications related to invasive IVF procedures such as oocyte retrieval and embryo transfer, predominantly involve infection and bleeding along with anesthesia problems.[274] The drawbacks associated with profound ovarian stimulation for IVF include considerable patient discomfort such as weight gain, headache, mood swings, breast tenderness, abdominal pain, and sometimes diarrhea and nausea. In this respect it is important to comprehend that after a first unsuccessful IVF attempt around 25% of patients refrain from a second cycle, even in countries where costs are covered by health insurance companies. The most common reason for treatment discontinuation is burden of treatment.[275]

OHSS is a potentially life-threatening complication characterized by ovarian enlargement, high serum sex steroids, and extravascular fluid accumulation, primarily in the peritoneal cavity. Mild forms of OHSS constitute around 20% to 35% of IVF cycles, moderate forms 3% to 6% of cycles, along with 0.1% to 0.2% severe forms.[66,225] To some extent, patients at risk of developing OHSS may be recognized by the following features: young age, PCOS, profound hyperstimulation protocols with GnRH agonist long protocol cotreatment, large numbers of preovulatory graafian follicles, high serum E_2 levels, high (greater than 5000 IU) bolus doses of hCG to induce final oocyte maturation, the use of hCG for luteal phase supplementation, and finally the occurrence of pregnancy. In fact, the incidence of OHSS is directly related to hCG concentrations with a twofold to fivefold increased incidence in case of multiple pregnancy.

Preventive strategies in case of imminent OHSS include cessation of exogenous gonadotropins for several days (coasting), follicular aspiration, prevention of pregnancy during the stimulation cycle by cryopreserving all embryos, or the prophylactic infusion of glucocorticoids or albumin. The risk of OHSS may also be lowered by using alternative strategies to induce oocyte maturation, such as inducing an endogenous LH surge by administration of a single bolus dose of GnRH agonist or the short half-life preparation of recLH instead of hCG. Finally, current efforts focus on individualized dosing of ovarian stimulation based on initial screening characterstics, such as female age, body weight, AMH concentrations, and antral follicle count.[276-278] The most important complication related to IVF treatment is multiple pregnancy. The magnitude of the problem has been discussed previously in this chapter (Fig. 30.5). (For recent reviews see Fauser and colleagues[69] and Verberg and colleagues[70]) Between the years of 1980 and 2000, twin birth

rates in the United States increased by 75%, and currently represent around 3% of total births.[69] Similar trends have been reported in European countries.[70] Although an association between increased female age and multiple gestation is clearly established, the delay in childbearing accounts for no more than 30% of the observed overall increase in multiple pregnancies.[69] Although the available data indicate that the majority of twin births are still unrelated to infertility therapies[69] up to 80% of higher order multiple births are considered to be due to ovarian stimulation and ART. Births resulting from infertility therapies account for around 1% to 3% of all singleton live births, 30% to 50% of twin births, and more than 75% of higher order multiples.

Pregnancy complications include increased risk of miscarriage, preeclampsia, growth retardation, and preterm delivery. Perinatal mortality rates are at least fourfold higher in twin, and at least sixfold higher in triplet, births compared with singleton births. Moreover, the risks of prematurity in twin and higher order multiple birth are increased sevenfold to fortyfold, and for low-birth-weight infants tenfold to seventyfivefold, respectively. Adverse outcomes among children conceived through IVF are largely associated with multiple gestation.

Recent data are reassuring with respect to possible long-term health consequences such as ovarian cancer, breast cancer, and advanced menopausal age.[279]

New Approaches to Mild Ovarian Stimulation for IVF

After the initial years of IVF, profound ovarian stimulation became the rule for more than 2 decades. The stimulation of growth of large numbers of follicles and the retrieval of many oocytes has been viewed as an acceptable marker of successful IVF treatment. Medication regimens to achieve profound ovarian stimulation are extremely complex and expensive, take many weeks of frequent injections, and require intense monitoring. Moreover, patient discomfort and chances for serious side effects and complications are considerable. In addition, this profound stimulation gives rise to greatly abnormal luteal phase endocrinology, and its impact on endometrial receptivity and therefore IVF success is mostly unknown.

Attitudes toward profound ovarian stimulation are changing,[204,280] particularly given the growing tendency to transfer a reduced number of embryos. It has previously been demonstrated on the basis of the U.K. national database that reducing the number of embryos tranferred from three to two does not diminish chances of birth despite a reduction in risk of multiple birth.[87] In Europe, an increasing number of centers are carrying out single transfer in younger women. Emphasis may therefore now be directed toward the development of more simple and milder stimulation protocols[29,216,224,281] or the improvement of natural cycle IVF outcomes.[83,211,213] The increasing quality of embryo cryopreservation programs will serve to encourage the transfer of one embryo at a time.[282]

Previous studies in normo-ovulatory female volunteers[125,126] confirmed that the development of multiple dominant follicles can be induced by interfering with decremental FSH concentrations during the midfollicular to late follicular phase. As shown previously, this decrease is required for the selection of a single dominant follicle.[11,12] These

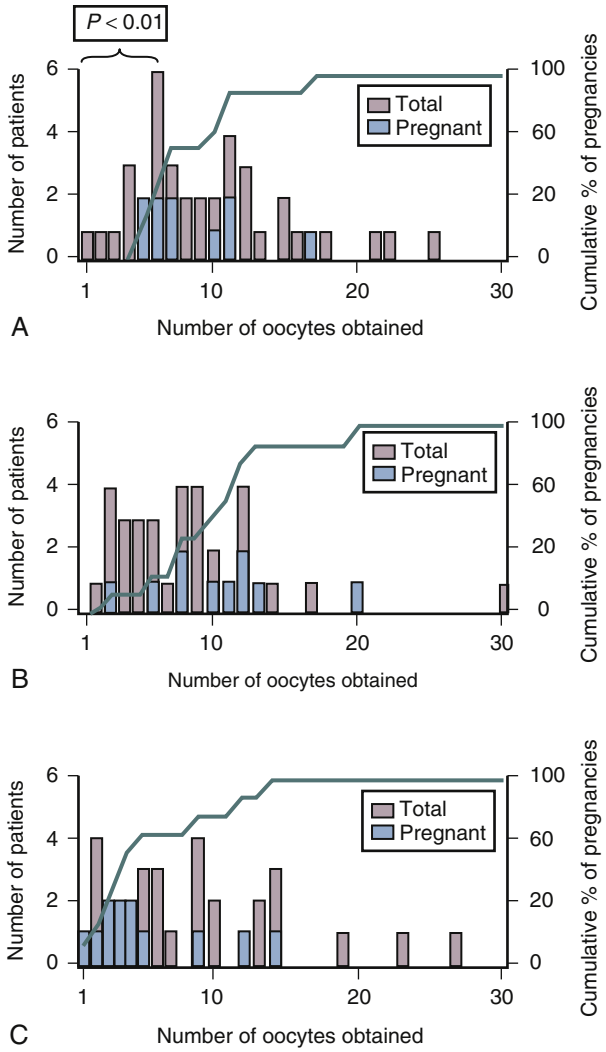
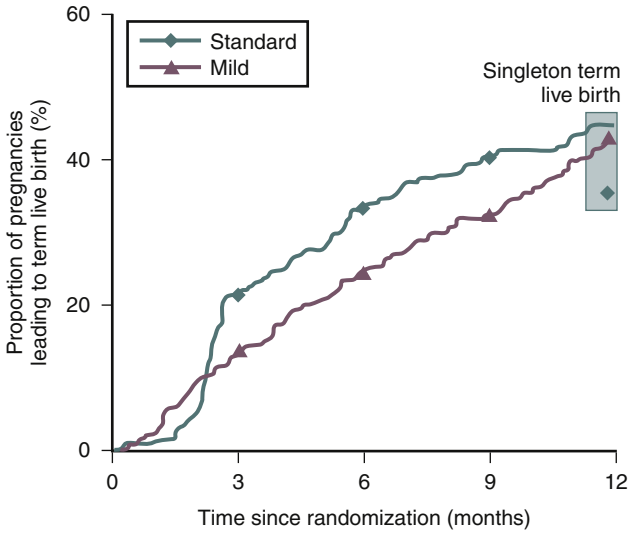


FIGURE 30.16 Number of women undergoing *in vitro* fertilization who did or did not achieve a pregnancy in relation to the amount of oocytes retrieved, comparing conventional hyperstimulation with gonadotropin-releasing hormone (GnRH) agonist long protocol **(A)** with two mild stimulation protocols employing GnRH antagonist cotreatment (**B** and **C**). *(Adapted from Hohmann FP, Macklon NS, Fauser BC. A randomized comparison of two ovarian stimulation protocols with gonadotropin-releasing hormone [GnRH] antagonist cotreatment for in vitro fertilization commencing recombinant follicle-stimulating hormone on cycle day 2 or 5 with the standard long GnRH agonist protocol.* J Clin Endocrinol Metab *88:166–117, 2003.)*

observations are in agreement with previous findings in the monkey model.[127,283] We were subsequently able to demonstrate that the initiation of exogenous FSH (fixed dose, 150 IU/day, GnRH antagonist cotreatment) as late as cycle day 5 results in a comparable clinical IVF outcome, despite a reduced duration of stimulation (number of ampules used) and increased cancellation rates[284] (Fig. 30.16).

To test the efficacy of this mild stimulation protocol in standard practice, a large randomized effectiveness study has been performed to analyze whether a strategy including the mild stimulation protocol in combination with single embryo transfer (SET) would lead to a similar outcome assessed over a 1-year interval after initiation of treatment, while reducing patients' discomfort, multiple



Number of patients

Standard	199	152	123	106	97
Mild	205	174	149	130	109

FIGURE 30.17 Proportions of pregnancies leading to cumulative term live birth within 12 months after starting *in vitro* fertilization. Mild: mild ovarian stimulation with GnRH antagonist and single embryo transfer. Standard: standard ovarian stimulation with GnRH antagonist and dual embryo transfer. The shaded area represents the singleton live birth rate after 12 months. *(From Heijnen EM, Eijkemans MJ, De Klerk C, et al. A mild treatment strategy for in-vitro fertilisation: a randomised non-inferiority trial.* Lancet *369[9563]:743–749, 2007.)*

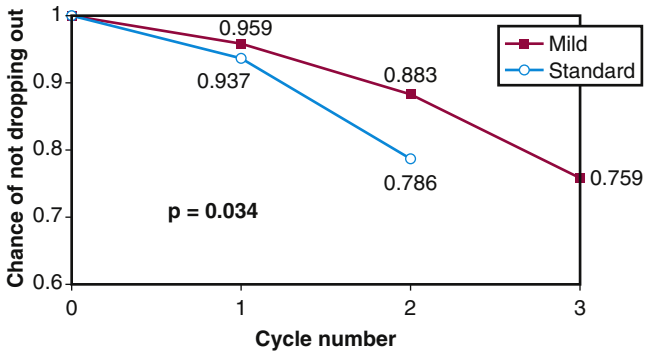


FIGURE 30.18 Chances of not dropping cut from successive IVF treatment cycles applying either mild IVF or standard IVF. *(Adapted from Verberg et al. Why do couples drop-out from IVF treatment? A prospective cohort study.* Hum Reprod *23[9]: 2050–2055, 2008.)*

pregnancies, and costs compared with standard treatment.[72] The study included a total of 404 patients and observed that because of the shorter duration of treatment per cycle, less medication needed, and a reduction in twin pregnancies, the mild approach led to an equal chance of live birth after a year of treatment while reducing the total costs (Fig. 30.17).

Apart from clinical efficiacy and costs (see later in the chapter), emotional stress should be considered an important negative side effect associated with IVF treatment. Following mild stimulation, patients reported fewer side effects and stress related to hormone treatment compared with conventional stimulation.[285] Consequently drop out rates have been reported to be significantly reduced during mild stimulation (Fig 30.18). Treatment-related stress has been found to be the most important reason patients drop out of IVF treatment.[286] The early drop-out of treatment deprives the couple of an optimal cumulative chance of achieving pregnancy, and therefore also impacts on the success of the respective IVF program. Mild stimulation might therefore have a positive impact on cumulative treatment success rates as it positively affects the chance that patients are willing to continue treatment following a failed attempt.[288]

Other novel protocols under investigation include the replacement of FSH by LH, an approach based on the acquired LH responsiveness of granulosa cells of dominant follicles. Besides the expected reduction of gonadotropin usage, this ovarian stimulation approach might also reduce the number of small, less mature follicles, possibly reducing the chance of OHSS, because smaller ovarian follicles are unlikely to be responsive to LH.[169] Several randomized controlled trials [222,289,290] have shown that this approach can result in a significant reduction in FSH needed and in the number of small follicles at final oocyte maturation. Pregnancy rates do not appear to be compromised. More extensive studies are required to determine the critical threshold for FSH replacement by LH stimulation and the most appropriate dosage of LH or hCG.

There are indications that the degree of ovarian stimulation affects both the morphological embryo quality and the chromosomal constitution of the developed embryos.[291-294] This phenomenon could be the result of interference with the natural selection of good quality oocytes or the exposure of growing follicles to the potentially negative effects of ovarian stimulation. A randomized trial concerning the chromosomal analysis of human embryos following mild ovarian stimulation for IVF showed a significantly higher proportion of euploid embryos compared to conventional ovarian stimulation, suggesting that through maximal stimulation the surplus of obtained oocytes results in chromosomally abnormal embryos.[295]

Toward Individualized Treatment Algorithms

As previously highlighted, the chance of achieving a spontaneous pregnancy is frequently underestimated by couples and their physicians.[99] An increasingly assertive patient population, who continue to delay childbearing for career, social, or other reasons is putting physicians under greater pressure to intervene in order to aid the couple in achieving their goal quickly and with minimal disruption to busy lives.[296] Time is increasingly an issue for couples seeking to conceive, and the commercial pressures and competition between IVF centers can lead to couples being accepted into IVF programs without a sound indication. Yet the virtue of patience can pay dividends for many who are now subject to premature and unnecessary intervention. Most couples seeking help will present with subfertility rather than absolute infertility. On the basis of a modest range of investigations and certain individual characteristics, the chances of an individual couple conceiving spontaneously over a given period of time can be calculated. It is known for instance that the spontaneous monthly fecundity rate declines with increasing duration of subfertility. After 3 years the residual

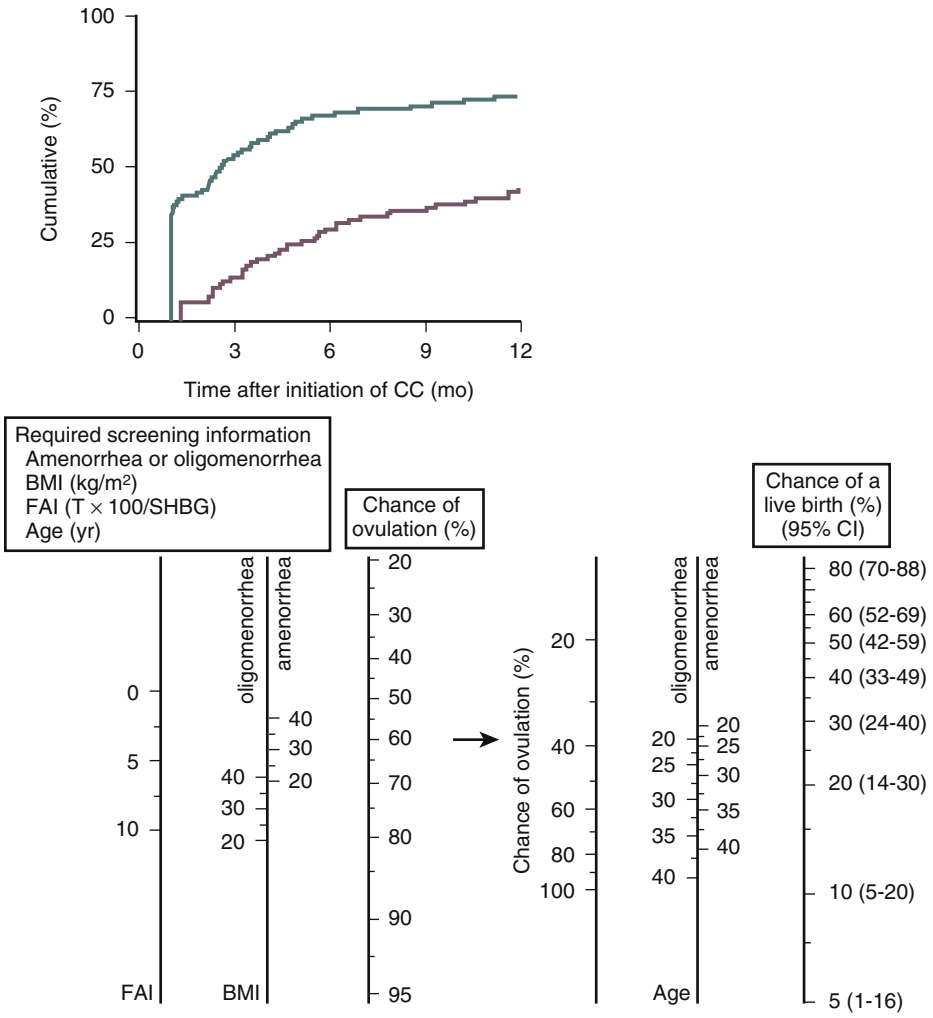


FIGURE 30.19 Cumulative percentage of patients who ovulate or conceive following the initiation of clomiphene citrate, CC *(top)*, and a two-step nomogram predicting chances of live birth following clomiphene citrate on the basis of initial screening characteristics *(bottom)*. BMI, body mass index; FAI, free androgen index. *(From Imani B, Eijkemans MJ, te Velde ER, et al. A nomogram to predict the probability of live birth after clomiphene citrate induction of ovulation in normogonadotropic oligoamenorrheic infertility.* Fertil Steril *77:91–97, 2002.)*

likelihood of spontaneous pregnancy in untreated couples with unexplained infertility falls to 40% and after 5 years to 20%.[99]

In recent years a number of prediction models for calculating individual chances of spontaneous conception in subfertile couples have been published.[297-299] The chance of conception over a given time frame can be calculated from the results of a number of fertility investigations and patient parameters such as age and duration of infertility. Caution is, however, required when applying a prediction model developed elsewhere to one's own patient population. Before a prediction model can be introduced into everyday clinical practice, prospective external validation is required. Furthermore, knowledge of the development cohort is important when selecting a model for application in one's own setting. The mean duration and degree of subfertility in a primary care population is less than in a tertiary population. As a result, the conclusions derived from models developed in academic centers may have limited relevance for primary subfertility management and vice versa.[99]

The majority of women undergoing ovulation induction have WHO class 2 anovulation. Although this is a highly heterogeneous group, the treatment for these women is the same.[300] The identification of patient characteristics predictive of ovulation induction outcome would allow the design of individual treatment regimens, and would provide useful information regarding the factors that determine the extent of ovarian dysfunction. In recent years a number of studies addressing these issues have been published. In one study the criteria that could predict the response of women with WHO class 2 anovulation to treatment with CC were identified.[122] Following multivariate analysis, the free androgen index (FAI), body mass index (BMI), presence of amenorrhea (as opposed to oligomenorrhea), and ovarian volume were found to be independent predictors of ovulation.[301] The area under the receiver operating curve in a prediction model using these factors was 0.82. By adding additional endocrine factors, the area under the curve increased to 0.86.[299] In a subsequent study, those factors which could predict conception following ovulation were studied. Multivariate analysis of a number of clinical, endocrine, and ultrasound characteristics revealed lower age and the presence of amenorrhea to be the only significant parameters for predicting conception. Initial LH levels were not found to be important. From these data, a nomogram was constructed[177] (Fig. 30.19) which may assist in the selection of patients for clomiphene therapy, and those for whom this first-line treatment will be of little value. In this latter group, early recourse to gonadotropin therapy is indicated [302]

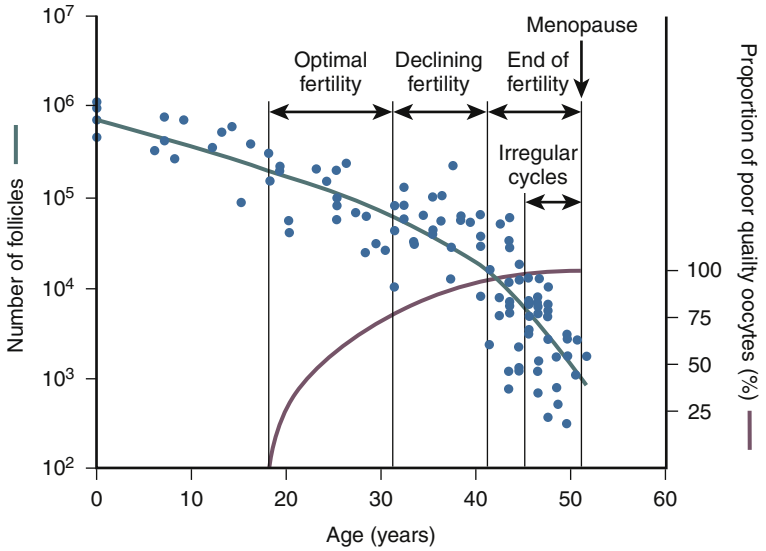


FIGURE 30.20 The decline in follicle number and the increase in poor-quality oocytes in relation to reproductive events with increasing female age. *(From Broekmans FJ, Knauff EA, te Velde ER, et al. Female reproductive ageing: current knowledge and future trends.* Trends Endocrinol Metab *18[2]:58–65, 2007.)*

When gonadotropin therapy for ovulation induction is selected, the duration of treatment, the amount of gonadotropins administered, the associated risks of cycle-to-cycle variability, multifollicular development, OHSS, and multiple pregnancy might all be reduced if the starting dose were individualized. This would require the means to reliably predict the dose of FSH at which a given individual will respond by way of monofollicular selection to dominance—in other words, their individual FSH threshold for stimulation. A prediction model has recently been developed which may be used to determine the individual FSH response dose (which is presumably closely related to the FSH threshold).[131] Women about to undergo low-dose step-up ovulation induction with recFSH, were subject to a standard clinical, sonographic, and endocrine screening. The measured parameters were analyzed for predictors of the FSH dose on the day of ovarian response. In multivariate analysis, BMI, ovarian response to preceding CC medication (CC-resistant anovulation [CRA], or failure to conceive despite ovulatory cycles), initial free insulin-like growth factor-I (free IGF-I), and serum FSH levels were included in the final model.[131] In a subsequent analysis of women with PCOS who had undergone ovulation induction with the step-down regimen, a correlation was observed between the predicted individual FSH response dose and the number of treatment days before dominance was observed.[302] Application of this model may enable the administration of the lowest possible daily dose of exogenous gonadotropins to surpass the individual FSH threshold of a given patient and achieve follicular development and subsequent ovulation. Refining ovulation induction therapy in this way offers the prospect of improving safety, reducing the risk of multiple pregnancies, and improving the efficiency of gonadotropin ovulation induction.

The ability to predict clinical outcome from ovulation induction with gonadotropins would also be of value in the individualization of treatment regimens. In a prediction model for outcome after FSH ovulation induction,[186] simple patient characteristics combined with endocrine factors were again shown to enable (limited) prediction of outcome following FSH ovulation induction.[303] The most important end point for ovulation induction is overall singleton live birth. Data are now available to allow the prediction of a given couple achieving this from conventional ovulation induction strategies over an extended period of time (Fig. 30.12).[55] This observation has been confirmed 10 years later.[302] Regarding IVF treatment, it appears that the most prominent factor determining outcome is the individual variability in ovarian response to stimulation. Rather than exhibiting the desired response, women can present with either a hyporesponse or a hyper-response to stimulation. Studies undertaken so far have been unable to demonstrate a beneficial effect of gonadotropin dose increase in patients who exhibit a poor response to standard dose regimens.[189,232] This may help in counseling the patient, because the chances of successful IVF in these women will be extremely low.

Poor ovarian response appears to be related to ovarian aging[191] and early menopause (Fig. 30.20).[192] In IVF, the association between poor ovarian response due to diminished ovarian reserve with cycle cancellation and poor success rates is well established.[304] Age is an important predictor of IVF outcome.[306] However, chronological age is poorly correlated with ovarian aging. A major individual variability exists in follicle pool depletion within the normal range of menopausal age, as complete follicle pool exhaustion may occur between 40 and 60 years of age. The quantity and quality of the primordial follicle pool diminishes with age, reducing ovarian reserve.[307] This results in a decline in both therapy-induced and spontaneous pregnancies.[308] However, some women older than 40 years of age will show a good response to ovarian stimulation and subsequently conceive with IVF, yet other women under 40 may fail to respond as a result of accelerated ovarian aging. In recent years, attention has been given to the identification of sensitive and specific markers for ovarian aging which may enable prediction of poor or good response. This would open the way to improved counseling and patient selection for IVF.

The first endocrine marker for ovarian reserve is the early follicular phase FSH level,[309] which has been shown to be an independent predictor to age of IVF outcome.[310] More recent studies have indicated that while FSH level

is a stronger predictor of cycle cancellation due to poor response and the number of oocytes collected at pick-up, age is more closely related to the chance of pregnancy.[310] In current practice, women with raised baseline FSH levels are usually advised against IVF treatment due to the anticipated poor outcome. However, although young women with high FSH levels demonstrate lower numbers of growing follicles and a high probability of cycle cancellation, normal ongoing pregnancy rates may be observed if oocytes and embryos are obtained.[310] Older women (older than 40 years old) with normal baseline FSH levels may demonstrate lower cancellation rates, but the implantation rate per embryo and the ongoing pregnancy rates are lower than those observed in young women with elevated FSH.[310] FSH has been suggested to be of greater value in predicting ovarian reserve than other ovarian markers such as inhibin B. However, in a meta-analysis, baseline FSH levels showed only a moderate predictive performance for poor response and a low predictive performance for nonpregnancy was observed.[311] Other markers may therefore have an adjunctive value when diagnosing diminished ovarian reserve. The ultrasound measurement of the number of antral follicles present on cycle day 3 has been shown in a number of studies to predict poor ovarian reponse. Addition of basal FSH and inhibin B levels to a logistic model with the antral follicle count appears to further improve the performance of this marker.[312] At present no single reliable marker for ovarian reserve has been identified.[310] Anti-müllerian hormone (AMH), a member of the transforming growth factor-β superfamily, has been proposed as another candidate in this context. It is produced by granulosa cells of growing preantral and small antral follicles and is directly involved in primordial follicle pool depletion in the rat. Serum levels decline with age[313] and recent studies have shown that poor reponse to IVF can be predicted by reduced baseline serum AMH concentrations.[310] Additional research firmly established AMH as the most useful marker for ovarian response prediction and individualized gonadotropin dosing.[313,314]

To some extent, women likely to present with a reduced response to ovarian stimulation can be predicted, but hyper-response (and the threat of OHSS) comes as a surprise in the great majority of cases. It is uncertain, as yet, which patients are likely to present with hyper-response (at risk for OHSS).[206] Early recognition of women at risk may give rise to effective, altered stimulation protocols and improved safety.[187]

The use of nomograms for individualising FSH dose for ovarian stimulation in IVF may optimize the risk to benefit dose of FSH in IVF. In recent years, several models have been developed based on multiple regression analysis.[315,316] Factors consistently observed to be predictive of the number of oocytes obtained were age, the total number of antral follicles, and smoking status.[317] Others have suggested that ovarian volume and and blood flow as measured by Doppler ultrasound are predictive factors.[312] A model combining all these factors has been developed to prescribe the optimal dose of rFSH that will yield 5 to 14 oocytes.[315] In a prospective randomized study, the application of this model increased the proportion of "appropriate ovarian responses" and decreased the need for dose adjustments during ovarian stimulation.[316]

In those who do respond to ovarian hyperstimulation for IVF and for whom embryos are available for transfer, individualizing treatment in order to optimize outcomes should involve consideration of the number of embryos to be transferred. As stated earlier, although a trend toward the transfer of fewer embryos is now clear, fear of a lower chance of pregnancy may discourage couples and their physicians from transferring two embryos or less. This applies particularly when single embryo transfer is being considered. The ability to identify those treatment cycles in which single embryo transfer would avoid the risk of twin pregnancy without reducing the chance of achieving a singleton pregnancy would certainly encourage the adoption of single embryo transfer into clinical practice. In recent years, a number of authors have tried to identify those factors that may predict the chance of birth and of multiple birth on the basis of key characteristics of the patient, the cycle, and the embryos available for transfer.[75, 318] Important determining factors thus far identified include the age of the woman, the duration of infertility, and the number of oocytes obtained following ovarian hyperstimulation. We have previously developed a prediction model to allow the chance of pregnancy and twin pregnancy to be assessed in a given transfer cycle should one or two embryos be transferred.[88] Application of this model allows a subgroup of young patients with good quality embryos to be identified for whom applying single embryo transfer could drastically diminish the twin rate without compromising singleton pregnancy rates.

As with models designed to predict spontaneous pregnancy, untested models for predicting IVF outcome can show a disappointing performance when used on patients from a different but plausibly similar population.[319] Therefore, before such a model can be applied in clinical practice, its reliability in predicting the selected outcomes should be validated on a different population to that on which it was developed. External validation of our model has demonstrated that it may be applied in different populations following a simple calibration process to adjust for local success rates.[89]

Health Economics of Ovarian Stimulation

Although a tendency to increased IVF consumption can be observed every year, IVF or IUI should not be routinely applied for all kinds of infertility problems. Assisted reproduction should not replace a proper infertility workup. A recent meta analysis involving six RCTs concluded that the effectiveness of IVF for unexplained infertility remains questionable.[320] Moreover, the economic implications of a more widespread use of assisted reproduction should be considered seriously when making decisions regarding treatment.[321,322]

The diagnosis by exclusion of unexplained infertility and subfertility is made in around 30% of couples in whom conventional diagnostic tests are normal. The prognosis for conception significantly decreases when the duration of infertility is at least 3 years along with an advanced female age beyond 35.[298] Again, chances of spontaneous conception are usually underestimated both by the doctor and the patient.[99] On the other hand, it appears that high costs prevent many couples with an indication for this treatment modality from undergoing IVF (i.e., undertreatment due to insufficient access to ART services). Data from the United States suggest that in states where IVF is not covered, only

one third of couples with a valid indication for IVF actually undergo treatment.[291] Moreover, IVF is available in only 25% of the countries worldwide.[322] In contrast, in a commercial environment couples may be exposed to risks associated with assisted reproduction too early (i.e., overtreatment under conditions in which expectant management might have been more appropriate). Indeed, in various European countries such as France, The Netherlands, and Sweden where IVF is covered by health insurance, a threefold higher use of IVF per capita compared to the United States can be observed.[323]

Cost-effective health care involves the achievement of a desired treatment goal at the lowest possible expenditure. IVF cost effectiveness should assess costs per live birth. So far, calculations of costs per live birth have only included direct costs related to neonatal care. The inclusion of indirect costs (i.e., including midterm and long-term health sequelae such as mental retardation, cerebral palsy, and learning disabilities) would presumably double the overall costs.

The financial consequences of multiple pregnancies are substantial for both parents and healthcare providers. However, the economic impact of a multiple pregnancy is not limited to increased costs of maternal hospitalization and obstetric and neonatal (intensive) care. Lifetime costs for chronic medical care, rehabilitation, and special education related to extreme prematurity must also be taken into account. For a low-birth-weight child, the average cost of heathcare and education up to the age of 8 years is seventeenfold higher than the costs for a normal birth weight child.[324] It has also been shown that multiple births contribute disproportionately to hospital inpatient costs, especially during the child's first year of life.[325]

Because of the limited use of ovarian stimulating medication, the per cycle costs of mild stimulation IVF cycles will be lower than conventional stimulation approaches. However, to analyze the cost effectiveness of mild stimulation, the total cost per live birth should be analyzed. Besides the costs for medication, medical consultations and visits, laboratory charges (general, hormone, and embryology), ultrasound procedures, IVF procedures (oocyte retrieval and embryo transfer), hospital charges, nurse coordinator costs, administrative charges, fees for anesthesia, costs for complications, travel expenses, and lost wages should be taken into account.[322]

Those who advocate milder strategies in IVF point to recent studies that show that the costs for IVF per year of treatment are comparable with conventional stimulation approaches, and the costs for the pregnancy and neonatal period are significantly lower following mild stimulation and single embryo transfer.[326]

Conclusions and Future Perspective

Any form of ovarian stimulation increases chances of pregnancy per cycle but at the expense of increased complication rates, most importantly multiple pregnancies (Fig. 30.8) and OHSS. This holds especially true for ovarian hyperstimulation aiming at maturing multiple dominant follicles for fertilization either *in vivo* (following intercourse or IUI) or *in vitro* by IVF. With IVF, the incidence of occurring multiple pregnancies can be controlled by the number of embryos transferred. Moreover, various strategies may significantly reduce chances for OHSS. In skillful hands and with proper ovarian response monitoring, chances for complications are lowest for ovulation induction. The aim of this intervention is to mimic physiological circumstances in anovulatory women, hence, single dominant follicle development and ovulation.

Special care should also be taken to carry out a proper infertility workup in order to diagnose other treatable infertility factors. This will also allow a proper assessment to be made of pregnancy chances for a given couple, either spontaneously or after infertility therapies. Along these lines, only patients with a proper indication will be exposed to the discomfort, risks, and costs associated with assisted reproduction and ovarian hyperstimulation.

Milder forms of ovarian hyperstimulation (or indeed none at all) may be considered for empirical treatment of unknown infertility (with or without IUI) due to the inherent risk of higher order multiple pregnancies. In general, however, the price to pay is a slightly lower pregnancy rate per cycle. Overall, assessment of cumulative pregnancy rates over a given period of time (which may involve multiple cycles) may be similar.

A trend can be observed toward hyperstimulation and assisted reproduction as first-line treatment in anovulatory infertility, especially PCOS. This shift in clinical practice is not based on sound scientific evidence. In fact, healthy live birth rates from conventional ovulation induction strategies are good, with acceptable rate of multiple pregnancies and OHSS.[64] Newly introduced compounds to the field of ovulation induction such as insulin sensitizers and aromatase inhibitors may further improve outcomes.

With regard to IVF, many new treatment modalities have been introduced over the years without proper preceding evaluation for efficacy and safety. The current most profound clinical challenge is to find the right balance between improving chances for success coinciding with an acceptable complication rate. The paradigm of so-called "controlled" ovarian hyperstimulation using maximum stimulation by exogenous gonadotropins together with the GnRH agonist long protocol has been taken for granted for more than a decade. Potential detrimental effects of this approach with regard to patient discomfort and safety, oocyte quality, corpus luteum function, and endometrial receptivity have been largely ignored. Large numbers of preovulatory follicles and oocytes subsequently retrieved have been applied as useful surrogate outcome parameters for successul IVF.[280] Maximum ovarian stimulation along with the transfer of large numbers of embryos in an attempt to maximize pregnancy rates per IVF cycle may by itself have a major impact on patient dropout rates, costs, and overall IVF outcome and should therefore be considered seriously. The introduction of GnRH antagonists allows for a careful reevaluation of current IVF strategies. We can now render stimulation protocols simpler, starting with a spontaneous menstrual cycle, allowing for more subtle interference with dominant follicle selection. Final stages of oocyte maturation can now also be stimulated, applying different drugs and strategies for the induction of an endogenous LH surge. Finally, effects of these altered follicular phase interventions on corpus luteum function and endometrial development (important for embryo implantation) should be assessed.

Especially in the light of a continued trend worldwide to reduce the number of embryos transferred, novel approaches of mild ovarian stimulation or even natural cycle IVF deserve reevaluation. It does not seem logical to stimulate the ovary profoundly in order to generate numerous embryos in case the aim is to transfer only one or two of them. Moreover, the possible relationship between quantity of oocytes stimulated and quality (i.e., genetic competence) of embryos obtained[295] should be studied in greater detail. Finally, in the light of a reduced number of fresh embryos being transferred, the continuing improvement of techniques to cryopreserve supernumerary embryos such as vitrification (allowing couples additional pregnancy chances without having to go through ovarian stimulation and oocyte retrieval) seems of pivotal significance.

Individualizing ovarian stimulation in order to optimize outcomes between risks and desired outcomes is likely to improve in the future with the development of pharmacogenetics. Clinical studies have shown that FSH receptor gene polymorphisms influence the ovarian response to stimulation in women undergoing IVF,[327] raising the possibility that genotyping could aid in tailoring FSH dosing based on individual ovarian sensitivity.[278]

Choosing the "best" embryo for transfer is still problematic, because the assessment of embryo morphology is still crude and inaccurate. More information is urgently needed regarding randomized controlled trials replacing a single embryo with or without preimplantation genetic aneuploidy screening. The paradigm of measuring success in terms of positive pregnancy test per IVF retrieval or transfer treatment should shift in future studies to take into account the balance between the chance of a healthy live (singleton) birth per started IVF treatment, which may involve multiple cycles in relation to risks and complications, patient discomfort, and costs.[101] Fortunately, large IVF centers now begin to report their cumulative IVF outcomes, independent from the number of cycles performed.[328-330] The health economics evaluation of IVF should be no different from other complex medical interventions. In this context, the impact of ovarian stimulation on embryo quality, corpus luteum function, and endometrial receptivity should be studied in greater detail.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 31

Assisted Reproduction

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Catherine Racowsky

The essence of mammalian reproduction is the fusion of a sperm and an egg resulting in a conceptus, which can grow and differentiate into a new organism. Mammalian reproduction is an efficient process that typically occurs entirely within the bodies of the male and female partners and requires no intervention or assistance from a third party. The essence of *assisted reproduction* is that a third party, the reproductive biologist, directly handles the oocyte and sperm to enhance the probability of achieving a pregnancy. In general, at least part of the manipulation of the sperm and oocyte occurs outside the body of the male and female partners.

Many techniques are currently utilized that involve manipulation of oocytes, sperm, and embryos to improve the probability of a successful outcome. *In vitro* fertilization and embryo transfer (IVF-ET) is the most commonly performed assisted reproductive technology (ART) procedure, involving the culture of aspirated oocytes and spermatozoa *in vitro*, followed by the transcervical replacement of the resulting embryo(s) into the uterine cavity. One variation of IVF-ET is intracytoplasmic sperm injection (ICSI), in which fertilization is achieved by injection of a sperm into the oocyte. In contrast, gamete intrafallopian tube transfer (GIFT) is the direct placement of aspirated oocytes and prepared spermatozoa into the fallopian tube, and zygote intrafallopian tube transfer (ZIFT) entails the laboratory culture of aspirated oocytes with spermatozoa followed by the direct placement of zygotes into the fallopian tubes. GIFT and ZIFT are rarely performed any more because live birth rates are higher with IVF. Therefore, this chapter focuses on IVF-ET, use of donor gametes, gestational carriers, and cryopreservation of embryos, and their indications, implementation. These techniques, along with intracytoplasmic sperm injection (ICSI) and other manipulation of gametes and embryos discussed in Chapter 32, are collectively referred to as the ARTs.

Assisted reproduction is more than 110 years old, with the first successful transfer of embryos from a donor to a recipient rabbit being achieved by Heape in 1890.[1] In 1959, Chang[2] successfully fertilized a rabbit oocyte *in vitro*. In the human, successful capacitation of sperm *in vitro* and the fertilization of human oocytes matured *in vitro*[3] were followed by the insight that preovulatory oocytes were optimal for *in vitro* fertilization.[4] These exploratory steps culminated in 1978 with a term birth resulting from the *in vitro* fertilization of a single preovulatory human oocyte obtained from a natural menstrual cycle, transferred to the uterus at the eight-cell stage.[5] Assisted reproduction is the jewel in the crown of reproductive medicine. It is one of the best examples in reproductive medicine of the transfer of knowledge obtained by laboratory scientists to an application that treats human disease. Assisted reproduction is a vast field encompassing both the sublime secrets of how two haploid cells combine to create a diploid zygote that contains all the information necessary to grow and develop into a complex mammal, and the pragmatically empirical application of the technology to treat infertility. As assisted reproduction becomes the standard treatment for infertility, the pace of knowledge transfer from bench to bedside is increasing, challenging our concepts of self, family, and society.

In sexual reproduction, unique haploid cells fuse at fertilization to form a diploid cell that divides to form daughter cells that undergo multiple subsequent divisions, and subsequent differentiation to develop into an entire organism. In turn, some diploid progenitor cells divide by the process of meiosis to produce unique haploid cells that can fuse to yield a new, totally unique diploid organism.

Sexual reproduction has two processes that maximize diversity in a species. One crucial process is that diploid cells give rise to unique haploid cells through genetic recombination between homologous chromosomes during meiosis. Exchange of genetic material between maternally and paternally derived chromosomes markedly increases the genetic diversity of the resultant haploid cells (Figs. 31.1 and 31.2). One theoretical advantage of sexual reproduction is that the process of meiosis permits the random recombination of genetic material, thereby increasing the range of traits displayed by members of the species. This diversity increases the chances of success of the species in adapting to an ever-changing environment. A second process of sexual reproduction is that the haploid cells fuse during fertilization to form a new and unique diploid cell. The single-cell diploid zygote has all the genetic information necessary to grow and develop into an adult organism.

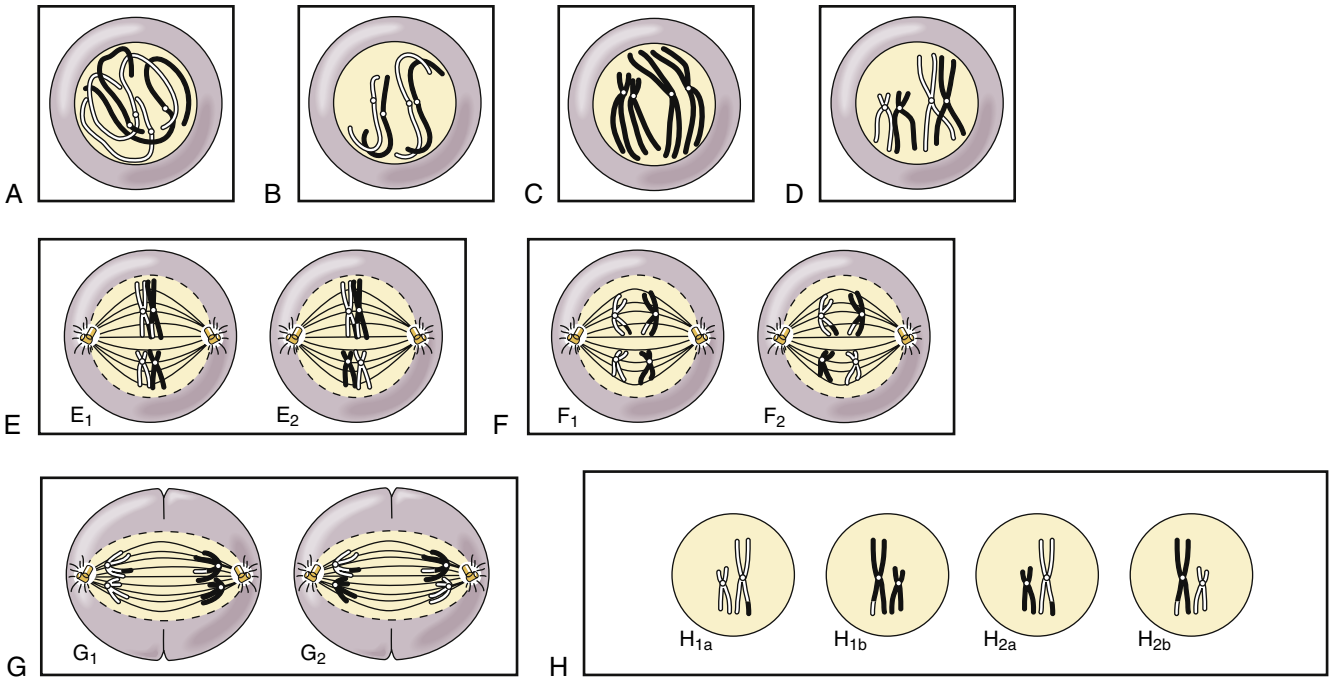


FIGURE 31.1 The first meiotic division. Two of the 23 chromosome pairs are shown to simplify the presentation. Chromosomes from the maternal source are shown in outline, chromosomes from the paternal source are shown in solid color. **A,** Leptotene. **B,** Zygotene. **C,** Pachytene. **D,** Diplotene. **E,** Metaphase (E_1, and E_2). **F,** Early anaphase (F_1, and F_2). **G,** Late anaphase (G_1 and G_2). **H,** Telophase (H_1 and H_2). One set of distributions of chromosome pairs is delineated in E_1 through H_1, an alternative combination in E_2 through H_2. Homologous recombination and random segregation increase the diversity of genetic material passed to the gametes. *(From Thompson JS, Thompson MW.* Genetics in Medicine. *Philadelphia, WB Saunders, 1986, p 19.)*

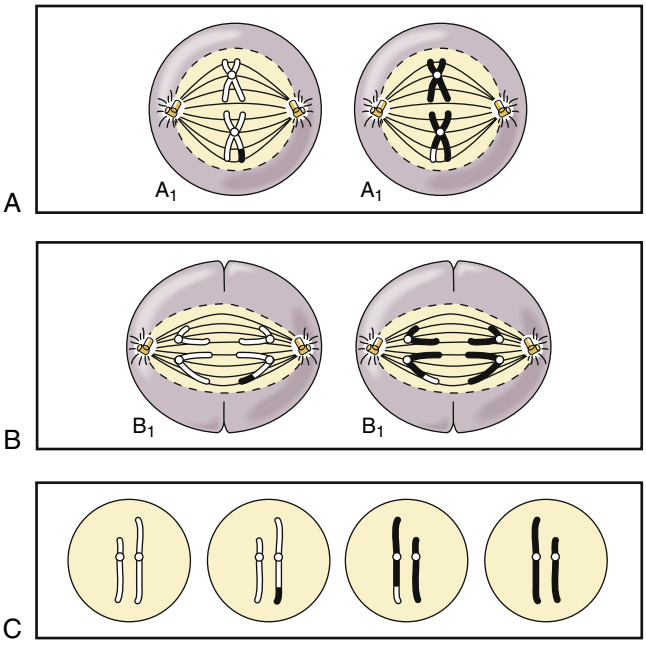


FIGURE 31.2 The second meiotic division. **A,** Metaphase. **B,** Anaphase. **C,** Telophase. A_1 and A_2 represent H_{1a} and H_{1b} from *Figure 31.1*. *(From Thompson JS, Thompson MW.* Genetics in Medicine. *Philadelphia, WB Saunders, 1986, p 20.)*

The Gametes

In most species that reproduce by sexual reproduction, two types of gametes are produced. The egg, or ovum, is large and nonmotile. The sperm or spermatozoa are small and motile. The developing egg is referred to as an *oocyte* and is one of the largest cells in the body. A mature human oocyte measures approximately 110 μm in diameter.[6] By contrast, the diameter of a human sperm head is 2 to 3 μm.[7] In some species, the oocyte is totipotent; once stimulated by artificial means, it can give rise to an entire adult organism. Such an artifical stimulus can occur by mechanical activation or chemical stimulation[8] and is called parthenogenesis.

The generation of the germ cells is achieved through meiosis (Figs. 31.1 and 31.2). During meiosis, duplication of diploid deoxyribonucleic acid (DNA) in the progenitor cell is followed by two successive cell divisions, which result in derivative packets of haploid DNA. As the progenitor cell enters prophase I, each duplicated chromosome consists of two joined sister chromatids; homologous chromosomes align in bivalents and genetic recombination occurs. In the first meiotic division, one homologue (consisting of linked sister chromatids) of each chromosome pair is distributed to each daughter cell (Fig. 31.1). No DNA replication occurs in the second meiotic division, and the strands of the sister chromatids are separated to the derivative haploid cells (Fig. 31.2).

The Oocyte

The process of formation of oocytes, known as *oogenesis*, begins when the primordial germ cells migrate into the

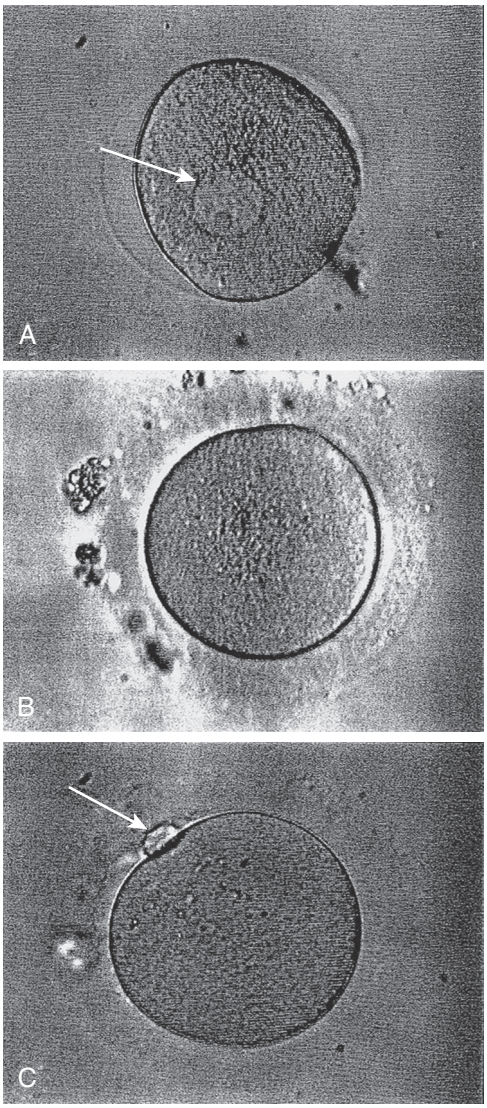


FIGURE 31.3 Oocytes at the three most commonly observed stages of meiotic maturation. **A,** Oocyte at the germinal vesicle (GV) stage; note the nucleus with its characteristic single nucleolus *(arrow)* and granular cytoplasm. **B,** Oocyte at metaphase I; note the absence of both a nucleus and a polar body and less granular cytoplasm compared with the GV-stage oocyte. **C,** Oocyte at metaphase II; note the presence of the first polar body *(arrow)* and the smooth cytoplasm. *(All photos courtesy of Dr. Gena Ratiu, Brigham and Women's Hospital ART Laboratory, Boston, MA.)*

embryonic gonad and become *oogonia* (see Chapter 8). The oogonia proliferate by mitotic division, become invested with a single layer of granulosa cells, and differentiate into primary oocytes. The primary oocyte duplicates its complement of DNA, enters the meiotic process, reaches diplotene of prophase I of meiosis, and then enters a state of prolonged meiotic arrest or "hibernation." Such an arrested so-called germinal vesicle (GV) stage oocyte (Fig. 31.3A), synthesizes a coat of glycoproteins, the *zona pellucida*, and remains arrested until the follicle in which it is enclosed either degenerates (atresia) or is recruited into the pool of growing follicles and ultimately is selected to become the dominant follicle. Under the influence of the luteinizing hormone (LH) surge, the fully grown GV-stage oocyte in the dominant follicle resumes meiosis to progress through metaphase I (Fig. 31.3B) and anaphase I, to reach telophase I. Upon extrusion of the first polar body at telophase I, the oocyte completes meiosis I to become a secondary oocyte. The secondary oocyte then rapidly proceeds to metaphase II of meiosis (Fig. 31.3C) to complete the so-called process of "meiotic maturation." Completion of meiotic maturation takes approximately 36 hours. The oocyte is ovulated at metaphase II, the stage at which it awaits subsequent fertilization. The progression through meiotic maturation from prophase I to metaphase II, and then to telophase II following fertilization, is shown diagrammatically in Figure 31.4.

A normal mature haploid human oocyte has a complement of 23 chromosomes. However, meiotic errors occur resulting in a high incidence of aneuploid oocytes. Such errors typically occur during the first meiotic division and involve a variety of mis-segregations including nondysjunction of homologous chromosomes and predivision of sister chromatids.[9] Resulting oocytes either possess too few chromosome copies and are referred to as hypohaploid, or have too many copies and are termed hyperhaploid. Maternal age is the main factor associated with the occurrence of aneuploidy[10,11] which, in turn, accounts for the majority of failed conceptions (reviewed by Hassold and colleagues[12]). The root cause of such age-related aneuploidy appears to relate to abnormalities in morphology of the meiotic spindle,[13] as well as a decrease in chromosome cohesion caused by an increase in inter-kinetochore distance between sister chromatids.[14]

Regardless of chromosome number, an oocyte that has reached metaphase II (i.e., completed nuclear maturation) has not necessarily achieved cytoplasmic maturation. Although the molecular processes underlying acquisition of cytoplasmic maturity remain poorly understood, it is clear that cytoplasmic maturation is critical for the oocyte to

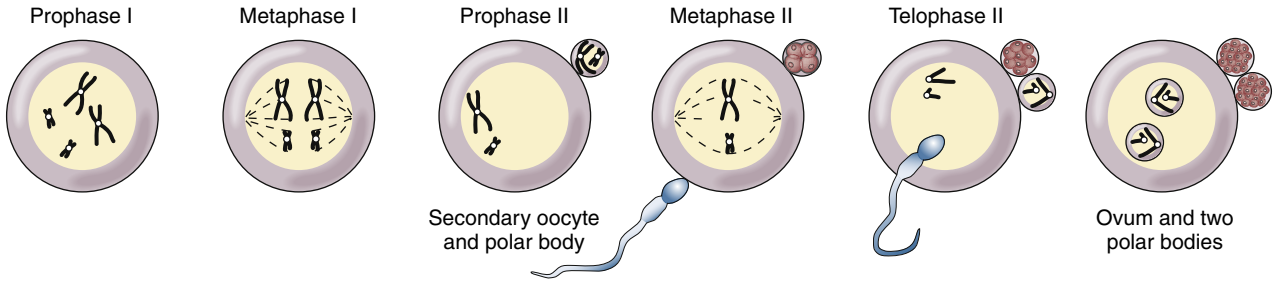


FIGURE 31.4 Stages of meiotic maturation and completion of the second meiotic division after fertilization. *(From Thompson JS, Thompson MW.* Genetics in Medicine. *Philadelphia, WB Saunders, 1986, p 23.)*

undergo "activation" at fertilization. Inadequate or absent oocyte activation leads to fertilization failure and/or abnormal syngamy and early developmental programming.[15]

The Sperm

In contrast to the egg, sperm are among the smallest cells in mammals. Sperm are highly specialized for the sole purpose of transporting DNA to an oocyte. Sperm consist of four key functional components:

1. The acrosome which contains enzymes that aid in digesting and penetrating the cumulus oophorus.
2. The nucleus which contains highly compacted DNA.
3. The midpiece, which includes the centrosome that is key in human embryogenesis, as well as mitochondria which provide the energy source for sperm.
4. The tail, which contains the axoneme and the dynein motor proteins.

To maximize transport efficiency, the sperm has no ribosomes, endoplasmic reticulum, or Golgi apparatus. However, small ribonucleic acids (RNAs) are transported within the sperm and may have a role in embryogenesis.[16]

Spermatogenesis differs significantly from oogenesis (Fig. 31.5) (see Chapter 13). In the embryo, primordial germ cells migrate to the testis and enter a state of arrest until puberty. Under the influence of testosterone and other hormones, the spermatogonia divide mitotically and generate two pools of derivative cells. The cells of one pool continue to divide mitotically and serve as the spermatogonial stem cells. The second pool of cells will enter meiosis and become primary spermatocytes (46 duplicated chromosomes in human). The primary spermatocytes proceed through the first meiotic division and then become secondary spermatocytes (22 duplicated autosomal chromosomes, plus a duplicated X chromosome or a duplicated Y chromosome). After the second meiotic division, the secondary spermatocytes become spermatids (haploid number of single chromosomes), which then differentiate into mature sperm. The process of meiotic reduction of the spermatogonia, as well as maturation of the spermatids (spermiogenesis), occurs inside the seminiferous tubule, with the precursor cells located at the outer border of the tubule and the mature sperm in the lumen of the tubule. The developing sperm cells undergo nuclear division but do not complete cytoplasmic division until near the end of sperm differentiation (see Fig. 31.5). Consequently, the developing germ cells are connected by cytoplasmic bridges in a syncytium, which allows the diploid spermatogonium to produce proteins and cellular materials for the haploid sperm.

Upon entry into the epididymis, the sperm undergo sequential steps of differentiation, largely related to the acquisition of motility, and the ability to bind to and penetrate the oocyte. Recent proteomic studies have revealed specific epididymal proteins thought to be directly related to these sperm maturation processes, and differences in the epididymal proteome have been reported in infertile men.[17,18] Thus, sperm from the caput epididymis are generally unable to undergo natural fertilization, but gain the ability to fertilize by the time they reach the cauda epididymis.

During ejaculation, the sperm are exposed to stabilizing factors from the seminal fluid that preclude the ability of the sperm to undergo the acrosome reaction. The sperm undergo a process of "capacitation" as they progress through the female reproductive tract that includes removal of cholesterol from the membranes and an influx of calcium, ultimately resulting in hyperactive motility, the ability to undergo the acrosome reaction in response to the zona pellucida proteins of the oocyte plasma membrane.[19] Sperm capacitation can be facilitated in the laboratory prior to ART or artificial insemination by sperm processing methods that remove the sperm from seminal plasma.[20] Defects of capacitation have been associated with male infertility.[21]

Sperm penetrate the cumulus cell layers surrounding the egg through their hyperactivated motility which is induced by cumulus cell progesterone secretion.[22] The sperm motility and hyaluronidase secretion allow the sperm to move through the cumulus extracellular matrix to reach the zona pellucida. On reaching the zona pellucida, the capacitated sperm then undergo the *acrosome reaction*, a process that is essential for fertilization. The acrosome is a large secretory cap on the sperm head that contains proteases and hyaluronidases. In the acrosome reaction, the outer acrosome membrane fuses with the plasma membrane of the sperm and the contents of the acrosome are emptied. In many

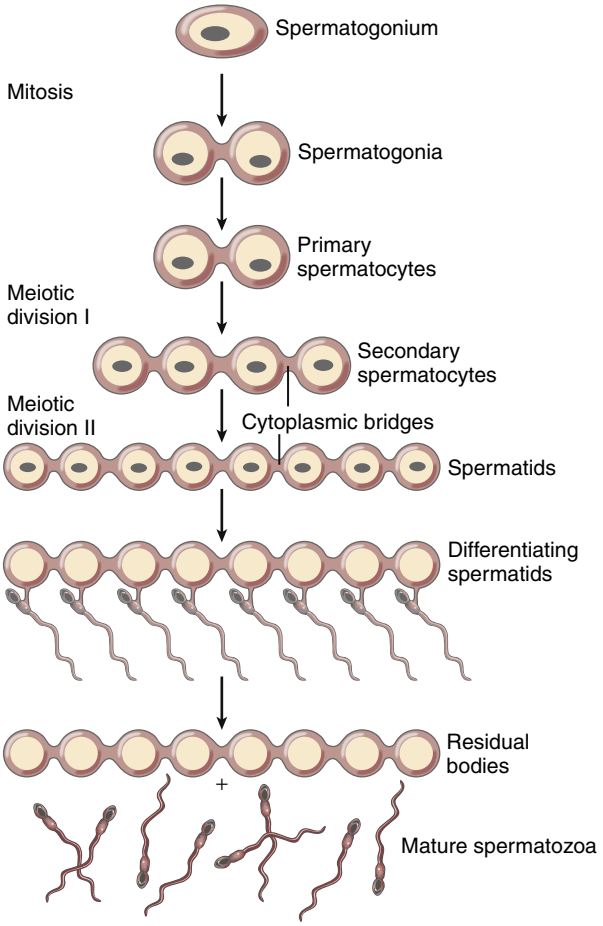


FIGURE 31.5 The progeny of a single maturing spermatogonium remain connected to each other by cytoplasmic bridges. The cytoplasmic bridges allow the spermatids to receive proteins from the parent cell apparatus without bearing the burden of protein machinery (e.g., endoplasmic reticulum). *(Redrawn from Alberts B, Bray D, Lewis J, et al.* Molecular Biology of the Cell. *New York, Garland Publishing, 1994, p W31.)*

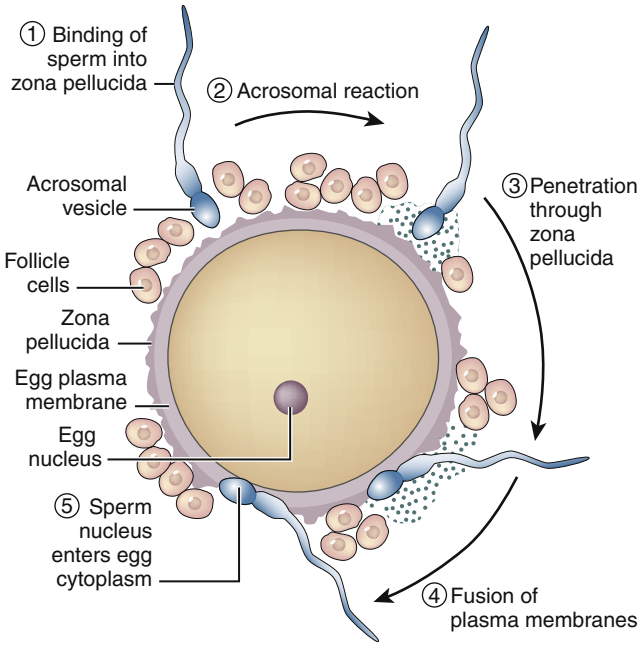


FIGURE 31.6 Key steps in the process of fertilization. *Step 1,* binding of sperm to zona pellucida involves zona protein ZP3 and a sperm protein, probably a carbohydrate-binding protein such as 1,4-galactosidase. *Step 2,* acrosome reaction. *Step 3,* penetration of sperm through zona pellucida. *Step 4,* fusion of plasma membranes of sperm and oocyte. Depolarization of oocyte membrane and secretion of cortical granules—the primary and secondary blocks to polyspermy. *Step 5,* sperm nucleus enters egg cytoplasm. *(Redrawn from Alberts B, Bray D, Lewis J, et al.* Molecular Biology of the Cell. *New York, Garland Publishing, 1994, p 1031.)*

species, the acrosome reaction is initiated by the glycoproteins of the zona pellucida[23,24] and can be accelerated by progesterone.[25-27]

Fertilization

The process of fertilization involves at least two key initial steps: interaction and penetration of the zona pellucida by the sperm, followed by fusion of the sperm and oocyte membranes (Fig. 31.6). Although the mechanisms that allow the human sperm and zona pellucida of the oocyte to interact are not fully characterized, use of antibodies and antagonists to candidate molecules has greatly advanced understanding in this area.[28] Prevailing data suggest that a dynamic array of zona pellucida receptor-sperm ligand interactions regulate sperm-zona binding.[28] In mouse, the zona pellucida contains three glycoproteins (ZP1, ZP2, and ZP3)[29] but the human zona has a fourth glycoprotein, ZP4.[30] ZP2 and ZP3 have a filament structure, and ZP1 appears to link ZP2 and ZP3 in a complex three-dimensional array, for the absence of either ZP2 or ZP3 prevents assembly of the zona matrix.[31] In the human, capacitated sperm can be induced to undergo the acrosome reaction by ZP1, ZP2, and ZP4, although downstream signaling events may be different with ZP3 compared to ZP1 or ZP4.[32] In an early study, purified ZP3 blocked the ability of sperm to bind to the oocyte zona pellucida in a dose-dependent manner, implying that ZP3 is the zona pellucida sperm receptor,[33] and data from several other studies have implied that O-linked carbohydrate moieties underly the interaction of ZP3 and the sperm receptor.[34] Recently, the nature of this carbohydrate ligand has been further identified as the sialyl-Lewis sequence, a member of the selectin family.[35] Nevertheless, the biological complexity of the sperm-egg recognition process appears to reflect participation by a series of sperm proteins,[28] and involve sperm binding to both ZP1, ZP3, and ZP4, but not ZP2, which appears to only bind previously acrosome-reacted sperm.[32]

The search for the sperm receptor to the ZPs has been difficult, and it appears that since knock-out studies of putative receptors have generally not precluded binding, there is likely a small number of proteins on the sperm surface that appear to be responsible for interaction with ZP3.[28] These proteins include fertilin,[36] galactosyltransferase, and cyritestin. βl,4-Galactosyltransferase on the sperm surface appears to be important for the ZP3-induced acrosome reaction.[37] Fertilin and cyritestin are members of the ADAM (*a d*isintegrin *a*nd *m*etalloprotease) family of proteins, which can bind to integrins but also have proteolytic activity. The sperm of mice lacking cyritestin (ADAM3) have defective binding to the zona pellucida.[38] Fertilin, a heterodimeric sperm surface protein composed of α and β subunits, was originally thought to be involved in fusion of sperm with the egg plasma membrane (the oolemma). However, mice lacking fertilin β (ADAM2) show defects in zona binding.[39] More recent studies have implicated involvement of a new candidate enzyme, mouse sperm lysozyme-like protein (mSLLP1), in sperm-oolemmal binding.[40] This protein is located in the equatorial segment of acrosome-reacted sperm in both mouse and human, and appears to bind to the entire oolemma except in that region where sperm normally do not bind, overlying the meiotic spindle.[40]

The roles of ZP3 and the galactosyltransferase and ADAM proteins in fertilization in the human are not fully characterized. Studies suggest that some metaphase II oocytes that fail to be fertilized have reduced ZP3 levels as determined by immunohistochemistry.[41] Another egg protein that is essential for successful fertilization is CD9, a member of the tetraspanin family. In mouse eggs lacking this oolemma protein, sperm-egg fusion fails to occur, resulting in infertility.[42]

After completion of the acrosome reaction, the sperm lose their affinity for ZP3,[43] and the continued attachment of the sperm to the oocyte appears to be dependent on ZP2.[44] The sperm penetrates the zona pellucida, in part, by the forward mechanical thrust provided by the flagellum, but likely is also dependent on the release of hydrolytic enzymes secreted by the acrosome, including the acrosomal 26S proteasome, that causes disruption of the continuity of the zona pellucida.[45,46]

Sperm binding and fusion to the oolemma is believed to be a complex process, possibly involving more than one receptor to sperm acrosomal proteins.[47,48] Once the sperm fuses with the oolemma, a depolarization of the oolemma occurs that acts as the primary block to polyspermy. Shortly thereafter, the inositol phospholipid cell-signaling pathway is activated and submembrane cortical granules release their contents.[49] The contents of the cortical granules change the glycoprotein coat of the zona pellucida, preventing sperm binding by hydrolyzing the oligosaccharides of ZP3 and by the proteolytic cleavage of ZP2. In the mouse at least, this proteolytic cleavage is achieved by exocytosis of the cortical

granule metalloendoprotease, ovastacin.[50] This process creates a secondary block to polyspermy.

After fusion of the sperm with the oolemma, the sperm phospholipase C (PLC) isoform PLC-zeta mobilizes the Ca^{2+} signal that induces egg activation and embryo development, with a tight association reported between the type of calcium oscillations and embryo development.[51,52] Accordingly, abnormalities in PLC-zeta negatively affect fertilization rates and possibly embryo development, confirming the key role of this factor in triggering oocyte activation. This finding suggests that certain types of infertility could be caused by failure of the sperm cell to properly activate the oocyte due to a defective PLC-zeta factor.[53-55]

Recent studies have begun to demonstrate the important role that sperm may play in post-fertilization events. Once in the oocyte, sperm chromatin and DNA integrity are necessary to ensure normal embryo development.[56,57] It is now clear that DNA damage in spermatozoa, in the form of single and double-stranded DNA breaks, has a negative influence on blastocyst development and ICSI outcome.[58] However, there is a strong need to standardize the specific tests used to quantify the extent of DNA damage.[56] Similarly, centrosome integrity is critical for successful fertilization and embryo development, and data indicate that the replacement of defective centrosomes, which are responsible for specific forms of male infertility, with functional donor sperm centrosomes can restore normal functionality.[59,60]

A great deal of research has recently emerged providing insight regarding the sperm epigenome and its potential role in early embryonic developmental programming.[61] Varying epigenetic modifications of the sperm genome occur, including histone retention and modification, DNA methylation and protamine incorporation into the chromatin.[57] Sperm from some infertile patients show different patterns of epigenetic marking compared with fertile men, particularly at certain imprinted and developmental loci.[62-64]

The sperm cell also contains various forms of RNA, (e.g., mRNA, miRNA, siRNA)[57,65,66] as well as more than 2000 proteins with unknown roles. Data are emerging which indicate that sperm-derived micro RNAs may be involved in normal embryogenesis.[16]

The Early Conceptus and Implantation

Sperm penetration into the oocyte triggers the oocyte to complete meiosis by rapid progression from metaphase II to telophase II. Telophase II is characterized by elimination of one of the sister chromatids of each chromosome through extrusion of the second polar body. The retained set of oocyte chromatids undergoes decondensation, followed by decondensation of the set of sperm chromatids, each to form a pronucleus. The zygote is therefore characterized by the presence of two pronuclei and two polar bodies (Fig. 31.7A). Following migration to the center of the oocyte, and then apposition of the two pronuclei, pronuclear membrane breakdown occurs, the maternal and paternal chromosomes intermingle at syngamy, and the diploid zygote is formed. Shortly thereafter, the chromosomes undergo condensation and the paired homologues (one maternal and one paternal) align on the metaphase plate of the first mitotic spindle, in preparation for the first cleavage division. The embryo then progresses through several cleavage divisions (Fig. 31.7B through D), giving rise to an embryo comprising multiple cells or blastomeres.

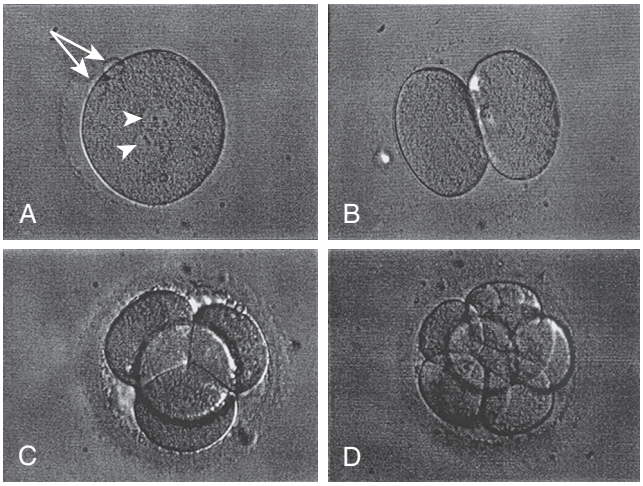


FIGURE 31.7 Development of human embryos during the first 72 hours after insemination. **A,** A zygote at 18 hours, showing two polar bodies *(arrows)* and two pronuclei in apposition *(arrowheads)* with several distinct nucleoli polarized toward the juxtaposed nuclear membranes. **B,** A two-cell embryo at 28 hours. **C,** A four-cell embryo at 44 hours. **D,** An eight-cell embryo at 68 hours.

In human embryos, the blastomeres are thought to be totipotent up to around the eight-cell stage[67] and removal of one, or even two, blastomeres from eight-cell embryos does not necessarily disrupt embryo development. However, a recent elegant study in which one of a pair of transferred embryos was biopsied and then DNA finger-printed showed that the implantation rate of the biopsied embryos was significantly lower than that of the non-biopsied embryo[68] (see Chapter 32). Beyond the eight-cell stage, the cells clearly begin to undergo differentiation. Compaction ensues to form the morula stage as blastomeres adhere because of secretion of cell adhesion molecules such as E-cadherin.[69] Tight junctions form between blastomere membranes, and pockets of fluid begin to accumulate among the blastomeres (Fig. 31.8A). Concomitant with onset of coalescence of these small fluid collections to form a fluid-filled cavity (the blastocoele), the first signs of overt cellular differentiation occur; the surface cells begin to undergo a change in shape from spherical to squamous epithelial-like to form trophectoderm cells, while a small cluster of inner cells retain their spherical appearance, ultimately to form the inner cell mass (Fig. 31.8B). The trophectoderm cells form the trophoblast, which gives rise to the extra-embryonic structures, such as the placenta, and the inner cell mass gives rise to the embryo. Following enlargement of the blastocoelic cavity, which causes expansion of the blastocyst (Fig. 31.8C and D), the blastocyst ultimately escapes from the zona pellucida in preparation for implantation (Fig. 31.8E and F).

Strictly speaking, the embryonic stage consists of the period from the development of the primitive streak through the initial steps in the development of all the major organs. As defined, in the human, the embryonic stage begins approximately 14 days after fertilization. In assisted reproduction, however, most authorities use the term "embryo" to describe the conceptus from the first cleavage through

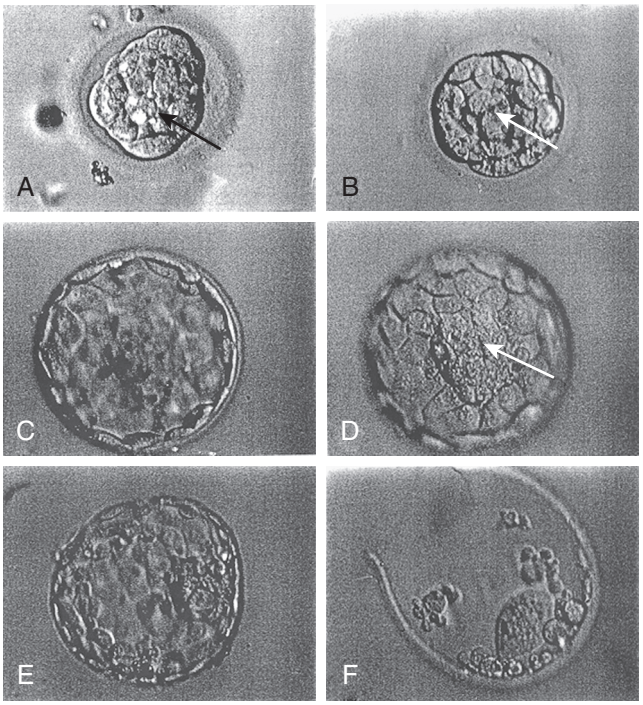


FIGURE 31.8 Development of human embryos between 90 and 120 hours after insemination. **A,** A morula transitioning to a very early blastocyst at 92 hours; note the characteristically compacted cells of a morula, yet the accumulation of small pockets of fluid accumulation *(arrow)*, and the first signs of cellular differentiation as reflected by the epithelial cells forming at the periphery that will develop into the trophectoderm cells. **B,** An early blastocyst at 100 hours; note the increasing size of fluid pockets, further differentiation of the trophectoderm cells, and the appearance of a small cluster of spherical cells that will form the inner cell mass *(arrow)*. **C** and **D,** A fully expanded blastocyst at 116 hours in two focal planes to show the trophectoderm layer surrounding the blastocoelic cavity **(C)** and the innercell mass **(D). E,** A hatched blastocyst at 118 hours and, **F,** its corresponding zona pellucida containing discarded cytoplasmic fragments.

the initial stages of organ development. This convention will be followed in the remainder of this chapter.

The molecular events underlying the process of implantation in humans are not completely understood (see Chapter 10). The human embryo enters the uterus approximately 4 days after fertilization, at the *morula stage*. Within 5 to 6 days of fertilization, the embryo reaches the *blastocyst stage* and begins the hatching process, thereby allowing the outer covering of syncytial trophoblast cells to interact with the endometrial surface. The trophectoderm nearest the inner cell mass, the polar trophoblast, plays an important role in the interaction of the blastocyst and the endometrium.[70] The interaction of the blastocyst with the endometrium involves phases of apposition, stable adhesion, and invasion. The blastocyst can attach to the endometrium only during a critical window of implantation corresponding to menstrual cycle days 19 to 24.[71] This "implantation window" is characterized by the formation of pinopodes which are fully developed for only 1 or 2 days. Although the molecules that control the attachment of the blastocyst to the uterine epithelium are not well characterized, recent transcriptomic analyses[72] have shown that several growth factors, cytokines, integrins and adhesion molecules are expressed in the trophectoderm and endometrium at the time of implantation. Differential secretion of some chemokines and growth factors attract the blastocyst to the pinopodes, while the repellent activity of the glycoprotein, MUC-1, appears to play an important role in repulsion of the blastocyst away from less desirable implantation areas.[73] Moreover, gene and protein expression studies have revealed that endometrial glands and stroma have distinct mRNA signatures that depend on the day of the cycle.[74] Interestingly, COS in general leads to disruptions in the transcriptional activity of genes involved in endometrial receptivity[75,76] and these distributions may be altered by different COS protocols.[76] These alterations in endometrial receptivity may partly explain the reduced birth weight in neonates conceived following fresh embryo transfer (i.e., under the influence of COS) versus that of neonates conceived following transfer of thawed embryos (i.e., into a "prepped" uterus).[77]

After attachment of the blastocyst to the uterine epithelium, the syncytiotrophoblast invades the endometrium and, by 12 days after fertilization, the conceptus is completely embedded in the endometrial stroma. Once this contact is established, human chorionic gonadotropin (hCG), secreted by the syncytiotrophoblast, can be detected in the maternal circulation.[78] Invasion of the syncytiotrophoblast then proceeds, ultimately to form the chorionic villi and establishment of a hemochorial placentation.

In Vitro *Fertilization: Clinical and Laboratory Aspects*

Pre-IVF Evaluation

As detailed in Chapters 23 and 24, testing to determine the etiology of infertility will help predict the likelihood of healthy pregnancy and delivery with various treatments, including IVF. Prior to IVF, the basic evaluation must include appropriate genetic testing, ovarian reserve testing, a uterine cavity evaluation, and semen analysis. As detailed below, the etiology of infertility has implications as to the prognosis for pregnancy and live birth with IVF.

Indications for IVF

In current clinical practice, the standard indications for IVF are tubal factor infertility, endometriosis, mild to moderate cases of male factor infertility, and idiopathic or unexplained infertility. Decreased ovarian reserve (i.e., depletion of the ovarian follicular pool), however, is seen with increasing frequency as women delay childbearing, and as women exposed to chemotherapy and other cancer treatments survive their disease and attempt to conceive. The most recent Center for Disease Control (CDC) detailed national report for the United States from 2009[79] shows that among couples who underwent ART using autologous oocytes, only 7.7% had tubal factor, with 4.2% having endometriosis, 18.8% having male factor, 13.5% unexplained infertility, 11.5% decreased ovarian reserve, 6.8% ovulatory dysfunction, 1.4% uterine factor, 7.7% other causes, and multiple female factors were diagnosed in 10.6%. Indeed, as the technology has improved, the application of IVF has broadened. IVF is now recommended for essentially all infertility conditions that

have not been successfully treated by other modalities, as first line treatment if this is the patient preference, or if clinical presentation indicates that it is the most appropriate therapy. For example, IVF has been advocated as a potential first line treatment of polycystic ovary syndrome (PCOS) in order to avoid ovarian hyperstimulation and multiple gestation. However, IVF is less successful if the female partner has diminished ovarian reserve. In this situation, donor egg has been demonstrated to be highly effective. Likewise, IVF is not likely to be successful in cases of severe male factor infertility; in this situation, ICSI is very effective (see Chapters 24 and 32). A good prognosis patient who would have a high risk pregnancy should she conceive a multiple pregnancy may be best treated with IVF and transfer of a single embryo, rather than with other modalities in which the multiple pregnancy rate is higher, and generally poorly predictable.

Tubal Factor Infertility

Surgical treatment and IVF are both possible therapeutic options for distal tubal occlusion, although no prospective trials have been reported comparing their relative efficacies. It is clear that IVF provides superior delivery rates to surgery. However, surgery is reasonable in women younger than 35 years old, with no other causes of infertility and mild tubal disease, as pregnancy rates in other patient populations are poor.[80] It is clear that the presence of hydrosalpinges (defined as fluid filled tubes on ultrasound, not merely occluded fallopian tubes) is detrimental to IVF outcomes. There are several meta-analyses of IVF outcomes in the setting of hydrosalpinges. A 2010 Cochrane review indicates that unilateral or bilateral removal of hydrosalpinges, or separation of the hydrosalpinges from the uterus result in higher delivery rates in IVF.[81] There is concern, however, that salpingectomy rather than division of the fallopian tubes and separating them from the uterus can result in elevated follicle stimulating hormone (FSH) levels, and reduced ovarian response during ovarian stimulation for IVF. [82] The Essure birth control device which occludes fallopian tubes proximally via hysteroscopy has been employed in a few small reports, with births reported. One study treated 20 women with the Essure device bilaterally or unilaterally (based on hydrosalpinx location).[83] There were 12 live births, with one complicated by premature rupture of membranes (PROM) and the other by placenta previa. In practice, Essure devices have beeen seen protruding into the endometrial cavity. Data are insufficient to recommend the Essure rather than laparoscopic salpingectomy or separation as first line treatment for hydrosalpinges. Given this, women who have documented tubal patency following tubal surgery, and who are younger than 35 years old and fail to conceive after 12 months, should undergo IVF, whereas those who are ≥35 years old and who fail to conceive after 6 months should proceed to IVF.

Endometriosis

For women presenting with infertility and early-stage endometriosis, IVF has not been definitively documented by a randomized clinical trial to be superior to other available treatments, such as expectant management, human menopausal gonadotropin (hMG) with intrauterine insemination (IUI), or surgical treatment (see Chapter 26).[84] However,

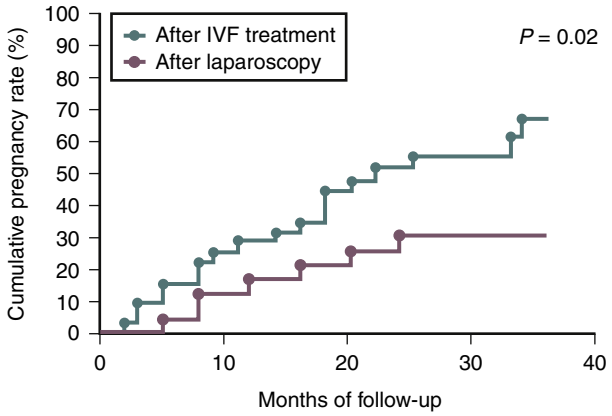


FIGURE 31.9 Cumulative conception rate curves during 36 months for women 32 years of age or older with infertility and endometriosis. *Top curve*, Cumulative conception rate after *in vitro* fertilization (IVF) treatment. *Bottom curve*, Cumulative conception rate with expectant management after laparoscopy. *(From Kodama H, Fukuda J. Karube H, et al. Benefit of in vitro fertilization treatment for endometriosis-associated infertility.* Fertil Steril *66:974-979, 1996, with permission of the American Society for Reproductive Medicine.)*

nonrandomized studies suggest that IVF treatment results in a higher pregnancy rate per cycle than conception attempts after surgical treatment, hMG with IUI, clomiphene treatment with IUI, or expectant management. For example, in one retrospective study of infertile women with advanced endometriosis, surgical treatment was associated with a cumulative pregnancy rate of 24% at 9 months after surgery. In a similar group of infertile women with advanced endometriosis, two cycles of IVF treatment were associated with a 70% pregnancy rate.[85] Another study of 313 women with endometriosis and infertility looked at crude pregnancy rates and estimated cumulative fecundity. The fecundity after one cycle of IVF was higher than that of 6 cycles of controlled ovarian hyperstimulation and IUI. After stratification for stage of disease, the benefit of IVF was more pronounced, and women with stage IV endometriosis and older than age 38 were much more likely to conceive from IVF than from COH and IUI treatment. Prior failure to conceive did not impact the likelihood of pregnancy from IVF.[86] It is controversial as to whether endometriosis is associated with lower IVF pregnancy rates than other infertility diagnoses. A recent cohort study from the Society of Assisted Reproductive Technologies database found that it is not.[87]

For infertile couples, the older the female partner, the more important is timely treatment with IVF. For example, Kodama and associates[88] demonstrated that in women 32 years of age or older with endometriosis, treatment with IVF resulted in a significantly higher pregnancy rate than that observed in the control group (Fig. 31.9). In women 32 years of age or older, the cumulative pregnancy rate was 59% in those women treated with IVF and 29% in women treated with expectant management. In contrast, in women under the age of 32, the cumulative pregnancy rate during the 3 years of the study was 64% in the women treated with IVF and 53% in the control group. Because IVF is often more expensive per cycle than other treatments (such as hMG and IUI), it is often reserved for couples who have failed to conceive after these other forms of treatment (see Chapter 30).

Retrospective analyses from many IVF centers indicate that IVF treatment of infertility associated with endometriosis is associated with a pregnancy rate similar to that observed with IVF treatment of tubal factor infertility. Olivennes and colleagues[89] reported that IVF treatment resulted in a live birth rate per cycle of 31% for women with endometriosis and 32% for women with tubal factor infertility. When the results were analyzed by surgical stage of the endometriosis (revised American Society for Reproductive Medicine classification),[90] the live birth rate per cycle was stage I, 27%; stage II, 31%; stage III, 36%; and stage IV, 33%. Another study of 31 women with stage I and II endometriosis compared to 67 women with stage III and IV endometriosis or 87 women with tubal disease were compared. The cumulative live birth rates after up to 4 cycles of IVF and ICSI were 40.3% for stage III and IV, 55.8% for stage I and II and 43.7% in tubal factor controls.[91] All stages combined, CDC data from 2009 indicate that the per cycle live birth rate for women with endometriosis as the primary infertility diagnosis was 35.3% compared to 33.7% for unexplained infertility and 32.2% for tubal factor.[92] However, some women with advanced endometriosis (stage III and IV) have undergone oophorectomy plus a contralateral ovarian cystectomy or bilateral ovarian cystectomies (for endometriomas); these women appear to have markedly reduced success with IVF due to decreased ovarian reserve from premature depletion of the oocyte pool.[93-97]

Patients often ask whether surgical treatment of active endometriosis prior to IVF improves the likelihood of pregnancy. There are no randomized trials on this issue. Retrospective studies are not adequately controlled, although there are retrospective data. One study of women with stage I or II endometriosis found that the implantation and live birth rates per oocyte retrieval were higher in 399 women who underwent laparoscopic treatment of visible endometriotic lesions compared with 262 women who underwent diagnostic laparoscopy (30.9% versus 23.9% [$P = 0.02$] and 27.7% versus 20.6% [$P = 0.004$] respectively).[98]

Male Factor

Male factor infertility is a broad category that ranges from minimally abnormal semen parameters to total sperm counts of less than 5 million per ejaculate. In general, men with severe semen abnormalities are probably best treated with ICSI (see Chapters 24 and 32). Very severe decreases in the number of motile sperm per ejaculate (less than 1.5 million) and decreases in the number of sperm with normal morphology are associated with poor pregnancy rates in standard IVF.[99,100] Typically, ICSI is indicated as front line treatment for men with fewer than 10 million sperm per mL in the whole ejaculate or less than 5 million/mL after processing. Prior to undergoing ICSI, men with severe male factor infertility (less then 5 million sperm per mL in unwashed ejaculate) should have a karyotype and Y chromosome microdeletion assessment because the incidence of karyotypic and genetic abnormalities is high in this group.[101] In a study of 1935 men with severe male factor infertility, 1214 with nonobstructive azospermia and 721 with severe oligoasthenospermia, the incidence of karyotypic abnormalities was 16.4% and 5.8%, respectively. The incidence of Y chromosome microdeletions was 9.5% and 1.9% respectively.[102] The incidence of chromosomal abnormalities increases with decreasing sperm count.[103] Nevertheless, such men are good candidates for IVF. ICSI in cases of severe male factor infertility provides fertilization and pregnancy rates comparable to those seen in standard IVF (see Chapter 32).

Idiopathic Infertility

For 10% to 17% of infertile couples, thorough evaluation reveals no identifiable cause of infertility. Data reported to the Society for Assisted Reproductive Technology (SART) in 2010 show that 13% of IVF and ICSI cycles initiated were for a primary infertility diagnosis of "unknown factor" infertility.[104] "Unexplained," or idiopathic, infertility is a term applied to those couples who have completed an infertility evaluation that includes laparoscopy. For the purposes of this chapter, however, idiopathic infertility refers to couples who have completed the basic steps of the infertility evaluation (semen analysis, documentation of ovulation, and tubal patency), but who may not have had laparoscopy, as laparoscopy is no longer part of the standard initial infertility evaluation in the United States.

Many couples with idiopathic infertility achieve successful pregnancies after a stepwise treatment approach (see Chapter 30), which may include empirical clomiphene treatment followed by empirical gonadotropin ovarian stimulation with IUI treatment.[105] One randomized trial has been performed seeking to answer the question of the most cost-effective path towards IVF treatment. In this trial, 247 couples with the female partner ages 21 to 39 year old and having unexplained infertility were randomized to undergo 3 cycles of clomiphene citrate and IUI therapy, and if not pregnant either 3 cycles of gonadotropin IUI followed by up to 6 cycles of IVF, or IVF directly following clomiphene IUI treatment.[106] Delivery rates per cycle of treatment were 7.6% for cc IUI, 9.8% for gonadotropin IUI, and 30.7% for IVF. Couples who underwent IVF after clomiphene IUI were pregnant on average 3 months earlier (8 versus 11 months). In addition, the cost per delivery was $9800 lower for the fast treatment arm.[106] It is important to note that if other treatments are attempted and fail prior to IVF, women are older by the time they undergo IVF, resulting in a higher likelihood that their ovarian reserve has diminished. This, in turn, may result in lower chances of conception with IVF due to older age at the time that IVF is initiated.

Polycystic Ovary Syndrome and Anovulation

Until recently, infertile women with polycystic ovary syndrome (PCOS) in whom both clomiphene and gonadotropin ovulation induction failed had few remaining treatment options except surgical procedures to reduce thecal and stromal androgen production. However, evidence has accumulated that IVF is often effective for such patients. A meta-analysis comparing IVF outcomes in PCO patients, diagnosed by Rotterdam criteria, to non-PCO patients found higher oocyte retrieval cancellation rates (presumably due to risk of ovarian hyperstimulation, higher oocyte numbers, lower fertilization rates), but equivalent pregnancy rates per embryo transfer. As the incidence of oocyte immaturity is high in PCO patients, this is not a suprising finding, and is reassuring in that pregnancy rates are acceptable, despite the ovarian mileu of PCO.[107]

Polycystic ovarian syndrome patients are at high risk of ovarian hyperstimulation syndrome. This risk may be mitigated by use of GnRH antagonists rather than agonists, and avoidance of hCG ovulatory triggering, with substitution of a GnRH agonist (see later in chapter). A prospective, randomized placebo-controlled trial of 120 PCO patients treated with metformin 500 mg three times per day during IVF treatment with a long GnRH agonist protocol until menses or a positive pregnancy test revealed that the relative risk of ovarian hyperstimulation was 0.28 (95% CI 0.11 to 0.67) with metformin use. The metformin arm also used somewhat more gonadotropins (1350 IU [range, 950 to 1800] versus 1275 IU [range 900 to 1750], $P = 0.018$), fewer nonperiovulatory follicles on the day of hCG administration (4.3 [range, 0 to 6] versus 5.5 [range, 2 to 9], $P = .034$) and lower estradiol levels at hCG (1951 pg/mL [range, 342 to 4021], versus 2346 [range, 709 to 4123], $P = 0.29$). Implantation rates (41% versus 31%) and live birth rates per cycle (29% versus 27%) were not different.[108,109]

For women with hypothalamic amenorrhea, as with PCO, treatment with IVF avoids a high risk of multiple gestation that is in turn associated with multiple follicular development with gonadotropins (see Chapter 30).

Uterine Factor

Fibroids

The uterus may be considered as abnormal and a contributor to infertility due to many factors including aquired defects such as fibroids, polyps, intrauterine adhesions, or congenital anomalies. Many investigators have reported that submucosal leiomyomas are associated with decreased pregnancy rates with IVF (see Chapter 27). In addition, a number of studies suggest that hysteroscopic myomectomy of submucosal myomas improves pregnancy rate with IVF.[110-112]

The effect of intramural myomas on IVF outcome is less certain. Some investigators have found that intramural myomas are associated with decreased pregnancy rates in IVF. In one report, 112 women with intramural myomas (the largest of which had a mean diameter of 2.3 cm) and 322 women without myomas undergoing IVF were prospectively studied.[113] The ongoing pregnancy rate was 15.1% in the women with myomas and 28.3% in the women without myomas ($P < 0.003$). Logistic regression demonstrated that intramural myomas were associated with a reduced OR for pregnancy (OR 0.46, 95% CI 0.24-0.88, $P < 0.02$) after controlling for age of the female partner and number of embryos available for transfer. Other investigators have reported that intramural myomas up to 7 cm in diameter that do not distort the uterine cavity do not substantially influence IVF outcome. The impact of myomas on IVF was studied in 141 patients with myomas and 406 women without myomas. The OR for pregnancy was 0.73 in the myoma group (95% CI 0.49-1.19, $P = 0.21$) after controlling for the age of the female partner.[114] Surrey and colleagues examined consecutive IVF cycles in 399 women undergoing IVF with and without leiomyomas. They found that the live birth rate was not affected by the presence of intramural leiomyomas provided that the endometrial cavity was hysteroscopically normal. They did not recommend prophylactic surgical intervention for intramural fibroids.[115] In contrast, a meta-analysis of 19 observational studies in 6087 IVF cycles supports the contention that intramural fibroids which do not distort the cavity are associated with decreased live birth rates (RR 0.79, CI 0.70 to 0.88, $P < 0.0001$).[116] If there is, indeed, an impact of intramural fibroids on IVF live birth rates, the question remains as to whether myomectomy returns the pregnancy rate to expected levels, or whether the biology of the uterus is different and somehow more hostile in women who develop intramural fibroids. Additional large-scale studies are needed to determine if intramural myomas reduce IVF success rates, and if so, whether myomectomy is of benefit.

Adenomyosis

Adenomyosis is typically diagnosed pathologically, at the time of hysterectomy. However, a study of first gonadotropin releasing hormone (GnRH) antagonist IVF cycles in women younger than age 39 with good embryo quality comparing 38 women with ultrasound diagnosed adenomyosis to 175 without, found that the clinical pregnancy rate was 23.6% compared to 44.6% in controls ($P = 0.017$). After adjustment for maternal age and duration of infertility the difference was significant, OR 0.417, CI 0.175 to 0.989, $P = 0.047$.[117] Another small study of 19 women with adenomyosis and 256 controls, also found lower ongoing pregnancy and higher miscarriage rates.[118]

Other Factors

Endometrial polyps may also decrease live birth rates with IVF.[119] However, studies are inconsistent. If polyps do play a role in miscarriage or lowering pregnancy rates it appears to be in large polyps 2 cm or more in size.[120] This may be due to the fact that polyps less than 1 cm have been found to regress.[121] Cervical stenosis can impair effective embryo transfer and thus lower pregnancy rates with IVF.[122] Placing a Malecot catheter after hysteroscopic evaluation in preparation for IVF appears to improve the efficacy of embryo transfer in women with cervical stenosis.[123] Other methods including cervical dilation and resection to improve ease of transfer have also been reported.[124]

Congenital Defects

Müllerian anomalies appear to be associated with a reduced pregnancy rate in IVF. In a study of 37 women with müllerian anomalies undergoing IVF, the live birth rate per initiated cycle was 8%, compared with 25% in a control group without müllerian anomalies. Following IVF, women with *in utero* exposure to diethylstilbestrol and uterine anomalies have very low pregnancy rates compared with those without anomalies.[125]

Decreased Ovarian Reserve

Decreased ovarian reserve is increasingly acknowledged as a significant infertility factor, and is likely second only to age as a predictor of IVF delivery rates. It has become increasingly clear that IVF is most successful for infertile couples when the female partner has an adequate ovarian follicular pool. As a woman ages, there is a decline in her ovarian follicular pool.[126] Some reports suggest that the decline in the oocyte pool accelerates after age 37. In addition to the chronological age of the female partner, the biological age of the ovary (a measure of the remaining ovarian

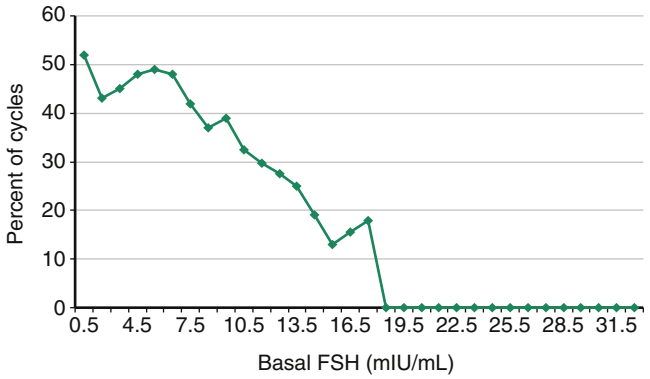


FIGURE 31.10 Delivery rates per initiated cycle after *in vitro* fertilization (IVF) treatment as a function of the basal follicle-stimulating hormone (FSH) concentration of the female partner. Data based on 18,018 couples. *(From Scott RT, Elkind-Hirsch KE, Styne-Gross A, Miller KA, Fratarelli JL. The predictive value for in vitro fertility delivery rates is greatly impacted by the method used to select the threshold between normal and elevated basal follicle stimulating hormone.* Fertil Steril *89:868-878, 2008, with permission of the American Society for Reproductive Medicine.)*

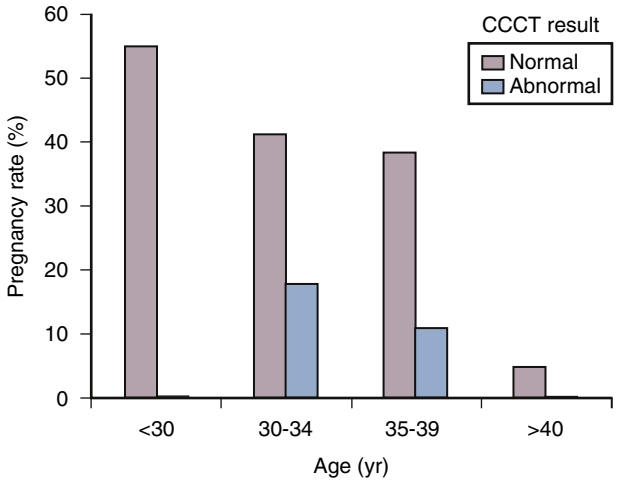


FIGURE 31.11 Clinical pregnancy rates as a function of both age and the response to a clomiphene citrate challenge test (CCCT) in a general infertility population observed prospectively. The illustration is based on data from 236 couples. In women with an abnormal CCCT result (suggestive of a depleted oocyte pool), pregnancy rates are low regardless of the age of the female partner. In women older than 40 years of age, pregnancy rates are low with *in vitro* fertilization even if the CCCT result is normal. *(From Scott RT, Hofmann CE. Prognostic assessment of ovarian reserve.* Fertil Steril *63:1-11, 1995, with permission of the American Society for Reproductive Medicine.)*

follicular pool) as determined by follicular phase cycle day 3 follicle-stimulating hormone (FSH) concentration[127] is also a strong predictor of IVF pregnancy rates. However, the method used to establish the threshold to separate "normal" from "abnormal" FSH levels has great impact on the use of day 3 FSH as a screening criterion.[128]

A large study of 18,019 IVF cycles of older patients (36.2 ± 4.8, mean ± SD) investigated the relationship between cycle day 3 FSH concentration and IVF delivery rates. FSH levels measured in a multitude of FSH assays were included, as this reflects actual clinical practice. A threshold between normal and abnormal FSH levels was then assessed. The study showed that no live births occurred in this older population with basal FSH levels of greater than 18.0 mIU/mL and that between 1 to 7 mIU/mL live birth rates were relatively constant, but underwent a decline between 8 to 11 mIU/mL which was more precipitious beyond 12 mIU/mL (Fig. 31.10).[128] Similar trends were observed for each age group. When the interaction of age and the results of a clomiphene challenge test were examined, in women with a normal clomiphene citrate challenge test response (suggesting an adequate follicular pool), the age of the female partner remained an important prognostic variable.[129] Similar results have been observed in a general infertility population (Fig. 31.11).

The ovarian antral follicle count (the number of ovarian antral follicles between 2 and 10 mm in maximal diameter measured on ultrasound) appears to be a better predictor of ovarian reserve than is FSH. Women with fewer than 7 total antral follicles have diminished ovarian reserve and have generally poorer outcomes in IVF.[130] In addition, ovarian volume, which reflects antral follicle count, also appears to correlate with IVF success, but is not useful in the setting of ovarian cysts. Women with small ovarian volumes also suffer lower pregnancy rates with ART.[131,132]

Müllerian inhibiting substance (MIS), also known as anti-müllerian hormone (AMH), is a glycoprotein growth factor synthesized in granulosa cells. A number of studies have demonstrated that AMH is reduced with increasing age and, as such, is a useful marker of ovarian reserve. Seifer and colleagues were the first to demonstrate that AMH is significantly reduced in women who responded poorly to ovarian stimulation in IVF cycles.[133] Pregnancy and delivery rates are also lower in ART in women with low serum levels of AMH.[134] AMH has the advantages of minimal cycle-to-cycle variation,[135] its levels do not change significantly across the menstrual cycle, and it is accurate in women on oral contraceptives.[136]

Inhibin B, a hormone secreted by the granulosa cells, has also been studied as a marker of ovarian reserve. Inhibin B directly inhibits pituitary secretion of FSH in a manner similar to that of estradiol, and thus was thought to be a marker of ovarian health.[137,138] However, the utility of inhibin B as a marker of ovarian reserve is limited by the lack of a uniform commercial assay.

Meta-analyses confirm that although ovarian reserve tests are helpful in predicting response to ovulation induction, and selecting ovarian stimulation protocols, they are not accurate independent predictors of live birth rates.[139,140] Age remains a major factor. Young women with decreased ovarian reserve have pregnancy and delivery rates substantially higher than older women with equally low ovarian reserve.[141] Yanushpolsky and colleagues found that women under age 40 undergoing clomiphene citrate challenge testing who had FSH levels greater than 10 mIU/mL on either day 3 or day 10, had approximately half the pregnancy and live birth rates of women with FSH levels less than 10 mIU/mL.

As shown in Figure 31.12, in assisted reproduction cycles the proportion of pregnancies that end in spontaneous abortion increases with age of the female partner due to increases in oocyte aneuploidy. Many authorities advise that women, particularly over age 40, with a depleted oocyte

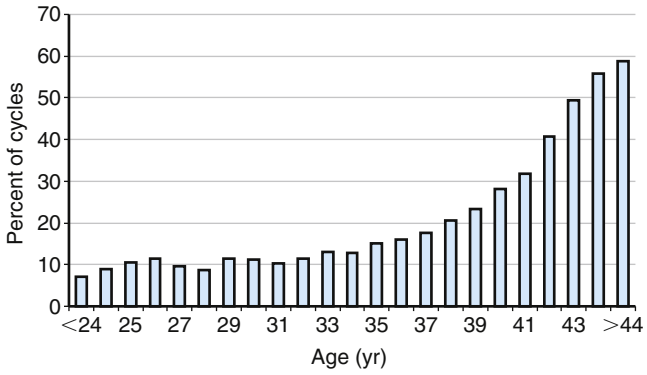


FIGURE 31.12 Percentage of ART (assisted reproductive technology) cycles using fresh nondonor eggs or embryos that resulted in spontaneous abortion, by age of woman in 2009. *(From CDC Assisted Reproductive Technology [ART] Report 2009. Available at www.cdc.gov/art/ART2005. Accessed Sept. 30, 2012.)*

pool and who have a poor response to gonadotropin stimulation should consider either treatment with donor oocytes or adoption.

Multiple Infertility Factors

Many couples have multiple factors contributing to their low fecundability. In general, the greater the number of infertility factors, the lower the success with IVF treatment. For example, in one study of the efficacy of IVF on couples for whom the only identifiable infertility factor was endometriosis, the live birth rate per cycle was 31%. In women with endometriosis and a male partner with an abnormal semen analysis, the live birth rate per cycle was 16%. For women with both endometriosis and tubal disease, the live birth rate per cycle was 8%.[89] Similarly, in a study of the effectiveness of IVF on women presenting with tubal disease with or without other infertility diagnoses, the live birth rate per transfer was 30% in women with tubal disease alone, 25% in women when tubal factor occurred in combination with male factor, and 20% and 19%, respectively, in cases of previous diethylstilbestrol exposure or immunological infertility.[142] In 2009, U.S. data reported to the CDC revealed that 10.6% of cycles involved more than one female infertility diagnosis, and 17.8% involved both male and female infertility factors[92]; delivery rates per stimulated cycle were 23.4% and 28.6%, respectively. In comparison, the delivery rate for cycles with unexplained infertility, endometriosis and male factor were 33.7%, 35.3%, and 37.6%, respectively, whereas those for poorer prognosis categories such as decreased ovarian reserve (14.5%) and uterine factor (25.4%) were more similar to cycles with multiple infertility diagnoses.

A recent study of data reported to SART found that per stimulation attempt delivery rates in women undergoing IVF/ICSI for uterine factor or decreased ovarian reserve were lower than all other causes of infertility.[87]

Preimplantation Genetic Testing (PGT)

Couples without infertility, but who are carriers of single gene defects or chromosomal translocations, are candidates for IVF with PGT so as to reduce the likelihood of transmission of disease or to avoid recurrent pregnancy loss. Good ovarian reserve and creation of fairly large numbers of good quality embryos are critical for optimizing the likelihood of pregnancy and delivery with PGT, as at least 25% of embryos are likely to be unsuitable for transfer (e.g., in cases of recessive gene defects). Chromosomal screening in order to avoid aneuploid pregnancy loss or miscarriage is currently under development; it is unclear if such screening will increase the likelihood of delivery per IVF stimulation initiated. PGT is detailed in Chapter 32.

Overview of IVF Statistics

As IVF has evolved as a scientific field, the reporting of treatment has continued to change and improve. Currently, most studies report the live delivery rate per IVF cycle initiated (i.e., per cycle in which gonadotropin therapy was begun). This statistic is helpful for patients deciding if the risk/benefit ratio is appropriate for them to undertake IVF. However, a more accurate representation may be the cumulative live birth rate per stimulation, which would include the number of deliveries for transfers of fresh and frozen embryos resulting from a single cycle of controlled ovarian stimulation.[143] This is a more difficult outcome to track reliably as it requires tracking the embryos used during cryopreserved embryo transfers back to the cycles in which they were created.

For the calculation of the delivery rate, multiple gestations are counted as one delivery, not two deliveries in the case of twins or three deliveries in the case of triplets. Unfortunately, many investigators use other numerators (clinical pregnancy, ongoing pregnancy) and denominators (pregnancy rate per oocyte retrieval or embryo transfer) when calculating IVF success rates. Many critics believe that these rates are overly optimistic and are not a realistic estimate of the success of IVF, as they do not include women who began stimulation but whose cycles were cancelled due to low response, failed fertilization or other causes.

One of the most remarkable features of IVF is the continuous improvement in its efficacy. From 1986 to 2009, there has been a significant increase in the deliveries per IVF cycle initiated, from less than 9% to 30% (Table 31.1).[104,144]

IVF is a highly effective treatment for most infertility diagnoses, with the exception of uterine factor, multiple concurrent female infertility factors, and decreased ovarian reserve (Fig. 31.13). Not surprisingly, advancing female age correlates inversely with successful IVF outcome regardless of infertility diagnosis (Table 31.2). As previously discussed, as a woman ages, follicles are depleted, as measured by decreased ovarian reserve[145] and her chances to achieve a pregnancy with IVF diminish. As seen in Figure 31.14, live birth rates in IVF are relatively stable between the ages of 25 and 31 years, at which point the birth rates gradually decline up to age 35. The decline in birth rates becomes steeper after age 35, and in women older than 44 years old, the live birth rate approaches 0%.

Patient Counseling

Counseling is critical for patients interested in proceeding directly to IVF. In virtually all outcome studies, age is the most critical predictor of live birth with IVF treatment,

Table 31.1 IVF Outcomes from the Society for Assisted Reproductive Technology—American Society for Reproductive Medicine Registries for 1986 and 1994, and from the CDC National Summary Reports for 1998, 2004, and 2009

	Fresh Cycles, NonDonor Egg				
	1986*	1994*	1998†	2004†	2009†
Cycles initiated	4867	26,555	61,650	89,533	96,233
Clinical pregnancies	485	6089	22,934	31,373	37,214
Deliveries	NR	4896	18,808	25,880	30,529
Delivery per cycle initiated (%)	<9%‡	18.4%	24.9%	28.9%	31.7%

*Adapted from Medical Research International, American Fertility Society Special Interest Group. *Fertil Steril* 49:212-215, 1968; Society for Assisted Reproductive Technology. *Fertil Steril* 66:697, 1996, with permission of the American Society for Reproductive Medicine; Society for Assisted Reproductive Technology. *Fertil Steril* 71:798-8907, 1999.

†From CDC Assisted Reproductive Technology [ART] Reports 1998, 2004 and 2009. Available at www.cdc.gov/art/ART. Accessed Sept. 30, 2012.

‡Assumes an abortion and ectopic rate of at least 10%.

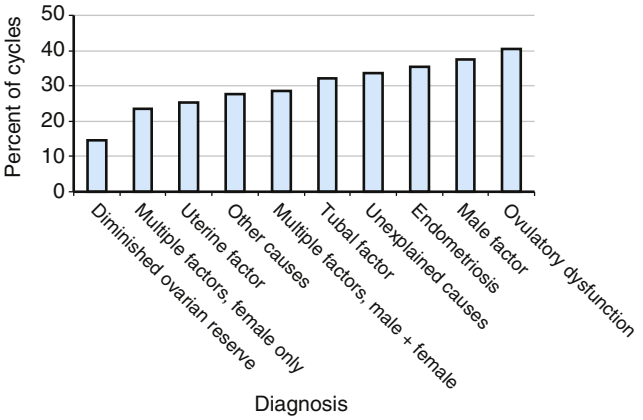


FIGURE 31.13 Percentages of ART (assisted reproductive technology) cycles using fresh nondonor eggs or embryos that resulted in live births, by diagnosis, 2009. *(From CDC Assisted Reproductive Technology [ART] Report 2009. Available at www.cdc.gov/art/ART2009. Accessed Sept. 30, 2012.)*

followed by ovarian reserve.[146,147] Unfortunately, normal ovarian reserve test results do not trump age; the unusual 44-year-old woman with normal ovarian reserve testing is likely to respond to gonadotropins but still has a delivery rate with IVF of less than 1% (Fig. 31.14)[92] due to the high incidence of aneuploidy in oocytes with increasing age.[148,149] Prior history of pregnancy and delivery, particularly with IVF, does increase the likelihood of success with IVF; however, again, age and ovarian reserve are still of critical importance.

Ovarian Stimulation

Natural cycle IVF and "mild stimulation" IVF are covered in detail in Chapter 30.

Table 31.2 2009 Assisted Reproductive Technology (ART) Report: National Summary*

Type of Cycle	Age of Woman				
Fresh Embryos from Non-Donor Eggs	<35	35-37	38-40	38-40	41-42
Number of cycles	42,384	21,860	22,144	22,144	9845
Percentage of cycles resulting in live births	41.4	31.7	22.3	22.3	12.6

*Using fresh embryos from nondonor eggs.
From CDC Assisted Reproductive Technology [ART] Report 2009. Available at www.cdc.gov/art/ART2009. Accessed Sept. 30, 2012.

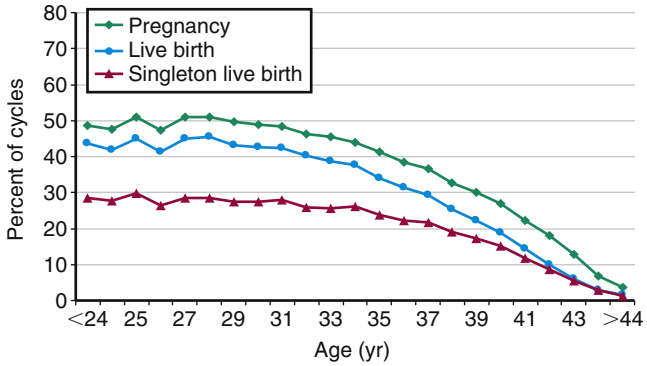


FIGURE 31.14 Percentages of ART (assisted reproductive technology) cycles using fresh, nondonor eggs or embryos that resulted in pregnancies, live births, and singleton live births by age of woman in 2009. *(From CDC Assisted Reproductive Technology [ART] Report 2005. Available at www.cdc.gov/art/ART2009. Accessed Sept. 30, 2012.)*

Natural Cycle IVF

Natural cycle IVF is possible through careful monitoring of the menstrual cycle with serum estradiol and transvaginal sonography, in order to identify the time when the dominant follicle can be aspirated to yield a fertilizable oocyte. Although conceptually appealing, natural cycle IVF is associated with low pregnancy rates. In one study of 74 such cycles, oocytes were harvested in approximately 50% of the cycles, and the pregnancy rate per cycle initiated was 3%.[150] In another study of 114 natural IVF cycles, the pregnancy rate per cycle initiated was 4%.[151] In true natural cycle IVF the spontaneous ovulation rate is high, the rate of obtaining no oocytes and embryos is high, and pregnancy and delivery rates are low.[150] IVF is a resource-intensive treatment, and pregnancy rates in the range of 3% to 4% per natural IVF cycle initiated are not cost effective.

Mild Ovarian Stimulation

"Mild" stimulation regimens, although poorly defined in the literature, have been gaining popularity. Such regimens may include the use of clomiphene or aromatase inhibitors with 75 to 150 IU of gondadotropins, but in some publications gonadotropin dosing is actually quite high. This treatment may be appropriate in selected young women with low expected rates of oocyte aneuploidy, and high

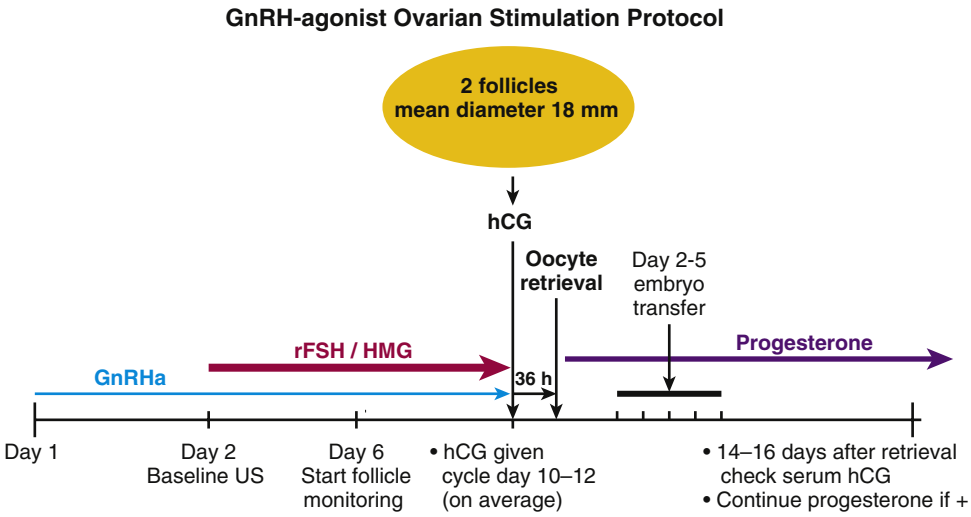


FIGURE 31.15 Schematic representation of the GnRH-agonist protocol for ovarian stimulation for *in vitro* fertilization (IVF). FSH, follicle-stimulating hormone; GnRHa, gonadotropin-releasing hormone-agonist analogs; hMG, human menopausal gonadotropin; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; US, ultrasound. *(From Cheong Y, Ginsburg ES, Macklon N. Protocols for ovarian stimulation. In Ginsburg ES, Racowsky C. New York,* Springer Science+Business Media, *In Press.)*

embryo implantation rates. In Europe, such treatment has been more widely adopted, perhaps in part due to the fact that many countries have legislation restricting the number of embryos that may be cultured and/or transferred, as well as lower maximal maternal ages that are considered acceptable.

The approach in the United States is largely based on data showing that higher numbers of oocytes and the ability to select the best embryos from a cohort have been shown to optimize pregnancy rates at all ages. Consequently, success is critically dependent on generating an adequate number of mature follicles that contain developmentally competent oocytes, while avoiding ovarian hyperstimulation syndrome. Large retrospective cohort studies suggest that obtaining enough embryos to allow for embryo selection is important in order to maximize pregnancy rates. One study of 7422 women treated with the long GnRH-agonist protocol found that pregnancy rates per cycle initiated were highest when 13 oocytes were obtained (28%).[152] Another study of 400,135 IVF cycles performed between 1991 and 2008 also found that the pregnancy rate per cycle correlated with the number of oocytes retrieved. The median number of oocytes retrieved was nine, with a live birth rate per initiated cycle of 21%. Live birth rates correlated with number of oocytes retrieved across all age groups, and were maximal when 15 oocytes were obtained, plateaued between 15 and 20 oocytes, and were lower if over 40 oocytes were retrieved.[153] Therefore, the predominant approach to ovarian stimulation for IVF in the United States is to aim for the development of multiple follicles in order to allow for embryo selection, particularly in women at advanced ages (i.e., older than age 38) when aneuploidy rates increase and embryo implantation rates decrease.

Medications used for ovarian stimulation include clomiphene, clomiphene-hMG; clomiphene-rhFSH (recombinant human FSH); hMG alone; immunopurified (highly purified) urinary human FSH (hpFSH) alone; rhFSH alone, and various combinations of the above. Compared to clomiphene alone, the combination of clomiphene plus low dose human gonadotropins (FSH or hMG) increases the number of follicles stimulated, but the number of oocytes retrieved, and the pregnancy rates are lower than expected with standard stimulation in normal responders.[154] Though such regimens do allow for lower cost stimulations,[155] the live birth rate per embryo transfer is low (19%). Therefore, in terms of the expected live birth rate per initiated stimulation and the cumulative live birth rate (which would include transfer of cryopreserved embryos), these regimens are likely inferior to standard dosing of gonadotropins alone[156] and may not ultimately be cost-effective.

Standard Ovarian Stimulation

Below we focus on controlled ovarian stimulation protocols as used for IVF in the U.S. In addition, we consider decision making in protocol selection.

GnRH-Agonist Down-Regulation Regimens

In ovarian stimulation for IVF, the main purpose of a GnRH-agonist analog or antagonist is to prevent a premature LH surge (triggered by the high concentrations of estradiol) while follicles are still immature. GnRH-agonist analogs differ from the native decapeptide GnRH in amino acid positions 6 and 10. They are resistant to degradation, giving them long half-lives and prolonged receptor occupancy. The initial administration of a GnRH-agonist analog is associated with an increase in LH and FSH secretion (agonist phase). Long-term administration causes down-regulation and partial desensitization of the pituitary GnRH receptor, resulting in the suppression of pituitary secretion of LH and FSH. The addition of a GnRH-agonist analog to regimens of ovarian stimulation for IVF-ET appears to be associated with an increase in the number of oocytes retrieved, the number of embryos transferred, and the clinical pregnancy rate.[157,158] In the standard down-regulation protocol, the GnRHa is begun in the midluteal phase of the prior menstrual cycle, and ovarian stimulation begins with or after the onset of the subsequent menstrual period (Fig. 31.15).

For example, one study demonstrated that treatment with a GnRH agonist (buserelin) plus hMG resulted in more oocytes retrieved (9.3 versus 6.2), more embryos (4.3 versus 2.8), and a higher clinical pregnancy rate (20% versus 14%) than did stimulation with clomiphene plus hMG.[159]

In another study, comparison of a stimulation regimen using the same medications also demonstrated that buserelin-hMG stimulation resulted in a higher pregnancy rate than that observed with clomiphene-hMG (36% versus 18%).[160] The type of GnRH-agonist analog used does not appear to be crucial to obtaining the improved outcomes. Studies with D-Trp6 GnRH-agonist analog also demonstrate improved pregnancy rates (21% versus 12%) compared with ovarian stimulation regimens that do not use GnRH agonists for IVF-ET. [161] The addition of a GnRH-agonist analog to a gonadotropin-stimulation regimen appears to suppress premature LH surges, reduces the chance of premature luteinization of granulosa cells, and reduces cycle cancellation due to premature luteinization or ovulation.

Among a smaller number of women, GnRH-agonist down-regulation protocols may excessively suppress endogenous pituitary LH secretion. If rhFSH is the only exogenous gonadotropin used, low serum levels of estradiol may result.[162] When follicular dynamics were compared between GnRHa and GnRH-antagonist cycles, it was found that the modulation of the FSH-induced protein kinase A pathway by protein kinase C was different in agonist versus antagonist cycles.[163] The changed modulation of this pathway results in lower levels of serum and follicular estradiol via lower levels of aromatase activity. It does not appear, however, that low levels of estradiol are, in fact, detrimental to pregnancy rates in these IVF cycles.[164] When LH levels are too high, premature ovarian luteinization may occur. It is unclear whether LH is required for optimal ovarian stimulation as studies do not uniformly indicate that the addition of LH to ovarian stimulation improves pregnancy rates, though estradiol levels are higher.[165-167]

It is important to note that women with normal ovarian reserve typically will produce optimal numbers of oocytes (discussed later in the chapter) from down-regulation regimens. However, women with decreased ovarian reserve may not respond even to high-dose gonadoptropin treatment after down-regulation. In some cases, employing lower doses of GnRH agonists will allow down-regulation without excessive suppression of response.[168,169] A randomized trial of a half dose of triptorelin (1.87 mg) adequately suppressed the pituitary, but required a lower dose of FSH ampules (42 ± 2 versus 59 ± 3) and there were more mature oocytes (10.1 ± 0.54 versus 7.4 ± 0.55), fertilized oocytes (8.24 ± 0.35 versus 6.34 ± 0.37) and embryos (7.8 ± 0.36 versus 5.9 ± 0.37) than when the standard 3.75 mg dose was given. There were no significant differences in pregnancy (38.8% versus 25.3%), implantation (22.6% versus 13.8%) or miscarriage (6.1% versus 5.0%) rates. However the cumulative pregnancy (fresh plus frozen embryo transfers) rate was significantly higher in the diluted agonist group (56.8% versus 35.4%).[170]

GnRH-Agonist Flare or Short-Course Regimen

In the down-regulation protocol, GnRH-agonist analogs are usually started in the luteal phase of the cycle preceding the IVF-ET cycle (long protocol). In the GnRH-agonist flare regimen, the GnRH agonist is begun in the early follicular phase of the IVF-ET cycle, typically between cycle days 1 and 3. Basically, the flare regimens take advantage of both the agonist and down-regulation properties of GnRH-agonist analogs. In the first few days of treatment, during the early follicular phase, the GnRH agonist stimulates endogenous LH (and FSH) secretion from the pituitary. The increased LH secretion stimulates more ovarian androstenedione production, which increases the substrate available for aromatization. However, the increased secretion of both LH and ovarian androstenedione may not be optimal, as some studies suggest that elevated circulating LH and intrafollicular androgen levels are not associated with optimal oocyte function.[171] In one study of women treated with a GnRH-agonist down-regulation or a GnRH flare regimen, follicular fluid androstenedione concentrations were 27 ng/mL versus 57.3 ng/mL ($P < 0.05$), respectively.[172] In any case, the elevated androstenedione results in higher estradiol levels, and the increased FSH secretion may increase follicular recruitment. With continuing administration of the GnRH-agonist analog, the down-regulation effects of the analog begin and premature LH surges are prevented.

In one study, women with a previous poor response to standard long-protocol GnRH-agonist down-regulation plus gonadotropin stimulation were treated with a flare regimen using microdose leuprolide acetate (20 μg every 12 hours), initiated in the early follicular phase of the treatment cycle, plus gonadotropin. Compared with a previous cycle of down-regulation, microdose GnRH agonist plus gonadotropin resulted in greater ovarian stimulation, higher serum estradiol concentration, and increased numbers of follicles and oocytes retrieved.[173] It should be noted, however, that women with normal ovarian reserve have higher cycle cancellation rates[174] and may have lower pregnancy rates with a flare as compared to down-reglulation protocol. In one large cohort study of women under 40 with normal baseline FSH levels, the GnRH flare regimen was associated with a lower delivery rate per cycle compared with the GnRH-agonist down-regulation regimen (15.1% versus 21.3%, $P < 0.05$).[175] Other investigators have reported similar trends.[176] The GnRH flare probably should not be the first choice ovarian stimulation regimen in women undergoing their first cycle of IVF or those who have a good prognosis. Flare protocols however are beneficial in poor responders who are unlikely to respond well to long or down-regulation protocols (discussed later in the chapter).

GnRH-Antagonist Regimens

A theoretical problem with the GnRH-agonist analogs is that LH secretion is stimulated at the initiation of treatment, which is not consistent with the physiology of the normal early menstrual cycle. The GnRH antagonists offer the possibility of acutely suppressing LH secretion without an initial increase in LH release.[177,178] Thus, pituitary LH secretion can be controlled within the stimulation cycle itself; such control is not possible with the GnRH agonists, which must be begun during the previous menstrual cycle to achieve full down-regulation by the time stimulation begins. GnRH antagonists are typically administered as a small daily dose (cetrorelix or ganirelix, 0.25 mg daily by subcutaneous injection), usually starting on cycle day 6 to 8 or when the lead follicle reaches 14 mm in diameter[179] or as a single large dose (cetrorelix, 3 mg subcutaneously, which has a 4 day duration of action) on approximately cycle day 8[180] (Fig. 31.16). Both protocols block a spontaneous LH surge.

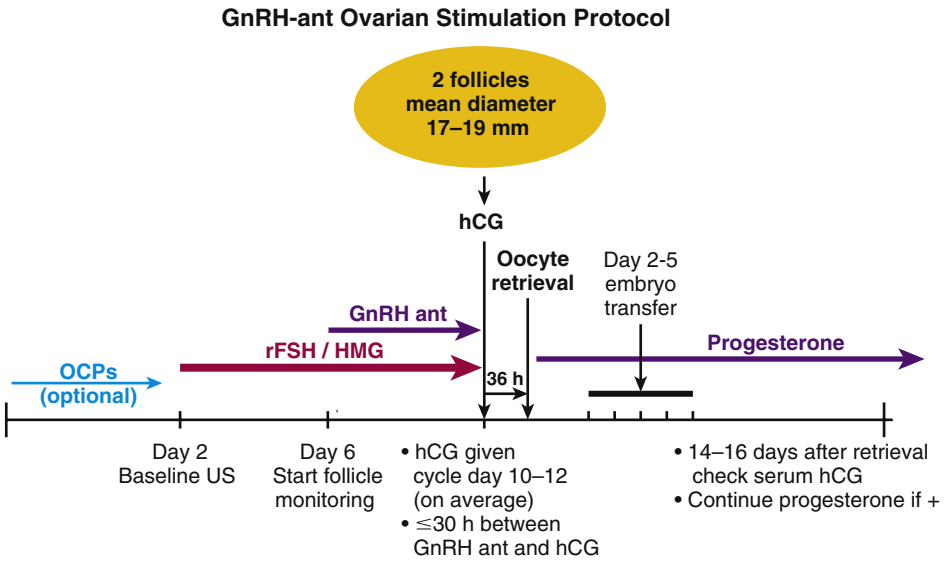


FIGURE 31-16 Schematic representation of the GnRH-antagonist protocol for ovarian stimulation for *in vitro* fertilization (IVF). FSH, follicle stimulating hormone; GnRH-ant, gonadotropin-releasing hormone antagonist; hCG, human chorionic gonadotropin; hMG, human menopausal gonadotropin; US, ultrasound. *(From Cheong Y, Ginsburg ES, Macklon N. Protocols for ovarian stimulation. In Ginsburg ES, Racowsky C. New York,* Springer Science+Business Media, *In Press).*

The impact of a GnRH antagonist on ovarian stimulation for IVF is heavily dependent on the dose of GnRH antagonist administered. At small doses, the suppression of LH is minimal. With large doses, near complete suppression of LH is achieved. In one study, the impact of six doses of the GnRH antagonist ganirelix on LH secretion and IVF outcomes was studied.[181] A fixed daily dose of recombinant FSH was administered. Ganirelix produced a dose-dependent suppression not only of LH (Table 31.3) but also of serum androstenedione and estradiol. The use of oral contraceptives prior to GnRH antagonist cycles is common in order to have some control over the start of cycles. Initial studies that lead to FDA approval used oral contraceptives for approximately 3 weeks prior to stimulation. In oligo-anovulatory patients it is preferable to ensure that the patient is not pregnant prior to starting stimulation; a course of contraceptive pills after an anovulatory progesterone level accomplishes this, and allows the patient and clinician to start the stimulation in a scheduled way. A three-arm randomized IVF trial comparing OCP pretreatment in GnRH antagonist (n =110) versus agonist (n =111) versus GnRH antagonist without OCP treatment (n =111) showed that the OC-scheduled antagonist regimen appeared more similar to the agonist regimen rather than the no-OCP GnRH antagonist regimen. Suppression was more profound at the start of stimulation ($P \leqq 0.001$), as manifested by slower follicular growth ($P \leqq 0.001$), longer stimulation (11.7 and 10.3 days respectively versus 9.4; $P \leqq 0.001$), and more rFSH utilized (2667 and 2222 IU versus 1966 IU; $P \leqq 0.001$). In the three groups, the number of oocytes was similar (13.1, 12.9, and 11.5, respectively); and the number of good quality embryos (5.1, 5.7 and 5.0) were similar.[182] One meta-analysis evaluated 4 RCTs in 847 patients and found no difference in ongoing pregnancy rates[183] though duration of stimulation and gonadotropin requirements were higher after OCP pretreatment. However, in a follow-up study the authors found that ongoing pregnancy rates were lower in OCP pre-treated GnRH-antagonist cycles (relative risk: 0.80, 95% CI 0.66-0.97), and again that duration of stimulation and gonadotropin requirements were higher.[184] Another meta-analysis including estradiol and progesterone pretreatment had similar findings, including slightly lower ongoing pregnancy rates, however concluded that no changes in clinical practice were indicated as numbers in trials were small.[185] A recent randomized trial showed longer stimulation and gonadotropin requirements but no difference in clinical pregnancy rates when 4 mg oral estradiol (comparable to 20 mcg ethinyl estradiol) versus no treatment was begun prior to stimulation. In our program we use 10 to 21 days of oral contraceptives. Not all patients have a withdrawal bleed after oral

Table 31.3 Efficacy of GnRH in Preventing Premature Luteinizing Hormone Surges in Women Undergoing Ovarian Stimulation

Dose of Ganirelix	0.0625 mg	0.125 mg	0.25 mg	0.50 mg	1.0 mg	2.0 mg
Number of women	30	65	68	69	64	26
FSH (IU/L)	9.1	9.0	9.1	10.2	9.8	8.8
LH (IU/L)	3.6	2.5	1.7	1.0	0.6	0.4
Androstenedione (ng/mL)	2.6	2.6	2.4	2.2	2.0	1.5
Estradiol (pg/mL)	1475	1130	160	823	703	430
Pregnancy rate per cycle	23.3%	23.1%	33.8%	10.1%	14.1%	0%

Women undergoing IVF received recombinant FSH (Puregon) starting on cycle day 2 and continuing daily until three follicles with a mean diameter greater than 17 mm (as determined) were achieved when hCG was given to initiate the ovulatory process. The women were randomized to receive the GnRH antagonist, ganirelix, on a daily basis starting on cycle day 7 at one of six doses (0.0625 mg, 0.125 mg, 0.25 mg, 0.5 mg, 1.0 mg, 2.0 mg). Serum FSH, LH, androstendione, and estradiol concentration were measured on the day of hCG administration. Large doses of ganirelix markedly suppressed serum LH, androstenedione, and estradiol concentrations. Pregnancy rate per cycle was maximal with a dose of ganirelix of 0.25 mg.

The Ganirelix Dose Finding Study Group: A double-blind randomized dose-finding study to assess the efficacy of the gonadotropin-releasing hormone antagonist ganirelix [org 37462] to prevent premature luteinizing hormone surges in women undergoing ovarian stimulation with recombinant follicle stimulating hormone [Puregon]. *Hum Reprod* 13:3023-3031, 1998.

contraceptives, so a baseline ultrasound is performed on day 2 of the menstrual period following pill cessation, or 4 days after the last pill, as the vast majority of women will have had a withdrawal bleed by that point.

GnRH Agonist vs GnRH-Antagonist Protocols in "Normal" Responders

Most studies of IVF have demonstrated that both GnRH agonists in a down-regulation protocol and GnRH antagonists are associated with similar rates of cycle cancellation due to a premature LH surge, as well as similar rates of ovarian hyperstimulation syndrome (OHSS). An older Cochrane review of five randomized controlled trials comparing GnRH antagonist (n = 1211) to GnRH agonists in a down-regulation protocol (n = 585) for IVF, revealed that the odds ratio (OR) for becoming pregnant in the GnRH antagonist group was 0.79 (95% confidence interval [CI], 0.63 to 0.99) compared with the GnRH agonist group.[186] However, a recent Cochrane review by the same first author found, in forty-five RCTs (n = 7511) comparing the antagonist to the long agonist protocols in women with normal ovarian reserve, that there was no evidence of a statistically significant difference in rates of live-birth (9 RCTs; odds ratio [OR] 0.86, 95% CI 0.69 to 1.08) or ongoing pregnancy (28 RCTs; OR 0.87, 95% CI 0.77 to 1.00). There was a statistically significant lower incidence of OHSS in the GnRH antagonist group (29 RCTs; OR 0.43, 95% CI 0.33 to 0.57). Therefore, it appears reasonable to use clinical judgement in choosing which type of protocol to use in patients with normal ovarian reserve.[187]

"Poor Responder" Protocols

"Estradiol Priming" GnRH-Antagonist Protocols

A variation of GnRH antagonist use in IVF utilizes a luteal estradiol patch or oral estradiol (e.g., 0.3 mg patches changed qod or 4 mg oral estradiol daily) followed by GnRH-antagonist suppression (Fig. 31.17). Following a menstrual period, gonadotropin stimulation with GnRH antagonist support is utilized. The hypothetical advantage of this approach is to improve the synchrony of the developing follicles by reducing antral follicle sizes and follicular phase heterogeneity[188] making it best suited for use in poor responders. In one report, patients using the "patch" protocol had a lower cycle cancellation rate, more oocytes retrieved and more embryos transferred than in a prior cycle.[189] A meta-analysis looking at GnRH antagonist cycles with and without pretreatment did find that more oocytes were retrieved after estradiol pretreatment compared to no pretreatment.[185]

Microflare Protocol

The "microdose" flare protocol employs diluted or low dose GnRH agonist typically starting day 1 or 2 of the menstrual cycle (Fig. 31.18). The goal is to provide stimulation with enough suppression so as to avoid a spontaneous LH surge, but not enough to suppress endogenous pituitary LH and FSH release so that ovarian stimulation is augmented by endogenous gonadotropins. The protocol is often used with oral contraceptive pretreatment starting day 1 to 2 of menses. The microdose leuprolide acetate protocol has been compared to the estrogen priming protocol in prospective randomized trials in poor responders, with no difference in clinical or live birth rates reported. However, numbers are quite small and likely not powered adequately to show any significant differences in outcomes.[190] In one larger trial of 116 cycles, the estrogen priming protocol used higher doses of gonadotropins, (3247.8 ± 634.6 versus 2994.8 ± 611 IU), yet similar numbers of oocytes were retrieved and fertilized. There was no significant difference between the two groups in terms of the implantation rates (9.8% versus 7.9%) and the clinical pregnancy rates per transfer (16.3% versus 15.6%).[191]

Another study randomized 270 poor responders to a standard flare protocol (0.05 mg/day of triptorelin) versus antagonist, with no hormonal lead-in for either treatment. Triptorelin was started at the time of stimulation on cycle day 2, and cetrorelix was started at a follicular diameter of ≥14 mm. A fixed dose of 400 IU r FSH was used. As expected, estradiol at hCG was lower in the antagonist group, but oocyte yield, fertilization, and implantation rates were not different. However, the ongoing pregnancy rate, was higher in the antagonist group (12.2 versus 4.4%,

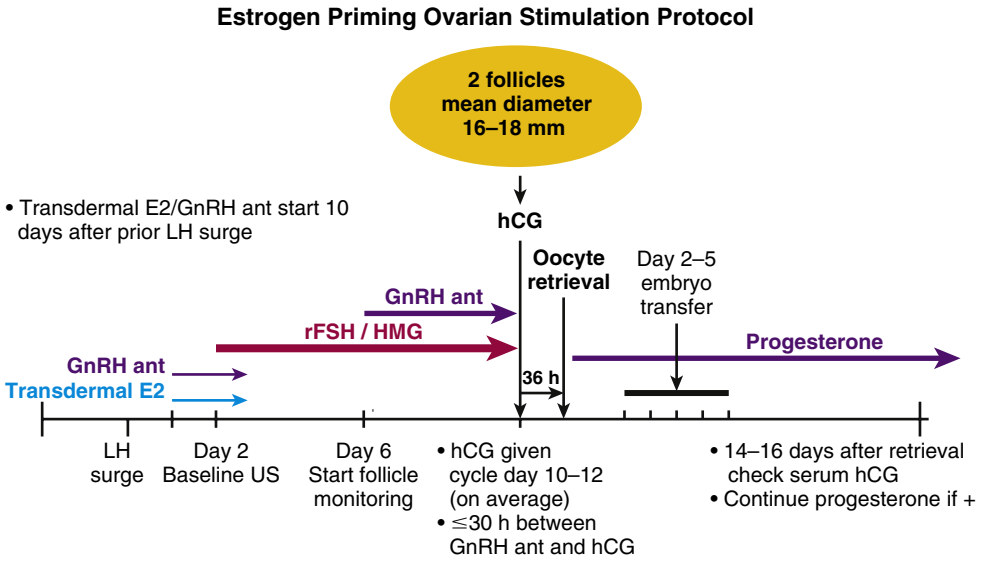


FIGURE 31.17 Schematic representation of the "patch" or "estrogen priming" protocol for ovarian stimulation for *in vitro* fertilization (IVF). FSH, follicle stimulating hormone; GnRH-ant, gonadotropin-releasing hormone antagonist; hCG, human chorionic gonadotropin; hMG, human menopausal gonadotropin; US, ultrasound. *(From Cheong Y, Ginsburg ES, Macklon N. Protocols for ovarian stimulation. In Ginsburg ES, Racowsky C. New York,* Springer Science+Business Media, *In Press.)*

$P < 0.048$; difference 7.8%; 95% CI: 0.2 to 14.0); however, the confidence interval does slightly overlap 1.0.[192] Another trial of 55 poor responders found equivalent pregnancy rates between the flare and antagonist groups. However, there were more mature eggs retrieved, and more high quality embryos transferred, as well as a higher fertilization rate in the flare group (84% versus 63%).[193]

Cycle Monitoring in IVF

In GnRH-agonist cycles, documentation of pituitary downregulation is performed by assessing serum estradiol and progesterone levels after menses has begun; levels should be suppressed, and depending on the assays used, estradiol levels are typically less than 50 pg/mL and progesterone less than 1.0 ng/mL. A baseline estradiol is also performed to rule out any large ovarian cysts which could grow in response to treatment, and to rule out potential malignancy. Simple ovarian cysts need not necessarily prevent the start of stimulation but persistent complex cysts greater than 3 cm warrant surgical treatment prior to ovarian stimulation, unless the patient is known to have endometriosis and the cyst has a typical endometriotic cyst appearance.

As the pituitary is not suppressed at the start of GnRH-antagonist cycles, only a baseline ultrasound is needed. After the first 4 or so days of stimulation, serum estradiol and ultrasound examinations are typically performed to assess for the development of mature sized follicles; programs typically use at least 1 to 2 follicles with a mean diameter of 18 mm as an indication for administration of a trigger hCG injection. Mature oocytes, however, are generally obtained from smaller sized follicles as well.

Final Follicle Maturation

Final follicle and oocyte maturation prior to retrieval requires stimulation by high concentrations of LH. Prior to the availability of recombinant hormones, the most accessible source of LH-like material was urinary human chorionic gonadotropin (hCG). Recently, recombinant hCG (rhCG) has become available.[194] Typically hCG is administered as 5000 or 10,000 units (250 μg of rhCG is approximately equivalent to 10,000 units of hCG) to stimulate final follicle maturation. In a clinical trial, hCG was compared with recombinant human LH (rhLH) for final follicle maturation. The investigators reported that hCG and rhLH had similar efficacy for maturing follicles and oocytes and performed similarly in terms of pregnancy rates. However, the rhLH was associated with slightly lower rates of OHSS, probably due to its shorter half-life.[195] It should be noted that the rate of OHSS is in part dependent on the dose of FSH administered during follicle stimulation.[196] rhCG is formulated for subcutaneous use whereas urinary hCG preparations are FDA approved for intramuscular use.

When ovarian stimulation is performed using a combination of GnRH antagonist plus rhFSH, it is possible to use a single dose of a GnRH agonist to stimulate the pituitary to release a surge of LH that will initiate final follicle maturation. For example, in one small clinical trial, women undergoing IVF were stimulated with rhFSH and the GnRH antagonist ganirelix, and then randomized to receive either hCG (10,000 IU), triptorelin (0.2 mg), or leuprolide (0.5 mg) to initiate final follicle maturation. In the GnRH agonist groups (triptorelin and leuprolide), the endogenous secretion of LH peaked at 4 hours after the injection and LH levels returned to baseline by 24 hours. After the hCG injection, circulating hCG peaked at 24 hours and remained elevated for 5 days. Estradiol and progesterone levels were similar up until the day of oocyte retrieval in all three groups. In the luteal phase, estradiol and progesterone levels were greater in the hCG group than in the triptorelin or leuprolide groups. Similar numbers of oocytes and mature oocytes were retrieved in all three groups, and fertilization rates were also similar.[197] Stimulation of an endogenous LH surge with a GnRH agonist is associated with a lower rate of early OHSS.

Oocyte Retrieval

Oocyte retrieval is typically performed 35 to 36 hours after hCG administration by a transvaginal sonography-guided technique using a 16 to 17G needle, and generally under

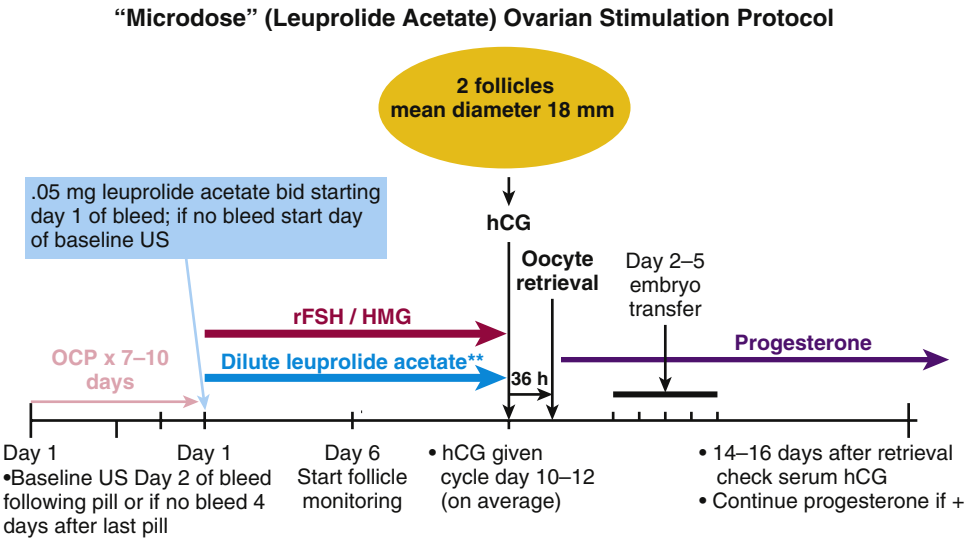


FIGURE 31.18 Schematic representation of the "microflare" protocol for ovarian stimulation for *in vitro* fertilization (IVF). FSH, follicle stimulating hormone; GnRHa, gonadotropin-releasing–hormone agonist; hCG, human chorionic gonadotropin; hMG, human menopausal gonadotropin; US, ultrasound. *(From Cheong Y, Ginsburg ES, Macklon N. Protocols for ovarian stimulation. In Ginsburg ES, Racowsky C. New York,* Springer Science+Business Media, *In Press.)*

intravenous anesthesia. Reports of oocytes in the human fallopian tube after hCG administration suggest that a time interval between hCG administration and oocyte retrieval of about 36 hours maximizes oocyte maturation and minimizes the chance of spontaneous ovulation (Fig. 31.19). Before 30 hours, oocytes in the lead follicles may not be mature. After 38 hours following hCG administration, ovulation begins and oocytes are lost into the peritoneal cavity. The number of oocytes retrieved is largely dependent on the number of large follicles present at the time of retrieval. In general, oocytes can be harvested with a high rate of success from follicles with a mean diameter greater than 12 mm (Fig. 31.20).[198]

Preparation of the vagina with saline, or antibacterial agents[199] followed by copius saline irrigation is typically performed prior to retrieval, and intravenous antibiotics often administered, though there are no randomized trials proving benefit.[200,201] In cases of abnormal anatomy when ovaries are not accessible transvaginally,[202] retrieval can be performed with transabdominal ultrasound guidance, or as in the early years of IVF, via a laparoscopic approach. Regardless of the route of oocyte retrieval, traps containing follicular fluid are passed off to the embryology laboratory which should be directly adjacent to the operating room.

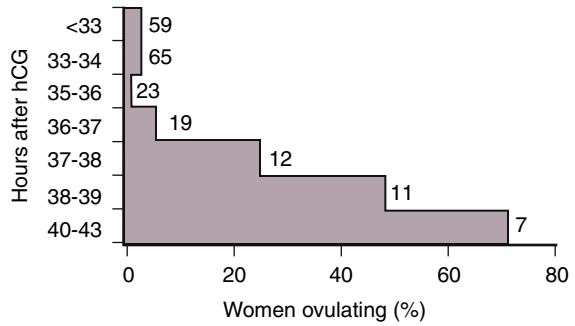


FIGURE 31.19 Interval between an injection of human chorionic gonadotropin (hCG) and the percentage of women in whom follicular rupture has occurred. The number of women studied is indicated to the right of the bar. *(From Edwards RG. Physiological aspects of human ovulation.* J Reprod Fertil *18[suppl]:87-101, 1973.)*

The embryologist immediately picks up and then assesses the oocytes for maturity.

Assessing Oocyte Quality

Ideally, both nuclear and cytoplasmic maturation should be assessed because both processes are required for an oocyte to fertilize successfully and support early embryonic development. Typically, only 70% to 80% of retrieved oocytes have achieved nuclear maturation (i.e., have emitted the first polar body and are in metaphase II); the remaining 20% to 30% are at either prophase I or metaphase I. As no overt morphological changes in the oocyte have yet to be associated with cytoplasmic maturation, the proportion of retrieved oocytes that are cytoplasmically mature remains unknown. However, this proportion is likely quite small since, even after consideration of the multiple factors involved in formation of a viable healthy embryo, the overall efficiency of oocyte to liveborn in ART is only about 5%.[203]

Current techniques for assessing oocyte quality fall into the two major categories, invasive and non-invasive. The invasive approach involves polar body biopsy and preimplantation genetic screening (PGS) which enables, by extrapolation, determination of the oocyte chromosomal complement,[204] as well as insight into the oocyte transcriptome[205] (see Chapter 30). Noninvasive assessment continues to rely on visual inspection of the morphological appearance of the surrounding cumulus oophorus with a light microscope. During the periovulatory period in response to the midcycle surge of LH or exogenous hCG, the cumulus cells secrete glycosaminoglycans,[206,207] resulting in expansion of the cumulus oophorus, and a radiating appearance of the inner corona radiata cells. Although the overall disposition of the surrounding cumulus-corona cells may thus provide some guide to quality of the enclosed oocyte,[208] this association is not perfect. Nuclear maturity and emission of the first polar body is most accurately determined by visualization of the oocyte itself (Fig. 31.2).

The imperfect prediction of oocyte maturity by morphological assessment of the cumulus-corona radiata cells has

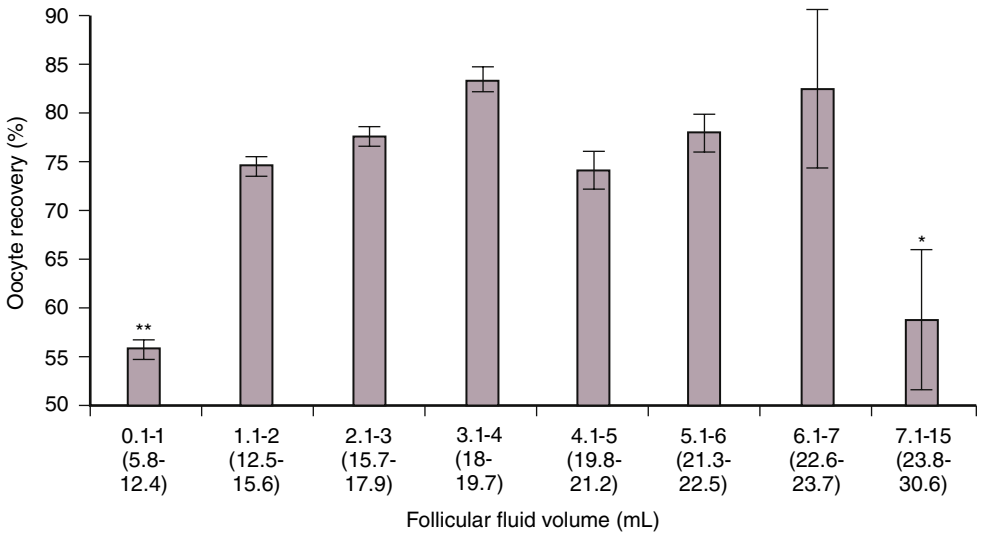


FIGURE 31.20 Percentage of follicles from which an oocyte was obtained at the time of retrieval as a function of follicle size. Mean, SEM; *$P < 0.01$; **$P < 0.001$. Numbers in parentheses represent corresponding follicular diameter in millimeters as determined by sonography. *(From Wittmaack FM, Kreger DO, Blasco L, et al. Effect of follicular size on oocyte retrieval, fertilization, cleavage, and embryo quality in in vitro fertilization cycles: a 6-year data collection.* Fertil Steril *62:1205-1210, 1994, with permission of the American Society for Reproductive Medicine.)*

led to development of alternative non-invasive approaches to assess oocyte quality. Use of polarized light to visualize the size and shape of the meiotic spindle,[209] and the thickness of the inner zona pellucida layer[210] have been correlated with outcome.[211] However, because of the cost of the polarizing system and the sensitivity of the results to oocyte positioning it is unlikely that this approach will be routinely adopted in the IVF laboratory. Alternatively, genomic approaches are being aggressively pursued. Expression of genes specifically associated with cumulus expansion such as *PTGS2*, *HAS2*, and *GREM.1VCAN* and *RPS6KA2*,[212-215] as well as those involved in the calcium signaling pathway[216] have all been found to have some predictive value. Other genes have been associated with oocyte meiotic stages and specific maturation conditions,[217,218] and cumulus cell gene expression has been used in multivariable models to predict embryo quality and implantation.[219] Furthermore, membrana granulosa cell genes involved in the luteinization process have been associated with oocyte quality,[220] and a recent study has revealed a correlation between mature (as opposed to propeptide) forms of *GDF9* in follicular fluid and oocyte nuclear maturation and embryo quality.[221]

Although such molecular studies provide insight into the differential expression of biomarkers associated with developmentally competent versus incompetent oocytes, much work remains to be done to identify a gene network algorithm that can be applied clinically for identification of oocytes that have acquired both nuclear and cytoplasmic competence.[216] Moreover, if ever established clinically, the laboratory protocols will need to be streamlined to ensure that each oocyte is individually tracked with fidelity of its association with corresponding cumulus and/or granulosa cells ensured.

Collection, Assessment and Preparation of Sperm

Semen is typically collected by masturbation or occasionally by sexual intercourse using a condom lacking spermicide. In cases of male factor infertility, retrograde ejaculation, electroejaculation, testicular biopsy or epididymal aspiration may be employed (see Chapter 24). Moreover, for men with markedly abnormal semen parameters, ICSI is recommended (see Chapters 24 and 32).

Assessment of ejaculated semen begins with determination of the time it takes for the specimen to liquefy. A normal specimen has liquefied within 60 minutes at room temperature, although usually this occurs within 15 minutes. The liquefied specimen is then examined for color, volume, viscosity, and pH. Initial assessment of the raw, unprocessed, specimen is then undertaken by phase-contrast microscopy to determine the concentration, motility, and agglutination of sperm, and presence of any cellular elements other than the sperm. Sperm morphology may be assessed from smears stained, for example, with Papanicolaou stain. Classification of sperm morphology typically involves assessment of the shape of the head, and defects in the neck, midpiece, and tail (World Health Organization [WHO] Manual), and is generally performed using Kruger's strict criteria.[99]

Further tests may be performed on a case-by-case basis [for example, sperm antibody assessment or viability assessment using the hypo-osmotic (HOS) test].[222] The WHO reference values of semen variables are shown in Table 31.4. If the semen parameters are abnormal, the number of sperm isolated from the specimen is related to both fertilization and pregnancy rate following routine IVF.[223] As the number of sperm in the unwashed specimen decreases below 10 to 20 × 10^6/mL, the incidence of chromosomal abnormalities increases.[224,225] Therefore, nonobstructive azoospermia and severe oligospermia (less than 5 million/mL) are indications for karyotype evaluation (see Chapter 24).[226] Interestingly, the incidence of aneuploidy in sperm appears to increase with increased incidence of sperm DNA fragmentation,[227] although as mentioned previously, there is a strong need to standardize the specific tests used to quantify the extent of DNA damage.[56]

Table 31.4 WHO Reference Limits for Semen Characteristics (2010)

Parameter	Lower Reference Limits
Semen volume (mL)	1.5 (1.4-1.7)
pH	≥7.2
Total sperm number (10^6 per ejaculate)	39 (33-46)
Sperm concentration (10^6/mL)	15 (12-16)
% Total motility (progressive and non-progressive)	40 (38-42)
% Progressive motility	32 (31-34)
Sperm morphology (normal forms %)	4 (3.0-4.0)
Vitality (live sperm, %)	58 (55-63)
Immunobead test (motile sperm with bound beads, %)	<50

Values are lower reference limits (5th centiles and their 95% confidence intervals).
WHO laboratory manual for the examination and processing of human semen, 5th edition, Cambridge University Press, 2010.

The liquefied semen specimen is processed for isolation of the sperm by a variety of techniques, including swim-up, and centrifugation techniques using a density gradient comprising a polymer (e.g., Isolate). For normal semen specimens, both techniques generate high-motility sperm for oocyte insemination.[228] For semen with abnormal sperm characteristics (e.g., low sperm concentration), however, most studies demonstrate that sperm prepared by the gradient centrifugation technique are superior to sperm prepared by the swim-up technique. For example, in one study on abnormal semen (defined by WHO criteria), density gradient centrifugation resulted in selection of sperm with higher scores for the acrosome reaction, the HOS test and nuclear maturity, than those selected with a swim-up procedure.[229]

In preparation for incubation with the oocytes, sperm prepared by either the swim-up or gradient centrifugation technique are incubated in a protein-supplemented medium for up to 4 hours to initiate the process of capacitation.

Incubation of Sperm with Oocytes and the Fertilization Check

For standard IVF, the number of sperm used for insemination will vary depending upon whether the oocytes are inseminated in microdrops of 100 to 200 μL medium or

in a larger volume (usually 1 mL). Typical numbers used for microdrop insemination range from 25,000 to 50,000 sperm, while those for insemination in a 1 mL volume, typically holding 3 to 5 oocytes, usually range from 100,000 to 300,000. For men with normal semen parameters, higher concentrations of sperm do not significantly improve the fertilization rate.

Approximately 16 to 18 hours later, the oocytes are examined for fertilization. The cumulus-corona radiata cells are gently removed from the oocyte which is then examined under an inverted microscope with Hoffman illumination at around 100× magnification. A zygote with two pronuclei (PNs) and two polar bodies is morphological evidence of normal fertilization. All oocytes with either less than or more than 2PNs are discarded. The 2PN zygotes are then set up in culture either separately or in groups.

Embryo Culture Media

Substantial improvements in culture systems for clinical IVF have occurred since the culture of the first viable human embryo in the 1970s. In contrast to the use of Ham's F10 medium or Earle's balanced salt solution supplemented with maternal serum for use in these early days of clinical IVF, a multitude of commercial culture media are now available for growing human embryos in culture.[230,230a]

Early attempts to culture human embryos to the blastocyst stage required the use of feeder cells to support embryo development.[231] However, significant improvements in human IVF culture media were made in the 1990s using two different approaches: the so-called "back to nature" approach and the "let the embryo choose" approach.[230] These approaches resulted in successful culture of human embryos to the blastocyst stage without feeder cell support.

The "back to nature" approach drew upon assessment of the energy substrate profiles in fallopian tube and uterine fluids. The results indicated that, in contrast to the fallopian tube, the glucose concentration in uterine fluid was relatively high, but the lactate and pyruvate concentrations were relatively low.[232] These observations led to development of a pair of media, reflecting these differences in energy substrate concentration, that are used in sequence for support of human blastocyst formation: the first medium, relatively high in lactate and pyruvate, is used from days 1 to 3 of culture, and the second medium, relatively high in glucose, is used from days 3 to 5.[233] The resultant paired media comprise the sequential media system.

The "let the embryo choose" approach is based on determination of blastocyst formation rates following systematic adjustments in medium composition in numerous experiments using the simplex optimization approach.[234] This approach has resulted in development of a single medium for the culture human embryos from day 1 to day 5 or 6. This system is referred to as the single-step system.

Numerous studies support efficacy of both sequential and single-step systems for growing human embryos to the blastocyst stage.[235,236] Although the overall weight of the evidence indicates that neither system is likely superior to the other,[237] the single-step system offers advantages to lab management including increased simplicity and requirement of less quality control testing, and therefore may be more economical.

Embryo Culture Systems

Standard systems used for culturing human embryos are not physiological. Typical systems comprise a culture dish containing: (1) 25-50 μL volumes of medium placed either onto the surface of the dish in microdrops or into wells sunk into the dish surface; or (2) a larger volume of medium that covers the entire surface area of the dish. Regardless of which system is used, all provide fluid, macro, relatively stagnant and chemically static environments on an inert substrate. In contrast, the embryo *in vivo* not only is exposed to a moist 3-D micro environment that is chemically dynamic, but also is in contact with epithelial surfaces rich in glycoprotein.[238] Due to the nonphysiological nature of current IVF systems, new systems and culture platforms are under development that are designed specifically to address physical and chemical requirements of the embryo. These include the Well of the Well or WOW system,[239,240] and a micro-fluidic, micro-channel approach which has the potential to perform almost all steps of the IVF process with minimal embryologist intervention.[241,242] However, none of these innovations have yet to be integrated into routine IVF, and it is unclear whether there is any increase embryo viability and thus implantation rates, over the traditional 2-D systems currently in use.

The one fact that is becoming accepted regarding human embryo culture is the beneficial use of low oxygen tension (5%), rather than atmospheric oxygen (20%). The detrimental effect of 20% oxygen on *in vitro* development of bovine embryos was shown 20 years ago,[243] and recent experiments in mice have revealed that this effect is irreversible.[244] Consistent with these observations, a prospective randomized trial showed that following culture of embryos in low versus high oxygen tension, there was a significant increase in both the conversion rate of human zygotes to blastocysts (47.8% versus 42.1%; $P = 0.02$), as well as the live birth rate (42.1% versus 32.2%; $P = 0.04$).[245] Of note, a recent meta-analysis of studies involving cleavage stage transfers showed no improvement in implantation ($P = 0.63$) rate for embryos cultured in 5% versus 20% oxygen, but a significant increase in implantation rate for the low oxygen tension group after days 5 to 6 transfer ($P = 0.006$).[246] Therefore, the beneficial effect of low oxygen tension may manifest only after activation of the embryo genome on day 3, although further studies are required to investigate this interpretation of available data.

Evaluating Embryo Quality

The goal of evaluating embryo quality is identification of an embryo marker that predicts pregnancy rate more accurately than the age of the female partner and the number of embryos transferred.[247] Such a marker should lead to the development of highly successful single embryo transfer protocols and reduce the risk of multiple gestations.[248]

As with approaches for assessing oocyte quality, those for evaluating embryo quality fall into the two broad categories of invasive and non-invasive. Invasive approaches entail PGS of one, or occasionally two, biopsied blastomeres

Table 31.5 The Timeline for the Development of the Conceptus from Pre-Zygote (Fertilized Egg) to Implanting Blastocyst and the Location of the Conceptus in the Reproductive Tract

Conceptus	Fertilization	Location of Stage of the Days after Conceptus in the Reproductive Tract
Pre-zygote—fertilized	0	Fallopian tube (oocyte)
2-cell embryo	1	Fallopian tube
4-cell	2	Fallopian tube
8-cell	3	Fallopian tube
Morula	4-5	Utero-tubal junction
Blastocyst	5	Intrauterine
Implanting, hatched blastocyst	5-6	Intrauterine

from a cleavage stage embryo, or several trophectoderm cells excised from the trophoblast (see Chapter 32). Non-invasive methods include morphological assessment paradigms, and screening of spent culture media for constituent changes that may relate to embryo viability.

Despite limitations of determining embryo quality using the light microscope, such morphological evaluation remains the first-line approach for assessing viability. Human embryos follow a specific developmental timeline,[249] during which milestones are reached in a coordinated sequence that are typified by characteristic morphological features (Table 31.5) Therefore, utilization of the Darwinian concept of "survival of the fittest" has proved useful. Historically, evaluation has been performed only once, within a few hours of transferring cleavage stage embryos on either day 2 or day 3. In this setting, evaluations have included assessment of cell number, degree of fragmentation (typically assessed as percentage of the volume of the embryo fragmented), cytoplasmic pitting, compaction, symmetry of blastomeres with respect to size and shape, and absence of vacuoles and multinucleation. Although each of these parameters has been shown to have some value in predicting quality and pregnancy rate,[250-252] cell number, fragmentation, and symmetry remain the most commonly assessed characteristics for day 3 embryos. Compared with eight-cell embryos lacking fragments and having perfect to near-perfect symmetry (i.e., blastomeres of uniform size and shape; Fig. 31.21A), those exhibiting low cell number and fragmentation, but with severe asymmetry (Fig. 31.21B), have significantly lower implantation rates,[252] which are further compromised when fragmentation is very high (Fig. 31.21C).

In addition to evaluations performed on day 3, assessments on day 1 have revealed a relationship between implantation potential and prezygote morphology at 16 to 18 hours after insemination (pronuclear size, size and number of nucleolar precursor bodies, and cytoplasmic appearance),[253-255] as well as the ability of embryos to complete the first mitotic division by 27 hours after insemination.[256,257] Moreover, embryos are typically considered of high quality if they are able to reach the blastocyst stage by 120 hours after insemination, exhibit a complete trophectoderm surrounding an expanded fluid-filled cavity, and have a tightly packed cluster of inner cell mass cells.[258]

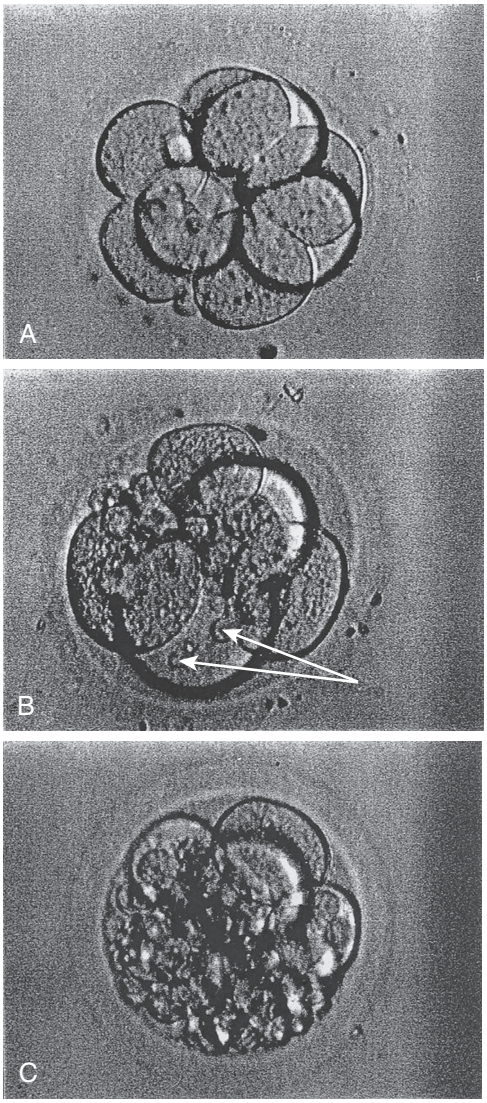


FIGURE 31.21 Day 3 embryos showing various degrees of asymmetry and fragmentation. **A,** An eight-cell embryo with minimal fragmentation but having blastomeres somewhat discordant for size and shape (i.e., exhibiting moderate asymmetry). **B,** A six-cell embryo with severely asymmetrical blastomeres but exhibiting slight fragmentation, with a few fragments interspersed among the blastomeres *(arrows)*. **C,** A five-cell embryo showing moderately asymmetrical blastomeres and a high degree of fragmentation, with more than half the volume of the embryo fragmented, likely resulting from the fragmentation of several blastomeres.

In view of the reported benefit of independent morphological evaluations on specific days of culture, numerous studies have investigated the possible additional value of undertaking morphological evaluations on sequential days in culture. This approach enables a developmental library of morphological information to be built on each embryo that might improve embryo selection. Although several studies do not show benefit in scoring embryos on days other than on day 3 immediately before transfer,[259] other studies indicate that multiscoring systems may enable improved embryo assessment.[260] Indeed kinetic parameters acquired from time-lapse imaging of embryos during very early development hold promise for improving embryo assessment.[261,262]

Early concerns that embryo development might be perturbed by the constant, albeit low level, light exposure required during such imaging have not been borne out by data that show comparable development of sibling zygotes cultured in a standard incubator versus a time-lapse imaging system.[263,264] Retrospective analysis of imaged embryos has shown that three parameters predicted development to the blastocyst stage with greater than 93% accuracy: duration of the first cytokinesis, time interval between the end of the first cleavage and initiation of the second cleavage (from 1 to 2 cells embryo) and synchronicity of the blastomeres in the second cleavage division, from the 2 to 4 cell stage.[265] A further retrospective study has corroborated the apparent significance of duration of the second cell cycle and synchrony of the second and third cell divisions with implantation potential.[266] Another recent study has also documented a relationship between times required to complete the second and third mitotic divisions and the potential to develop to high scoring blastocysts.[267] Of note, however, as all the above analyses were retrospective, it is currently unknown what utility these developmental kinetic markers have for prospective embryo evaluation and selection, and whether evaluation based exclusively on these time-sensitive markers would result in superior implantation rates compared with those obtained following evaluation by conventional morphological evaluation alone. Further research is required in this area, and the ultimate combination of morphological features required for optimum evaluation of developmental competence remains to be identified.

Several studies have investigated possible marker candidates in the medium used to culture the embryos. These markers include soluble human leukocyte antigen (sHLA-G),[268] markers for amino acid metabolism,[269,270] and specific protein peaks associated either with implantation failure or success using proteomic profiling.[271] Each of these approaches hold some promise for identifying a marker(s) for embryonic developmental competency, but their standard application in the IVF laboratory awaits further research and confirmation of efficacy.

Cleavage Stage versus Blastocyst Transfer

Early attempts of clinical IVF in the late 1960s and 1970s began with transfer of cleavage stage embryos on day 2. At that time, *in vitro* systems were rudimentary and so it seemed prudent to remove the embryos from culture and place them in the maternal environment as soon as possible.[5] In addition, because only a handful of oocytes were retrieved and few embryos were created, there was no need for embryo selection, which is the main objective of extending the culture interval in current IVF approaches. Today, with more embryos typically created than will be transferred, the decision regarding whether to transfer at the cleavage (days 2 and 3) or blastocyst (days 5 and 6) stage remains a central challenge in most IVF programs. This challenge is complicated by our continuing inability to predict with certainty which cleavage stage embryos will develop into viable blastocysts.

Transferring human embryos at the blastocyst stage offers several theoretical advantages: (a) improved temporal synchronization between embryo and uterus at the time of transfer; cleavage-stage embryos typically undergo development in the fallopian tube with traversal of the uterotubal junction being at the morula stage, 4 to 5 days after fertilization (Table 31.5); (b) the possibility of selecting developmentally more competent embryos, because blastocyst stage embryos have proved their ability to progress through normal developmental milestones *in vitro* although, of note, selection against aneuploid embryos progressing to blastulation appears to be weak[272]; and (c) the rationale to transfer fewer embryos with higher implantation potential, thereby reducing the risk of multiple gestations.[233,273]

Despite these theoretical advantages to blastocyst transfer, there are several potential disadvantages. Extending culture to the blastocyst stage appears to result in attrition of some embryos that otherwise would support viable pregnancies following cleavage-stage transfer. In one study of blastocyst transfer, when there were no eight-cell embryos present on day 3, the pregnancy rate was higher when transfer occurred on day 3 as compared with day 5 (33% versus 0%).[274] Moreover, for some patients with healthy-appearing day 3 embryos, all of the embryos underwent developmental arrest or degeneration before day 5, leaving no embryos for transfer.[274] The inability to predict which cleavage-stage embryos will develop into blastocysts *in vitro* has led to a significantly higher incidence of canceled embryo transfers in patients randomized to day 3 versus day 5 culture.[275] Blastocyst transfer has also been associated with an increased risk of monozygotic twinning[276,277] and the obstetrically more serious condition of monochorionic twinning.[278] Moreover, a high rate of dizygotic twinning can be expected in good prognosis patients even following transfer of only two blastocysts.[279,280] Finally, concerns have been raised regarding possible adverse effects of longer durations of culture on the risks of epigenetic mutations in offspring,[281,282] particularly as animal data indicate that developmental programming is modifiable by *in vitro* manipulations,[283,284] and children born from blastocyst transfer may be at a slightly increased risk for preterm birth[285] and adverse neonatal outcomes compared with those conceived following cleavage stage transfer.[286]

Randomized controlled trials comparing the efficacy of blastocyst transfer with cleavage-stage transfer have revealed the importance of patient selection (based on patient age, number and quality of embryos, etc.) and the time during treatment for selecting patients to proceed with blastocyst transfer. In a recent Cochrane review of 12 randomized controlled trials involving 759 day 2 and 3 transfers and 751 day 5 and 6 transfers, clinical pregnancy rates were similar for the two groups (OR, 1.14; 95% CI, 0.99 to 1.32), but live birth rates were significantly higher in the day 5/6 group (OR, 1.40; 95% CI, 1.13 to 1.74).[275] However, these results were corroborated only when restricting the analysis to those trials involving women with good prognosis for success (6 RCTs: OR, 1.43; 95% CI, 0.99 to 2.07), and not for those with poor prognosis or who were unselected for trial participation.[275] Moreover, the time point during treatment for selection of patients to blastocyst transfer is important: live birth rates were only significantly increased when randomization occurred on days 2 and 3 (OR, 2.17; 95% CI, 1.42 to 3.33), rather than at cycle start (OR, 1.25; 95% CI, 0.90 to 1.73) or on the day of or after egg retrieval (OR, 0.97; 95% CI, 0.37 to 2.58).[275] This observation

highlights that the overall quality of the cleavage stage embryo cohort directly correlates with the likelihood of successful development of at least one viable blastocyst.

Regardless of prognostic group, fewer surplus embryos are frozen in blastocyst versus cleavage stage cycles, and available data show significantly increased cumulative pregnancy rates in the group of patients having day 2 and 3 transfers (4 RCTS: OR, 1.58; 95% CI, 1.11 to 2.25).[275] This observation is likely due not only to the decreased number of embryos frozen on day 5/6, but also to the historically poor survival of frozen blastocysts experienced by many programs. With the recent establishment of highly successful vitrification methods for blastocyst cryopreservation,[287] it may be that cumulative pregnancy rates with blastocyst transfer will meet, or even exceed, those of cleavage stage transfer.

Collectively, available data indicate that the best candidates for blastocyst transfer are those women with good prognosis (based on age, number and quality of embryos). Serious consideration should be given to single blastocyst transfer in this group because of the risk of dizygotic twinning after transfer of two blastocysts.[288] Indeed, the first IVF human pregnancy occurred after the transfer of a single blastocyst-stage embryo to the uterus, although it resulted in an ectopic implantation.[289] It is possible that for patients with good prognosis, the IVF technique will eventually go full circle, returning to the transfer of a single, blastocyst-stage embryo.

Embryo Transfer

The embryo transfer is conceptually a simple procedure, involving loading the embryo or embryos to be transferred into a catheter and transferring them into the midfundal portion of the uterus. However, it is a critical part of the ART cycle; there may be significant differences in pregnancy rates between clinicians, even when only those transfers that had high quality embryos are included.[290] One study found a range of pregnancy rates from 17% to 54.3% in the same program.[291] The mechanical aspects of embryo transfer appear to influence implantation and pregnancy rates. For example, traumatic embryo transfers resulting in blood on the embryo transfer catheter are associated with a decreased rate of embryo implantation and clinical pregnancy.[292] In addition, transfers made in the presence of high-frequency uterine contractions (as detected by ultrasound) appear to be associated with lower pregnancy rates.[293] Embryo transfers performed with soft, flexible catheters lead to higher pregnancy rates than do rigid catheters.[294,295] This was confirmed in a meta-analysis of published randomized trials.[296]

Performance of transabdominal ultrasound guidance during transfer, if feasible based on patient body habitus, confirms depth of transfer and may reduce trauma to the endometrium, however it does require the patient to have a full bladder, which is uncomfortable for her. Several investigators have reported that ultrasound guidance of the catheter during embryo transfer slightly improves implantation rate and may improve pregnancy rates.[297-299] Although randomized trials have not consistently found that US guidance improves implantation or live birth rates, meta-analyses suggest a benefit. The most recent meta-analysis found that published trials are of poor quality, with 42 of 59 trials excluded from analysis.[299a] Nevertheless the authors concluded that, overall, the ongoing pregnancies per woman randomised to ultrasound guided ET (441/1254 or 35.2%) was significantly higher than for clinical touch (350/1218 or 28.7%); (OR 1.38, 95% CI 1.16 to 1.64, $P < 0.0003$). The majority of studies show that the optimal depth of transfer from the fundus is approximately 15 mm, and pregnancy rates were lower at 1.5 to 2.0 cm as compared to 1.0 cm or greater than 2 cm.[300] Therefore US guidance for ET is appropriate when feasible.

Table 31.6 ASRM/SART Recommended Limits on Number of Embryos to Transfer

Cleavage-Stage Embryos				
Prognosis	**<35y**	**35-37y**	**38-40y**	**>40y**
Favorable*	1-2	2	3	5
All others	2	3	4	5
Blastocysts				
Prognosis	**<35y**	**35-37y**	**38-40y**	**>40y**
Favorable[a]	1	2	2	3
All others	2	2	3	3

*Favorable means first cycle of IVF, good embryo quality, excess embryos available for cryopreservation, or previous successful IVF cycle.
In frozen embryo transfer cycles, the number of good-quality thawed embryos transferred should not exceed the recommended limit on the number of fresh embryos transferred for each age group.
ASRM Practice Committee. Guidelines on number of embryos transferred. *Fertil Steril* 92:1518-1519, 2009.

Number of Embryos to Transfer

The issue of multiple gestations, particularly high-order multiple gestations (triplets and above), is a significant problem in assisted reproduction, associated with poorer obstetric outcomes and the neonatal complications of prematurity and low birth weight. The social and economic impact of such gestations on the families involved and on society in general cannot be underestimated. As a result, numerous approaches have been tried to reduce the rate of multiple gestations. In some Western European countries a legislative approach has severely restricted the number of embryos transferred in IVF cycles. Several countries have included significant disincentives for physicians who exceed the mandated number of transferred embryos. For example, such physicians face loss of license in Sweden and the United Kingdom and potential imprisonment in Germany and Switzerland. The United States has taken a largely voluntary approach, with a series of guidelines on the number of embryos transferred published by SART and the ASRM (Table 31.6). These guidelines, based on age and prognosis, have gradually reduced the acceptable number of transferred embryos, particularly in women age 37 and younger.[301] The result has been a decline in the percentage of triplet births from ART from 11.4% to 7.4%, a 35% decline between 1995 and 2001, with the largest decrease (20.8%) from 1998 and 1999, the year following the publication of the first SART guidelines.[302] The triplet live birth

rate has dropped to 1.5% or less in all age categories in the 2010 national SART data report.[104]

Elective Single Embryo Transfer

Since the 1950s, the incidence of twin gestations has doubled and the number of triplet gestations has tripled. Currently, approximately 40% of twin gestations and 80% of triplet gestations are the result of infertility therapy; however, only 8% of twins and 14% of triplets result from ART.[303] Moreover, since 1998, the percentage of triplets resulting from IVF has progressively decreased.[302,304] Nevertheless, an important question remains: How many embryos should be transferred to maximize the live birth rate and minimize the number of high-order multiple gestations? Overall, there are correlations among the number of embryos transferred and both the pregnancy rate and the high-order multiple gestation rate,[305] with the quality of the embryos transferred an important determinant in both. As discussed earlier, most centers take advantage of this relationship by stringent morphological grading to assess embryo quality. However, in general, scoring of cleavage-stage embryos is not a foolproof predictor of implantation rate. The pregnancy and multiple gestation rates are also dependent on the age of the female partner. For women older than 36 years, more embryos must be transferred to produce the pregnancy rates achieved in younger women,[306] a trend that is even more pronounced in women over the age of 39.[307]

To date the most significant impact on reducing multiple gestations, not only high-order multiples but also twins, has come from the use of elective single embryo transfer (SET). This practice has been more widely accepted in Europe; single embryo transfer represented only 6.7% of U.S. cycles versus 13.7% of European cycles in 2002,[308] and only 9.6% of cycles in women less than 34 years old in the United States in 2009.

Several groups have investigated the use of SET in women undergoing IVF. In the first randomized clinical trial, Gerris and colleagues randomized patients younger than 34 years old undergoing their first IVF cycle with at least two "top-quality" embryos to either SET or double embryo transfer (DET). Within this group with excellent prognosis, those women with a DET achieved a higher ongoing pregnancy rate than those with a SET (74.0% versus 38.5%, $P = 0.013$); however, the multiple gestation rate of 30% was higher in the DET group than in the SET group (30% versus 4%).[309]

A large Scandinavian multicenter study randomized 661 women under 36 years old undergoing IVF treatment to either a single cycle of DET or a cycle with SET, followed by cryoembryo transfer for those patients who failed to conceive in their SET cycle. The live birth rate was 38.8% in the SET group versus 42.9% in the DET group ($P > 0.05$).[310] The authors concluded that the live birth rate was not substantially lower in the SET group. That conclusion, however, was based on the fact that to achieve such a pregnancy rate, two cycles (one fresh and one frozen) were necessary in 158 patients (48%), and the live birth rate for SET-only patients was 27.6%, substantially lower than that in the DET group (42.9%, $P < 0.001$). It is not clear how acceptable to patients two cycles of ART would be to achieve similar results to those in a single DET cycle.

The challenge in balancing the risks of multiple gestations and the lower pregnancy rate with elective SET may lie in defining an appropriate patient population for SET. The studies to date show that SET is a viable option for women younger than 36, undergoing their first or second IVF cycle who have at least two excellent-quality embryos which have high implantation potentials, as blastocyst stage transfer of embryos on day 5 results in higher implantation rates than does cleavage stage transfer and may optimize delivery rates with eSET. Morphologically top quality blastocysts (those expanding by day 5 after oocyte retrieval with a high quality inner cell mass and trophectoderm) may implant at rates of 70% or greater[258,311] with high quality cleavage stage embryos implanting at rates of 50% or more.[312] However, triplets may occur in 2% of double blastocyst transfers,[279] indicating the risk of high order multiple pregnancy after monochorionic twinning. Of note, the SART and ASRM guidelines recommend that strong consideration be given to SET for patients younger than 35 years old having the most favorable prognosis, defined as the first IVF attempt with good-quality embryos and excess embryos for cryopreservation (Table 31.6).

A major contributor to the high-order multiple pregnancy rate may be patients' pressure on physicians to transfer more than two embryos to increase the probability of a successful cycle.[313] This pressure is especially strong when patients pay for their IVF treatment; where patients pay for their IVF therapy, the triplet pregnancy rate is higher than in areas where insurance pays for IVF therapy.[314,315] Shared risk programs, in which young, good prognosis patients pay a fairly high fixed up-front cost, but have a money-back guarantee if they do not take home a baby, may increase acceptance of eSET. [315]

Luteal Phase Support in Fresh Embryo Transfers

In IVF cycles, the ovary and endometrium are buffeted by numerous countervailing forces that are not controlled by normal feedback mechanisms. The endometrium, for example, is exposed to extremely high levels of estradiol and abnormally high levels of androgens. These high hormone levels may result in abnormal epithelial and stromal maturation, especially if luteal progesterone does not adequately balance the estrogen effect. Because oocyte retrieval is associated with the removal of a large number of granulosa cells, the adequacy of ovarian progesterone production after oocyte retrieval has been a major concern since the inception of IVF. Two major methods of luteal-phase support have been used in IVF cycles: progesterone supplementation, usually by intramuscular injection at doses of 25 or 50 mg daily,[316,317] and intramuscular hCG at doses ranging from 1500 to 10,000 IU administered once or more during the luteal phase.[318] Both hCG and progesterone appear to be associated with an increased pregnancy rate compared with no luteal support.[319] In general, hCG is associated with a higher rate of OHSS.[320]

The timing of the initiation of luteal phase support with progesterone affects the pregnancy rate. In a well-designed clinical trial, women undergoing IVF were randomized to receive progesterone supplementation starting either after oocyte retrieval or 12 hours before oocyte retrieval.

The pregnancy rate with progesterone supplementation after oocyte retrieval was significantly better than with progesterone 12 hours before oocyte retrieval (24.6% versus 12.9%, $P < 0.011$).[321] In another well-designed study, women were randomized to start luteal phase support with vaginal progesterone either 3 or 6 days after oocyte retrieval. The clinical pregnancy rate per transfer was significantly better in the group that started luteal phase support at 3 days rather than 6 days.[322] These studies demonstrate that the optimal window for initiating luteal phase support begins after oocyte retrieval but ends by the third day after oocyte retrieval.

The length of time necessary for luteal phase support has not been clearly delineated. Most centers continue support until it is clear whether the patient is pregnant. In one study, discontinuing progesterone support after a positive hCG resulted in pregnancy outcomes similar to continuing progesterone for an additional 3 weeks after a positive hCG.[323] A recent randomized trial in 220 women using micronized progesterone vaginally found no difference in miscarriage or ongoing pregnancy rates when progesterone was discontinued at 5 versus 8 weeks of gestation. However, there was substantially more bleeding in the group discontinuing treatment at 5 weeks.[324] Given the anxiety first trimester bleeding causes patients, treatment beyond 5 weeks seems reasonable.

A major problem with the use of progesterone for luteal phase support is the pain and sterile abscesses associated with numerous daily intramuscular injections. Alternative routes have been explored, although oral administration does not appear to be as effective as intramuscular injection.[325] Randomized trials using vaginal progesterone administered in a polycarbophil gel preparation suggest equivalent pregnancy rates to intramuscular progesterone[317] with one study showing superiority of vaginal gel in young patients.[326] Meta-analyses have not found any difference in delivery rates between different vaginal progesterone preparations including vaginal micronized progesterone suppositories,[327] or between vaginal preparations and intramuscular progesterone.[328]

Another controversy regarding luteal phase support concerns estrogen. Though the importance of the effect of luteal progesterone on the endometrium in implantation and early pregnancy maintenance is well established, the role of supplemental estrogen is less clear. In cycles utilizing GnRH agonists or GnRH antagonists, the profound suppression of gonadotropins in the follicular phase may carry over to the luteal phase. Several groups have investigated the benefit of the addition of estrogen in the luteal phase in IVF. A prospective randomized study involving 166 women undergoing 231 IVF cycles with long-protocol GnRH-agonist suppression examined the effect of luteal phase estrogen support. Participants were given 0 mg, 2 mg, or 6 mg estradiol over the entire luteal phase. Pregnancy rates were improved by estrogen supplementation in a dose-related manner, with the highest rates in the 6 mg group (23.1% versus 32.8% versus 51.3%, $P < 0.001$).[329] A recent randomized trial found that in patients on long GnRHa protocols the addition of 4 mg oral estradiol to vaginal progesterone was associated with lower miscarriage rates than progesterone alone. Other studies have found no benefit, including a randomized trial in poor responders.[330,331] A recent meta-analysis could not confirm a benefit in clinical pregnancy rates with luteal estradiol supplementation.[320]

Adjuncts to Standard IVF Medications

Baby Aspirin

Another area of controversy concerns the utilization of low-dose (baby) aspirin as an adjunct to IVF-ICSI treatment. Aspirin inhibits cyclooxygenase in platelets as well as thromboxane A2 (a potent vasoconstrictor) and prostacyclin (a vasodilator). However, in low doses the vasoconstrictor inhibition predominates. Therefore, use of low-dose aspirin has been proposed to increase ovarian blood flow, improving folliculogenesis and promoting implantation. Some practitioners begin aspirin during ovarian stimulation, while others utilize it only in the luteal phase, believing that it may contribute to excessive bleeding during oocyte retrieval. A meta-analysis of seven trials included 1241 women undergoing ovarian stimulation for IVF or ICSI.[332] There was no significant improvement in IVF and ICSI outcome for women taking aspirin in terms of clinical pregnancy (relative risk [RR] 1.11, 95% CI 0.95 to 1.31) or live birth (RR 0.96, 95% CI 0.64 to 1.39). There were no differences between the groups with respect to miscarriage or ectopic pregnancies. The authors concluded that the currently available data do not support the use of aspirin in IVF cycles.

Acupuncture

Acupuncture has been used as part of traditional Chinese medicine for thousands of years. It is based on the premise that energy (Qi) flows through the body in particular pathways, which are abnormal in disease states, and which can be manipulated through acupuncture. Acupoints, particularly auricular points, are targeted in infertile women to change energy flow in the uterus and ovaries. Electroacupuncture involves attachment of electric cables to the acupuncture needles, whereas manual acupuncture involves inserting and rotating the needles. Studies have used acupuncture before and during stimulation, before and after embryo transfer, and during the luteal phase of the cycle.

In general, studies employing acupuncture before and after embryo transfer have tended to find positive effects on pregnancy rates. A number of studies have been performed. An early study randomized 160 patients with good embryo quality to acupuncture 25 minutes before and 25 minutes after embryo transfer versus no acupuncture.[333] No differences in uterine artery pulsatility were found, and the clinical pregnancy rate was higher in the acupuncture group (42.5% versus 26.3%; $P = 0.03$). Another study employed a similar design, but controls lay quietly for a period of time equal to that of acupuncture.[334] Pregnancy rates in patients with good embryos were equivalent.

In a single blind randomized trial of 228 IVF patients, acupuncture was performed using acupoints determined by the acupuncturist, based on eastern medicine tenets, for each individual patient on day 9 of stimulation and before and after embryo transfer.[335] Control subjects had acupuncture needles placed near but not at true acupoints. The two groups were equivalent for ovarian stimulation and baseline parameters. Ongoing pregnancy rates were significantly higher in the acupuncture group (30.9% versus 22%; $P = 0.08$). A large, well-designed double blind randomized trial

of 370 patients employed acupuncture 25 minutes before and after embryo transfer.[336] The placebo group was treated with retractable needles (sham acupuncture) at the same acupoints. Pregnancy rates were higher in the sham group (55.1 versus 43.8%, $P = .038$) though the difference in ongoing pregnancy rates did not reach significance. It is possible that sham acupuncture had an effect, or that acupuncture has no effect. Another even larger sham-controlled randomized trial of 635 patients[337] found no differences in implantation, clinical pregnancy or live birth rates. A recent study of electoacupuncture in 309 women undergoing fresh or cryopreserved embryo transfer were randomized to "mock" treatment which included weak electrical stimulation and two other groups with higher doses of electrical stimulation once or twice after embryo transfer.[338] Pregnancy rates were lower in the "mock" group; though the groups were equivalent, the study population was poorly defined overall.

Four large meta-analyses have been performed.[339-342] Manheimer and colleagues evaluated 7 trials in which 1366 women were randomized to acupuncture within 1 day of embryo transfer, and controls either underwent no treatment or sham acupuncture.[339] Of those trials reporting clinical outcomes, ongoing pregnancy rates were higher (5 trials, RR 1.87; 95% CI: 1.40 to 2.49) as were live birth rates (4 trials, RR 1.91; 95% CI: 1.39 to 2.64). El-Toukhy and colleagues analyzed five trials, including 877 patients, with acupuncture performed around the time of oocyte retrieval, and found no difference in clinical pregnancy rates (RR = 1.06; 95% CI: 0.82 to 1.37, $P = 0.65$).[340] When they analyzed trials including 1623 patients with acupuncture performed around the time of embryo transfer there was also no difference in pregnancy rates (RR = 1.23; 95% CI: 0.97 to 1.58; $P = 0.1$). Cheong and colleagues analyzed 14 randomized trials involving 2670 subjects.[342] Analyses were performed based on the timing of acupuncture: near the time of ooctye retrieval, on the day of embryo transfer, and repeat acupuncture performed on and after the day of embryo transfer. Six trials included live birth data. Acupuncture around the time of oocyte retrieval had no impact on IVF pregnancy rates, and acupuncture on the day of embryo transfer was only potentially beneficial when therapy was repeated subsequently. The most recent meta-analysis included 24 trials with 5807 total participants.[341] This analysis pooled clinical pregnancy rates from 23 studies and found that they were higher in acupuncture versus control groups. Only six studies included live birth rates, and these did not differ between treatment and control groups.

In summary, the weight of the evidence indicates that acupuncture at the time of oocyte retrieval appears not to be beneficial, but when performed at embryo transfer, the procedure may marginally improve clinical pregnancy rates. However, as discussed by Cheong and colleagues,[342] studies with highly disparate designs are difficult to pool for analysis, and results may be confounded by the fact that sham acupuncture is not necessarily inert.

DHEA Supplementation

Dihydroepiandrosterone, an androgen secreted by both ovaries and the adrenal glands, has been studied as an adjunct to gonadotropins to improve ovarian stimulation in poor responders. Pregnancy rates have been reported to be higher in patients with basal testosterone levels greater than 20 ng/dL.[343] DHEA, via conversion from DHEAS, is responsible for 48% of follicular fluid testosterone synthesis during gonadotropin stimulation.[344] DHEA may also increase ovarian IGF-1 expression and induce FSH receptor up-regulation, amplifying gonadotropin action.[345,346] There are multiple small non-randomized trials of DHEA use before and during IVF in poor responders, some showing increased mature oocyte numbers,[347] and some showing increases in pregnancy rates.[348,349] However, the patient populations and ovarian stimulation regimens differ widely and there is no consistency of dose or duration of DHEA utilized. There is thus far only one small prospective randomized trial. In this, 33 women with diminished ovarian reserve and a prior failed IVF cycle were randomized to receive DHEA 75 mg or placebo daily before and during a similar protocol as used in the prior failed cycle.[350] Day 3 embryo quality was significantly improved and live birth rates were significantly increased (23.1% versus. 4.0%; $P = 0.006$) after DHEA administration compared to placebo.

The mechanism underlying a potential beneficial effect is unclear. It has been postulated that because levels of hypoxic inducible factor 1 levels were significantly lower in poor responders treated with 75 mg DHEA during ovarian stimulation, that improvements in the follicular microenvironment may be induced.[347] One study supplemented women who had decreased ovarian reserve with DHEA 25 mg orally three times per day for 30 to 90 days, and found that serum AMH levels improved by approximately 60% during treatment, particularly in women younger than 38 years old; women who conceived with IVF also showed greater AMH improvements.[351] Therefore there is some biological evidence supporting a potential role of DHEA supplementation in improving IVF outcomes for poor responders, however it is clear that large, controlled trials in well defined patient populations are needed to confirm a benefit, and to define proper dose and duration of treatment.

GH Supplementation

Growth hormone (GH) has been studied for many years as an adjunct to gonadotropins in an effort to improve ovarian response. Evidence indicates that GH directly stimulates the development of small antral follicles to gonadotrophin-dependent stages, as well as maturation of oocytes. GH stimulates insulin like growth factor I. Though IGF-I has no apparent effect on primordial follicle development, both IGF-I and IGF-II stimulate growth of secondary follicles, and stimulate granulosa cell proliferation and steroidogenesis.[352]

The co-administration of GH with gonadotropins to anovulatory hypogonadotropic women reduces the gonadotropin dose required to achieve ovulation.[353]

The literature regarding the use of GH in poor responders suggests an improvement in response and live birth rates, however the definition of poor response and ovulation induction protocols as well as GH dosing vary widely. For example, one study of prior poor responders used a long GnRHa protocol with GH used from the start of agonist at a dose of 4 IU per day and reported a higher number of fertilized oocytes and a trend toward a higher clinical pregnancy rate.[354] In a larger study of 100 women older than 40 years old, not defined as poor responders, GH 8 IU was used daily starting stimulation day 7 through

the day of hCG administration.[355] Peak estradiol levels (1523 ± 203 pg/mL versus 912 ± 129 pg/mL, $P < 0.05$), implantation rates (6.2% versus 1.7%; $P < 0.05$), and live birth rates (22% versus 4%; $P < 0.05$) were higher after GH co-administration. A Cochrane review in 2010 included 10 studies of 440 subfertile couples. A meta-analysis demonstrated a statistically significant difference in both live birth rates (OR 5.39, 95% CI 1.89 to 15.35) and pregnancy rates (OR 3.28, 95% CI 1.74 to 6.20) supporting the use of adjuvant growth hormone in *in vitro* fertilization protocols in women who are considered poor responders.[356] However, the authors caution that the included studies are small, and vary greatly, so it is still unclear which dose of GH should be used, and which subgroups of poor responders will in fact benefit from co-treatment.

Embryo Cryopreservation

Protocols for Embryo Cryopreservation

IVF often generates substantial numbers of excess embryos that cannot be safely transferred fresh in the oocyte retrieval cycle because of the risk of high-order multiple gestation. The development of successful embryo cryopreservation techniques allows multiple transfer cycles after just one ovarian stimulation and oocyte retrieval, as first reported by Zeilmaker and colleagues in 1984.[357] An important issue in embryo cryopreservation is the use of an optimal cryoprotectant and freezing protocol. Although slow cooling methods using cryoprotectants such as dimethyl sulfoxide (DMSO) or 1,2-propanediol have been successfully used for years for cryopreserving both 2PN zygotes and cleavage stage human embryos, vitrification protocols are now commonly used instead. Comparable results for slow-freezing versus vitrification have generally been reported, although prospective randomized trials are limited.[358]

The challenges associated with freezing blastocysts are different from those associated with freezing pronuclear or cleavage stage embryos due to the large fluid-filled blastocoel cavity that presents an increased risk of intracellular ice crystal formation. Early attempts at successful blastocyst cryopreservation using slow-freezing methods varied widely.[359] Although the recent introduction of blastocyst vitrification has provided promising results[287] with some programs achieving implantation rates comparable to those of fresh transfers,[358] more research is needed to evaluate the need and optimum methods for collapsing blastocysts before vitrification,[360] as well as safety issues such as storage, transport and the use of the very high cryoprotectant levels involved.

Protocols for Uterine Preparation

The endometrium appears to be more receptive when endometrial preparation is achieved with a controlled estradiol and progesterone regimen, as compared to that which occurs in an ovarian stimulation cycle. One randomized trial found an 84.0% versus a 54.7% clinical pregnancy rate per transfer after cryopreserved versus fresh cycle transfer.[361] Cryopreserved embryos may be replaced during a natural menstrual cycle, however some studies indicate that uterine preparation using a controlled sequential estradiol and progesterone regimen results in superior pregnancy and live birth rates. Nevertheless a recent Cochrane review did not find a difference in clinical outcomes among different methods of endometrial preparation, or after transfer in a natural cycle.[362] The critical factor may be to ensure synchronization of embryo development with the start of progesterone (i.e., with luteal endometrial development).

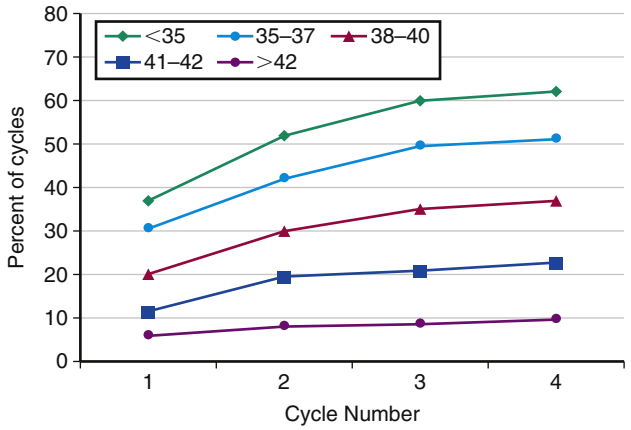


FIGURE 31.22 Plots of the cumulative delivery rates per woman (n = 14,265) for up to four *in vitro* fertilization–embryo transfer cycles according to the age of woman at first cycle using autologous oocytes. *(From Stern JE, Brown MB, Luke B, Wantman E, Lederman A, Missmer SA, Hornstein MD. Calculating cumulative live-birth rates from linked cycles of assisted reproductive technology (ART): data from the Massachusetts SART CORS.* Fertil Steril *94:1334-1340, 2010, with permission of the American Society for Reproductive Medicine.)*

Repeat *In Vitro* Fertilization Cycles

Because the majority of patients completing a single IVF cycle do not become pregnant, many will request a repeat IVF cycle. A specific cause of the IVF cycle failure may be identified (e.g., poor oocyte and embryo yield related to elevated cycle day 3 FSH concentrations, or poor oocyte/embryo quality). In most cases, however, a specific cause cannot be identified. Pregnancy rates appear to drop with repeated IVF attempts, however pregnancies still occur out to eight attempts or more. In one center, pregnancy rates were equivalent in cycles 1 and 2, 3 and 4, and 5 through 8.[363] At some centers, repeated IVF cycles after cycle 3 are associated with lower per-cycle pregnancy rates.[364]

A large study of 14,265 women of various ages from the SART database of Massachusetts residents who underwent up to four ART cycles demonstrated that the cumulative pregnancy rate per woman increased for all five age groups (Fig. 31.22).[365] Another investigation from a single center estimated cumulative pregnancy rates for women up to six cycles. Conservative estimates show a plateauing of pregnancy rates after the fourth attempt. For example, even in young women younger than 34 years old, the cumulative live birth rates after the first, second, third, fourth, fifth, and sixth cycles were 33%, 49%, 59%, 63%, 65%, and 65% respectively.[366] However, data concerning the pregnancy rate in repeat IVF cycles are confounded by decisions made by clinicians and patients. For example, when a patient has had a poor response to ovarian stimulation, the clinician may suggest discontinuation of IVF treatments. This would select patients with a "better prognosis" to repeat IVF. In fact, according to 2009 data from the CDC, 61.2% of U.S. ART cycles were in women undergoing their first cycle,

20.3% in women undergoing their second cycle, 11.3% in women undergoing their third cycle, and 6.1% in women in their fourth and 7.2% who had four or more previous treatments. These data suggest a selection bias in who receives prolonged ART treatment.[367] In every age bracket, women with prior ART cycles but no live births have a lower pregnancy rate than women undergoing their first ART cycle.[368]

IVF Risks and Their Management

Risk of Oocyte Retrieval

Oocyte retrieval can be associated with pelvic bleeding that requires transfusion or surgical exploration, or pelvic infection. Both complications are rare, occurring in fewer than 1 in 500 cases. A large prospective study of 2670 oocyte retrievals reported vaginal hemorrhage in 8.6% of cases, with a significant loss (estimated at greater than 100 mL) in 22 (0.8%). There were two cases of post procedure bleeding from an ovary causing hemoperitoneum. One case required emergency laparotomy. There was one case of pelvic hematoma formation which did not require intervention. Postoperative pelvic infection occurred in 18 (0.6%) of the cases and included 9 with pelvic abscess formation. Examination of the pus from these cases suggested inoculation of vaginal organisms into the peritoneal cavity by the oocyte retrieval needle.[369] Another prospective study of over 1058 cases assessing complications related to ultrasound-guided transvaginal retrieval reported vaginal bleeding requiring tamponade (2.8%) but no suturing was needed. There were no infections. There were no complications related to anesthesia (95% had general anesthesia and 5% had sedation with midazolam), 0.7% of patients had severe postprocedural pain requiring hospital admission, and it is possible that these patients did have postoperative bleeding as hemoperitoneum after retrieval causes significant pain. There was one ureteral injury.[370] A survey questionnaire study of over 10,000 IVF cycles in several centers reported a 0.5% complication rate after oocyte retrieval.

Risk of Fertilization Failure

Total fertilization failure (TFF) after standard IVF is an infrequent and unpredictable phenomenom, with the reported incidence ranging from 5% to 20%,[370a-c] in couples with otherwise unexplained infertility. Some studies in which idiopathic infertility couples had their oocytes split between ICSI and IVF suggest that such couples may have improved fertilization with ICSI.[371,372] However, since transfers involved embryos from both IVF and ICSI, it is difficult to determine the efficacy of one treatment over the other. Of note, at least two randomized trials of ICSI versus IVF for couples with unexplained infertility have shown no benefit of ICSI in terms of fertilization, implantation, clinical pregnancy, or live birth rates.[373,374]

Following TFF with standard IVF insemination, ICSI in the next cycle overcomes TFF in more than 95% of cases,[375,376] thus implicating failure of normal sperm and oocyte interactions including the abnormal release of sperm proteasomes resulting in inhibition of sperm binding to the zona pellucida.[377] In those rare cases that fail to fertilize following ICSI, studies with heterologous gametes have been undertaken to help determine the contributing factor.[378] Although an instance of spontaneous activation has been reported,[379] post-ICSI failure to fertilize is most likely due to the inability of the oocyte to activate.[15] Artificial oocyte activation (AOA) protocols using calcium ionophores,[380,381] strontium chloride,[382] or electrical impulses[383,384] have therefore been used. However, despite reassuring reports on obstetric, neonatal, and 1-year-old children, the routine use of AOA warrants caution, as alterations in calcium oscillations are associated with alterations in gene expression and embryonic development in a mouse model.[385,386]

Ovarian Hyperstimulation Syndrome (OHSS)

Current regimens of ovarian stimulation for IVF typically produce major increases in follicle numbers as well as circulating estradiol, with estradiol concentrations routinely reaching in the range of 1200 pg/mL or higher. The severity of OHSS is correlated most closely with the number of small to medium sized follicles at the time of hCG administration.[387] The intensive stimulation of ovarian follicle growth increases the risk of OHSS (see Chapter 30). Circulating androgen levels also increase substantially in women undergoing ovarian stimulation for IVF (Fig. 31.23)[388] though the impact on the ovary and endometrium remains to be fully characterized. However, in many model systems, excessive follicular androgen secretion is associated with suboptimal follicle and oocyte function. Although GnRH-agonist analogs have few adverse effects in ovarian stimulation protocols for IVF-ET, cases of OHSS caused by the administration of the GnRH agonist alone have been reported.[389]

Ovarian stimulation with gonadotropins for IVF is associated with up to a 5% risk of severe OHSS. Risk factors seem to be peak estradiol concentrations greater than 2000 pg/mL, the presence of more than 15 follicles greater than 12 mm in diameter, and the establishment of a successful pregnancy.[390] OHSS that develops during IVF cycles typically resolves with conservative management (see Chapter 30).

A number of strategies have been proposed to prevent OHSS. Withholding hCG and cancellation of the cycle is effective but fails to achieve a pregnancy. Continuing the cycle and freezing all embryos created is largely effective but requires a second cycle (albeit a simpler, cryoembryo transfer) to achieve a pregnancy. As previously noted, early OHSS incidence is reduced in GnRH antagonist cycles where an ovulatory dose of a GnRH-agonist is administed, even with very elevated estradiol levels. However, if a patient at high risk of OHSS conceives, placental hCG generally causes the syndrome to worsen and be more prolonged.

Another strategy involves "coasting," in which the doses of gonadotropins are withheld (sometimes for days) until estradiol levels decline to safer levels along with less follicular stimulation, likely decreasing the cohort of growing follicles.[391] The number of oocytes obtained, not suprisingly, is generally lower than the number of follicles measured on the day of hCG administration. A randomized trial comparing coasting to GnRH antagonist treatment in women at high risk of OHSS found fewer eggs and fewer high quality embryos in the coasting arm, but pregnancy rates were not different; no OHSS occurred in either arm.[392] A Cochrane review identified 16 studies related to coasting and OHSS, however only 4 met study criteria, and the authors concluded that a benefit of coasting for OHSS prevention could not be documented.[393] Intravenous administration of albumin has not been shown to reduce the incidence of OHSS

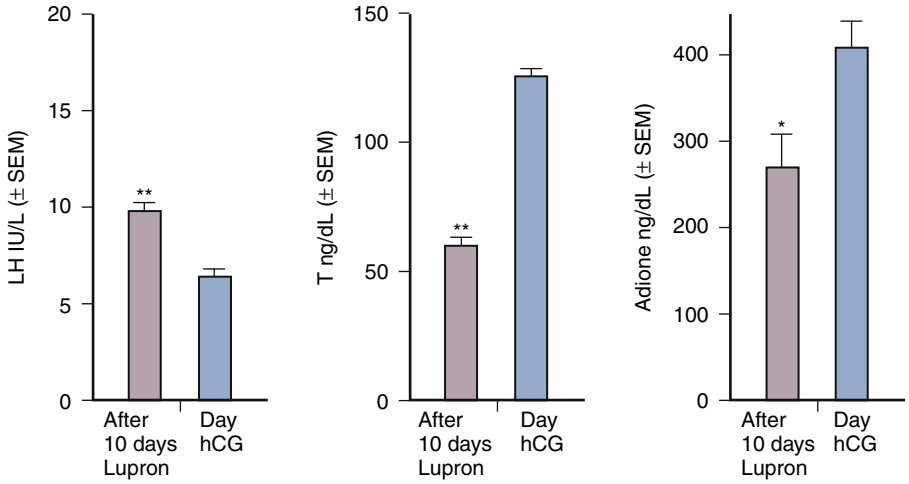


FIGURE 31.23 Androgen levels in normal ovulatory infertile women undergoing *in vitro* fertilization and receiving leuprolide (Lupron) and hMG (human menopausal gonadotropin) for ovarian stimulation in preparation for oocyte retrieval. Luteinizing hormone (LH), testosterone (T), and androstenedione (Adione) were measured after 10 days of leuprolide acetate treatment, 1 mg daily by subcutaneous injection, and then again after leuprolide plus hMG stimulation, just before the administration of human chorionic gonadotropin (hCG). After 10 days of leuprolide treatment, LH concentrations were in the normal follicular phase range. Testosterone and androstenedione concentrations increase significantly with ovarian stimulation. Mean, SEM; *$P < 0.05$; **$P < 0.001$. *(From Martin KA, Hornstein MD, Taylor AE, et al. Exogenous gonadotropin stimulation is associated with increases in serum androgens in IVF-ET cycles.* Fertil Steril *68:1011-1016, 1997, with permission of the American Society for Reproductive Medicine.)*

in randomized trials; a meta-analysis including 1999 subjects found the OR for OHSS to be 0.8 (CI 0.52 to 1.22) and pregnancy rates were unaffected.[394]

OHSS does appear to be at least partially preventable by the administration of the dopamine agonist cabergoline to at-risk patients.[395] The enzyme tyrosine hydroxylase, critical in the production of dopamine, is down-regulated eightfold in OHSS.[396] By adding the dopamine agonist cabergoline, Alvarez and colleagues hoped to restore dopamine and at the same time reduce vascular permeability through the vascular endothelial growth factor (VEGF) pathway. They randomized 82 oocyte donors at risk of developing OHSS to 0.5 mg daily cabergoline for 8 days or placebo. The incidence of moderate OHSS was reduced to 20% in the cabergoline group compared with 44% in the placebo group ($P = 0.04$). Hematocrit, hemoglobin, and ascites were all significantly lower in the group treated with cabergoline compared to placebo.[395]

There are no randomized trials of cabergoline for prevention of OHSS in women undergoing embryo transfer that are powered to evaluate ongoing pregnancy rates, and in one randomized trial of women undergoing embryo transfer, only early OHSS was reduced.[397] Therefore, at this time we suggest limiting use of cabergoline to cycles in which no embryo transfer is planned. To date, one randomized trial compares cabergoline to GnRH antagonist for treatment of early onset OHSS, with improved resolution on antagonist.[398] Another method to reduce the severity of OHSS is the use of GnRH agonists after hCG administration.[399,400]

For patients with established severe OHSS including tense ascites, paracentesis may both ameliorate symptoms and lessen duration and severity.[401] Paracentesis should be performed with sterile technique using either a transvaginal or transabdominal approach, based on the location of the largest pockets of ascitic fluid. It is safe to drain liters of ascitic fluid in one procedure. This is also a more cost effective approach than is hospitalization.[402]

Ectopic Pregnancy

IVF is known to carry the risk of ectopic pregnancy, with the incidence in some reports double to triple that in the general population. The Bourne Hall Clinic, site of the first successful human IVF treatment, reported an ectopic pregnancy rate in 1995 of 4.5% (135 of 3,000 clinical pregnancies in IVF patients).[403] However, a more recent report of SART clinic data found an ectopic pregnancy rate of 1.8% of cycles initiated, and 2.2% of clinical pregnancies.[404] The high prevalence of tubal disease and endometriosis in women undergoing IVF is associated with ectopic risk. It has also been suggested that factors intrinsic to the IVF process such as high volumes of culture medium used for embryo transfer may contribute.[403,405] There does not seem to be a difference in the incidence of ectopic pregnancies between women undergoing a day 3 or day 5 embryo transfer.[406]

Heterotopic pregnancies, involving a concomitant intrauterine and extrauterine gestation, occur in as many as 1 in 100 pregnancies after IVF. This is due to both the transfer of multiple embryos and the underlying fertility problems of the IVF population, including tubal disease. In the general population, the rate of heterotopic pregnancies is approximately 1 in 3500 conceptions.[407,408] The ectopic pregnancy can be treated either by surgical removal or by the selective injection of potassium chloride or hyperosmolar glucose.[409,410] If the ectopic pregnancy is to be treated surgically, salpingectomy rather than salpingostomy is appropriate; in a heterotopic pregnancy, it is not possible to determine whether the salpingostomy has been successful, because the intrauterine pregnancy maintains elevated hCG levels, so that documentation of complete resolution by following hCG levels down is not possible. If the ectopic pregnancy is removed surgically, vasopressin should be avoided, and sutures or the harmonic scalpel may be preferable to electrocautery

to minimize the chance of diminished blood flow to the intrauterine pregnancy.[411,412]

Cancer Risk in Women Undergoing IVF

Although some studies have suggested that drugs used to induce ovulation may be associated with an increased risk of ovarian cancer, the data are weak. Infertility and nulliparity are risk factors for ovarian cancer.[413] Most recent studies have reported no relationship between cancer and ovarian stimulation in women who have had IVF. A Swedish registry study reported that of 1,394,061 women, 95,775 had cancer. Of 24,058 women who had been treated with IVF, 1279 appeared in the cancer register. Cancer risk was elevated prior to IVF treatment (OR 1.37, 95% CI 1.27 to 1.48) and was especially high for ovarian cancer (3.93). The risk for cancer after IVF was significantly lower (OR 0.74, 95% CI 0.67 to 0.82), mainly due to a lower than expected risk for breast and cervical cancer. The risk of ovarian cancer was increased, but lower than the risk before IVF, with an OR of 2.13. The authors hypothesized that there may be a joint etiology for both infertility and ovarian cancer risk.[414] A recent meta-analysis of infertile patients concluded that the risk of ovarian cancer does not appear to be increased by treatment with ART compared to nontreatment.[415] A cohort of 9175 women who purchased medications for IVF performed between 1996 to 1998 in Finland were compared to age and residence-matched controls and further adjusted for socio-economic position and marital status. A linkage to the Finnish Cancer Registry for 1996 to 2004 was perfomed. The overall cancer incidence and combined incidence of hormonal-related breast, uterine and invasive ovarian cancers were similar among IVF women and controls. IVF women had less cervical cancer (OR 0.51, 95% CI 0.30 to 0.85), but more skin cancers other than melanoma (OR: 3.11, 95% CI: 1.02 to 9.6). IVF women had more invasive ovarian cancers and fewer breast than controls, but the numbers of cases were small and not significantly different. Larger studies are needed.[416]

There are conflicting data concerning an increased risk of breast cancer and IVF treatment. Late maternal age at first pregnancy is a well described risk factor for breast cancer, or in the offspring derived from IVF pregnancies.[417] In a recent cohort study of 21,025 Australian women, 7123 of whom underwent IVF, there were 384 breast cancers (55 were *in situ*). IVF utilization in the cohort was fairly low, with 50% of those who underwent IVF having only 1 to 2 cycles. There was no overall increase in the rate of breast cancer in women who had IVF (HR 1.10, 95% confidence interval [CI] 0.88 to 1.36). However, there was an increased rate in women who began IVF treatment when younger than 24 years old compared with those of the same age who did not undergo IVF, even after adjusting for late age at first delivery and multiple gestation (HR 1.56, 95% CI 1.01 to 2.40).[418] Of note, however, other covariates such as socioeconomic status and age at menarche were not taken into consideration in this analysis.

Management of High Order Multiple Gestations

Couples with a multiple gestation have the choice of expectant management or elective reduction of one or more fetuses. The most widely used method of elective reduction is transvaginal or transabdominal injection of potassium chloride into the fetal heart. The transabdominal approach is usually performed at 10 to 13 weeks of gestation; the transvaginal approach is typically used earlier.

For those couples who choose expectant management, approximately 15% will spontaneously lose one fetus, resulting in a twin pregnancy.[419] The risk of spontaneous miscarriage of the entire triplet pregnancy was 25% in one study of 12 expectantly managed triplets compared to 6% in 143 triplet pregnancies that underwent reduction to twins.[420] The risk of significant prematurity was 25% in the nonreduced triplets and 4.9% in those reduced to twins. In a retrospective series of 185 triplet pregnancies that underwent reduction to twins and 70 that were expectantly managed, median birth weight was 500 g higher and gestation was about 3 weeks longer in the reduced pregnancies. The rates of preterm delivery were significantly lower in the reduced group (11.17 versus 36.76% for delivery at ≤32 weeks and 40.58 versus 83.82% for delivery at ≤35 weeks, reduced versus nonreduced group).[421] Likewise the expectantly managed triplets were more likely to have very low birth weights (10.98% versus 28.44% for birth weight ≤1500 g and 68.55 versus 92.89% for birth weight ≤2500 g, reduced versus nonreduced group). Total fetal loss was significantly higher in the reduced group than in the nonreduced group (15.41% and 4.76%, respectively), mainly due to the higher miscarriage rate in the reduced group (8.11% versus 2.86% in the nonreduced group). The authors extrapolated the results to predict that severe handicaps would be expected in 0.63% of children due to extreme prematurity in the reduced group and 1.64% in the nonreduced group. A review of data pooled from 6 studies had 482 expectantly managed triplets and 411 that were reduced to twins. The rate of miscarriage was higher (8.1% versus 4.4%; relative risk [RR] =1.83, 95% confidence interval [CI] = 1.08 to 3.16, $P = 0.036$) and the rate of early preterm delivery was lower (10.4% versus 26.7%, RR= 0.37, 95% CI = 0.27 to 0.51, $P < 0.0001$) in the reduced group. Reduction of a triplet pregnancy to a singleton rather than a twin increases the likelihood of term live birth without increasing fetal loss rates.[422]

Some researchers maintain that it is "fairly well established" that multifetal reduction for quadruplet pregnancy improves pregnancy outcomes. Although no randomized studies have been reported, some series observe an increase in the length of gestation from 31 weeks to 35 weeks, in quadruplet pregnancies that had been successfully reduced to twins.[423] Nevertheless, reduction in the number of quadruplet pregnancies after IVF treatment is undoubtedly best achieved by transferring no more than two high-quality embryos in those women at highest risk for multifetal pregnancy.

Birth Outcomes in IVF Pregnancies (Multiple and Singleton Gestations)

The majority of twin births are naturally conceived, however the incidence of multiple birth delivery is 20 times higher with IVF treatment.[424] Transfer of multiple embryos is the primary reason for the high incidence of multiple gestation in IVF pregnancies,[302] though the incidence of

monochorionic twinning has been reported to be increased by procedures such as ICSI, assisted hatching, and blastocyst transfer.[278] The outcome of pregnancies produced by IVF is directly related to the number of fetuses established. Of the live births resulting from IVF in the U.S. in 2009, 29% were twins, and 3% were triplets or higher-order gestations.[425]

Pregnancies complicated by multiple gestations are associated with increased prematurity, low birth weight, gestational hypertension, diabetes, a higher incidence of birth defects, and increased perinatal mortality rate. Early studies proposing an association between IVF and poor obstetric and perinatal outcomes often did not adequately control for the increased rate of multiple gestations associated with IVF treatment. Several recent large studies and meta-analyses have reviewed the obstetric outcomes associated with ART. The FASTER (First and Second Trimester Evolution of Risk) Research Consortium, sponsored by the National Institute of Child Health and Human Development (NICHHD) prospectively investigated singleton pregnancies from an unselected obstetric population between 1999 and 2002.[426] A total of 36,062 pregnancies, including 1222 that used ovulation induction and 554 that used ART, were evaluated. Ovulation induction was associated with a greater risk of placental abruption, total loss after 24 weeks, and gestational diabetes, and IVF was associated with an increase in preeclampsia, gestational hypertension, placental abruption, placenta previa, and risk of cesarean delivery compared with naturally established pregnancies. There was no association between IVF and total growth restriction or aneuploidy for fetal anomalies. Significantly, this study adjusted for age, race, marital status, years of education, prior preterm delivery, prior total anomaly, body mass index, and bleeding with the recent pregnancy. A meta-analysis of 15 studies comprising 12,283 IVF and 1.9 million spontaneously conceived singletons concluded that IVF singleton pregnancies were associated with increased rates of perinatal mortality (OR 2.2, 95% CI 1.6 to 3), preterm delivery (OR 2, 95% CI 1.4 to 2.2), low birth weight (OR 1.8, 95% CI 1.4 to 2.2), very low birth weight (OR 2.7, 95% CI 2.3 to 3.1), and small size for gestational age (OR 1.6, 95% CI 1.3 to 2.0).[427] In a more recent large cohort study in 242,715 women with singleton pregnancies of whom 4111 used ovulation induction, 2351 used IUI and 4570 underwent IVF, all the ART procedures were associated with increases in preterm delivery, abnormal placentation and small for gestational age babies, suggesting that the causes of infertility rather than the type of fertility treatment underly the findings.[428] A systematic review of controlled studies concluded that IVF singleton pregnancies were at elevated risk for very preterm and preterm births as well as very low birth weight, low birth weight, and small-for-gestational-age infants. The IVF pregnancies were associated with more neonatal intensive care unit (NICU) admissions, a higher incidence of cesarean delivery, and an increased perinatal mortality rate.[429] A large meta-analysis examining 27 studies resulting in singleton births concluded that the risk of preterm births in singleton pregnancies resulting from IVF-ET and GIFT was twice that of naturally conceived pregnancies.[430]

An NICHD workshop on infertility, assisted reproductive technology, and adverse pregnancy outcome summarized the areas of increased risk as follows: Increased perinatal risks included preterm birth, low birth weight (less than 2500 g), very low birth weight (less than 1500 g), small size for gestational age, NICU admissions, stillbirths, neonatal mortality, and cerebral palsy. Maternal risks identified were preeclampsia, placenta previa, placental abruption, gestational diabetes, and cesarean delivery.[431] An American College of Obstetricians and Gynecologists Committee opinion concluded that "a growing body of evidence suggests an association between pregnancies resulting from ART and perinatal morbidity (possibly independent of multiple births), although the absolute risk to children conceived through ART is low."[432] Some have argued, however, that it is not possible to separate the ART-associated perinatal risks from those due to the underlying reproductive disease.[431] Infertile couples may also be at risk for a wide range of adverse reproductive outcomes not necessarily related to their treatment. A large cohort study from Belgium examined the perinatal outcome of 12,011 singleton and 3108 twin births from non-IVF assisted reproduction treatments compared with outcomes from natural conceptions.[433] The treatments consisted of controlled ovarian stimulation with and without intrauterine insemination. Among singletons resulting from infertility treatment, there was a higher incidence of prematurity, low and very low birth weight, and transfer to the NICU; among twin pregnancies resulting from infertility treatment they found an increased rate of neonatal mortality, assisted ventilation, and respiratory distress syndrome. The study demonstrated that both singletons and twins resulting from non-IVF fertility treatment have worse perinatal outcomes than naturally conceived children.

Another clue as to why IVF pregnancy outcomes may be worse than naturally conceived pregnancies comes from two Scandinavian studies examining the effect of vanishing twins on pregnancy outcomes from IVF.[434,435] The Danish study included 642 survivors of a vanished co-twin, 5237 primary singletons, and 3678 primary twins. The rate of small-for-gestational-age (SGA) infants was significantly higher in survivors than in singletons (OR 1.5, 95% CI 1.03 to 2.2), as was the risk of low birth weight (OR 1.71, 95% CI 1.06 to 2.74). The Swedish study included 8941 IVF singletons, and also found a significantly higher rate of SGA in those singletons associated with a vanishing twin.

Because ART cycles produce more multiple gestations than naturally conceived pregnancies, it is likely that they also produce more vanishing twins. The data suggest that the survivor of a vanishing co-twin has a worse obstetric prognosis than a fetus conceived as a singleton. Whether or not the vanishing twin phenomenon accounts for all or some of the increased perinatal risk seen in IVF singleton, but not IVF twin gestations, requires future investigation. A study evaluating 12,021 singleton and 3108 twin births compared those naturally conceived to those conceived through ovulation induction with or without IUI (OI and IUI). Cases were matched for maternal age, parity, fetal sex, and year of birth. There was a significantly higher incidence of prematurity (less than 32 and less than 37 weeks), low and very low birth weight, transfer to the neonatal intensive care unit and most neonatal morbidity parameters for the OI and IUI singletons, and twin pregnancies resulting from OI and IUI showed an increased rate of neonatal mortality, assisted ventilation and respiratory distress syndrome.

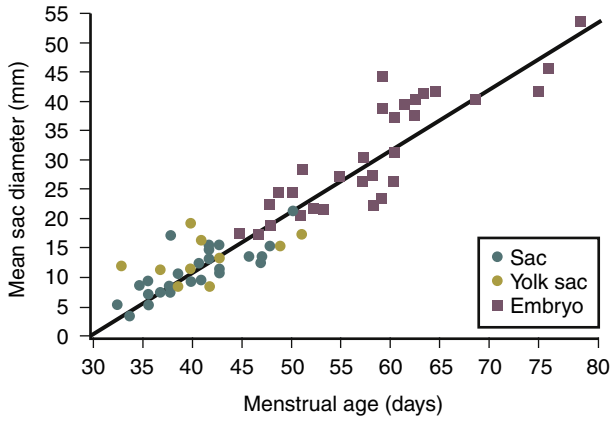


FIGURE 31.24 Relationship between pregnancy dating as determined by menstrual age and gestational sac size as measured by the mean sac diameter. *(From Nyberg DA, Mack LA, Liang FC, Patten RM. Distinguishing normal from abnormal gestational sac growth in early pregnancy* J Ultrasound Med *6:23-27, 1987.)*

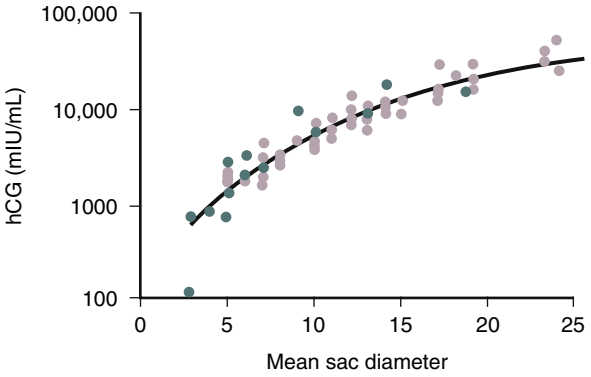


FIGURE 31.25 Relationship between mean gestational sac diameter as measured by the mean sac diameter and the serum human chorionic gonadotropin (hCG) concentration. *Green dots represent present series; gray dots represent previously published data. (From Nyberg DA, Mack LA, Liang FC, Jeffrey RB. Early pregnancy complications: Endovaginal sonographic findings correlated with human chorionic gonadotropin levels.* Radiology *167:619-622, 1988.)*

These data suggest that subfertility rather than procedures used to treat it underlie the poorer neonatal outcomes seen in IVF pregnancies.[433]

Pregnancy Outcome

Among women who achieve pregnancy after IVF, approximately 20% will experience a spontaneous abortion, and as previously reviewed 2% to 4.5% will have an ectopic pregnancy. Clearly, the incidence of miscarriage is highly associated with maternal age (Fig. 31.12) due to increasing aneuploidy as women age. Studies have not shown that paternal age contributes significantly to miscarriage or birth defects.[436] Early pregnancy should be monitored both by transvaginal sonography and by serial hCG levels to ensure that an abnormal pregnancy is diagnosed as early as possible (Figs. 31.24 and 31.25).

Birth Defects

The issue of IVF and the risk of birth defects is complex and the analysis of this subject has been limited by several factors including additional testing performed on ICSI offspring, underreporting of malformations in national birth registries, the effect of maternal age, the effect of multiple gestations, and the inclusion of severe male factor patients, whose children may be subject to an increased rate of chromosomal anomalies due to paternal transmission.[437,438]

The picture is further complicated by the fact that congenital malformations are more common in offspring from multiple pregnancies than from singleton pregnancies and are more common in children of women of advanced reproductive age. Because multiple gestations in older women are more common in IVF pregnancies, a potential bias may be introduced. Therefore, analyses restricted exclusively to singleton births in age-matched women make most sense. One of the difficulties in designing such studies is that pregnancies resulting from IVF are often scrutinized more carefully than other pregnancies. For example, the frequency and intensity of ultrasound testing might be higher in such pregnancies. In one of the larger and better-controlled matched cohort studies, Hansen and colleagues reported a twofold increase in major malformations from IVF (8.6%) compared to normal control subjects (4.2%) from a birth registry in Western Australia.[439] A strength of this particular study was that a single-blinded pediatrician determined which anomalies would have been detected independent of the surveillance method used. However, even this approach has been criticized on the presumption that a pediatrician could hypothesize which malformation might be ICSI-induced.[440]

A study from the Danish national birth cohort compared the incidence of congenital malformations in children born from three groups of couples: fertile couples, infertile couples who conceived without treatment, and infertile couples who required treatment to conceive.[441] Compared to children conceived by fertile couples, singletons conceived by infertile couples without treatment had a higher rate of malformations, hazard ratio (HR 1.70, 95% CI 1.07 to 1.35); and for those conceiving with treatment, the (HR 1.39, 95% CI 1.23 to1.57). Although this study did not specifically examine the types of infertility treatment used, and thus did not address the effect of IVF on birth defects, it did demonstrate that infertility in and of itself confers a higher risk of birth defects, even without treatment.

In the largest U.S. study to examine congenital malformations associated with infertility treatments, Olson and colleagues compared offspring from naturally conceived pregnancies, IVF, and intrauterine inseminations (IUI).[442] The malformation rate among the IVF children (6.2%; OR 1.44, 95% CI 1.12 to 1.85) was higher than among control subjects (4.4%), though the rate among IUI children was not statistically higher than the control group (5.0%; OR 1.14, 95% CI 0.7 to 1.87). The authors speculated that they were not able to show an increase in malformations among the IUI-conceived children because the number of infants was so small ($n = 343$) compared to that in the IVF group ($n = 1462$) or the control group ($n = 8422$).

A study of 15, 045 babies born after ART from 7 centers in China found a much lower incidence of birth defects than in other studies, with one or more malformations in 1.25% of all ART babies combined, compared to 1.35% in the general population. The malformation rate was higher in ICSI than in

IVF babies, but failed to reach statistical significance (1.58% versus 1.11%; OR, 1.42; 95% CI,0.99 to 2.03; $P = 0.52$).[443]

A recent cohort study of 308,974 births, of which 6163 were from assisted reproduction, was unique in that it included couples with infertility who conceived without treatment, couples who underwent IVF, and couples who underwent IVF and ICSI for male factor and nonmale factor infertility. The incidence of malformations was 8.3% in the ART group and 5.8% in babies not involving ART (unadjusted OR 1.47; 95% CI 1.33 to 1.62). After multivariate adjustment, the OR was 1.28 (1.16 to 1.41). When IVF and ICSI were analyzed separately after multivariate adjustment, the OR was no longer significant for IVF (7.2% birth defect rate; OR 1.07, 0.90 to 1.26) but was for ICSI (9.9% birth defect rate; OR 1.57, 1.30 to 1.90).[444] Of note, couples with a history of infertility, with or without ART treatment, were also at increased risk of having children with malformations.

Several meta-analyses have been performed to investigate any association between ART and malformation rate in offspring. One reviewed 25 studies comparing birth defects in ART and non-ART offspring.[445] The overall OR of malformations was 1.29 (95% CI 1.21 to 1.37) and reached statistical significance. The statistical significance remained in subgroup analysis of singleton births and major malformations. The authors concluded that there is an overall 30% to 40% increased risk of birth defects associated with ART. A more recent meta-analysis identified studies published into 2011 and included 56 studies, 46 of which compared IVF/ICSI babies to those spontaneously conceived and found a pooled OR of 1.37 (CI 1.26 to 1.48). Twenty four studies compared IVF and ICSI babies and found no difference in malformation rates 1.05 (0.91 to 1.20).[446] However, many of the included studies had crude, rather than adjusted risks. Tests for heterogeneity found that studies differed widely, with some assessing birth defects only at birth, some up to age 2, some were population based and some clinic based. In addition, the absence of infertile control groups conceiving without ART was a major deficiency, as discussed by the authors.

Another way to consider birth defect data is to analyze specific malformations. Although cardiovascular, musculoskeletal, and urogenital malformations seem more common in IVF offspring than non-IVF offspring, no single defect seems to predominate.[447]

Taken together, the literature suggests that infertile couples are at higher risk of having children with congenital malformations, and that this may be increased further by the use of ICSI.

Risks of Cancer in IVF Children

A Swedish cancer registry study of the years 1982 to 2005 was compared to a registry including data on IVF. An increased risk of Langerhans cell histiocytosis (LCH) was found in children born from IVF prior to 2002. The OR for LCH children born after IVF was 3.2 (CI 1.4 to 7.3), with a higher incidence prior to 2002 (OR 5.2, CI 2.3 to 11.9). The reason for the association was unknown.[448] A recent case control study performed in Greece and Sweden found an association between early childhood acute lymphoblastic leukemia (ALL) (age under 3.8 years) and ART.[449] Another Swedish study followed a cohort of 26,692 children born after IVF 1982 to 2005 and compared cancer incidence in them and children not conceived with ART. Factors including premature delivery, respiratory diagnoses, and low Apgar scores were risk factors for cancer in children and young adults. There were 53 cases of cancer in IVF children compared to 38 expected; 18 were leukemias and of them 15 were ALL. There were six cases of Langerhans histiocytosis (1.0 expected). The risk estimate for cancer was 1.42 (CI 1.09 to 1.87). The authors concluded that the risk factors could be preterm birth and neonatal hypoxia rather than ART procedures themselves.[450]

Overall, recent evidence raises questions about an increase in childhood cancers in children conceived through IVF. However, further research is needed to investigate this possibility.

Epigenetic Abnormalities

It has recently been postulated that reproductive technologies may be associated with epigenetic changes in one of the two inherited alleles for a particular imprinted gene. Such modifications, which may result from changes in differential DNA methylation or modifications in the histones, lead to either activation or suppression of a gene.[451] Attention has particularly focused on the possibility that ART is associated with an increase in imprinting defects resulting in rare congenital disorders such as Beckwith-Wiedemann and Angelman syndromes. In 2003, DeBaun and colleagues were the first to report an association between an imprinting disorder and ART.[452] Among ART offspring, they found a 4.6% prevalence of Beckwith-Wiedemann syndrome, a congenital disorder involving growth and neoplasia, compared with a background rate of 0.8% in the U.S. population. An increased incidence of several other imprinting syndromes has been reported in children conceived through assisted reproductive technologies.[281]

Although one study from the Netherlands reported five cases of retinoblastoma in children from IVF, a much higher prevalence than seen in the general Dutch population,[453] the Boston Collaborative Drug Surveillance Program identified no children born from IVF with retinoblastoma compared with 6.7 cases per 100,000 births in the general population.[454] A very large study from the Swedish national health registry involving more than 16,000 children born after ART failed to find an increase in childhood cancers or infant tumors in either IVF or ICSI offspring.[455] A study from the Netherlands National Retinoblastoma Center identified retinoblastomas in seven children conceived by IVF or ICSI but no hypermethylation of the RB1 promoter was seen; this study reached the conclusion that an association between IVF and ICSI and retinoblastoma was unlikely to be due to an epigenetic mechanism.[456]

Aside from specific imprinting diseases as discussed above, attention has recently been focused on subtle genome-wide epigenetic alterations in phenotypically normal children conceived from ART.[457] Widespread changes in DNA methylation have been identified, including genes linked to adipocyte development, insulin signaling, and obesity. These observations may link to reports that metabolical parameters and body composition may differ between children conceived via IVF and spontaneous conception. From 8 to 18 years of age, IVF children appear to have altered body fat composition, though they also appear to have a

more favorable lipid profile and higher IGF-I and IGF-II levels than non-IVF children.[458,459]

Although the etiology of these alterations is unknown, it appears that the infertility of the couple per se may be responsible. Nevertheless, further research is required to determine whether there is any association between the ART procedures themselves and an increased risk of imprinting disorders. As the overall absolute incidence of these diseases is, thankfully, extremely small, routine screening for imprinting diseases in ART children is not recommended.

Psychological and Cognitive Development in IVF Offspring

Another concern regarding pregnancies resulting from ART is the neurological development of children conceived through IVF. Although the majority of such studies are reassuring, several studies have demonstrated an increased incidence of cerebral palsy in children born through ART. One study found an increased risk for cerebral palsy in IVF children (OR 3.7, 95% CI 2 to 6.6); some of the increased risk was associated with multiple gestations, but the higher risk persisted when singletons alone were analyzed (OR 2.8, 95% CI 1.3 to 5.8). No difference in neurological development was seen between IVF twins and control twins.[460]

A second study also showed an increased risk of cerebral palsy among IVF offspring even after accounting for maternal age, educational level, parity, gender of offspring, and small size for gestational age. Importantly, however, the differences disappeared with adjustment for multiple gestations and prematurity.[461]

Long-term developmental, psychological, and cognitive studies on offspring of ART pregnancies are now available and generally show normal cognitive and motor development, as well as appropriate neurological function up to age 8.[462-465] Psychological development has also been demonstrated to be normal in children conceived via ART up to age 5.[466,467] However, IVF offspring appear to be at elevated risk of hospitalization in childhood, perhaps reflecting their higher rate of medical problems in the neonatal period.[468]

Paternal Age and IVF Outcome

In men undergoing ART treatment, increasing age is associated with lower ejaculate volume, worse semen parameters and a higher incidence of DNA fragmentation.[469] There is a significant body of literature indicating a paternal age-related association with infertility,[470] which appears to be more pronounced in men ≥40 years or older.[470,471] The impact of paternal age on IVF outcomes has also been analyzed, particularly using the oocyte donor model, in which oocyte-related factors (e.g., female age, female infertility diagnosis, etc.) and endometrial receptivity issues are more easily controlled. However, conflicting results exist even when using this model. Some studies have found either no association,[472] or a very limited association,[473] between paternal age and live births, while other studies have found significant age-related declines in live birth rate or other outcome parameters.[474,475]

One retrospective study evaluated the impact of paternal age in 672 IVF cycles with ovum donors who were ≤35 years old.[474] Not suprisingly, as men aged, semen parameters worsened, and the utilization of ICSI increased. Fertilization rates were lower with conventional insemination in men older than 60 years old. The number of cryopreserved embryos was significantly lower as men aged ($P < 0.05$), as also was the number of embryos with more than seven cells on day 3, as well as the proportion of embryos developing to the blastocyst stage ($P < 0.05$). As blastocyst formation is dependent upon successful paternal genome activation (which occurs around the four to eight-cell stage),[67] any inverse association between paternal age and blastocyst formation likely reflects perturbation of this fundamental developmental event. In this same retrospective study of 672 cycles,[474] implantation rates were significantly lower in cycles where the man was older than 60 years old, compared to those with men 50 to 59 years old (22% versus 43%, $P = 0.022$), and there was a trend toward lower clinical pregnancy rates (50% versus 72%, $P = 0.14$). Of note, the age of the recipients increased as paternal age increased and no multivariate analysis was performed to control for this potential confounder. As there is some evidence that recipient age may be inversely related to implantation rates,[476] it is unclear as to whether the observations in this study should be exclusively attributed to paternal age.[474]

Another study of 484 ovum donation recipient couples was analysed. The mean paternal age in cycles achieving clinical pregnancy was lower than in cycles that did not result in pregnancy (43.2 ± 8.1 years versus 46.8 ± 7.8 years; $P = 0003$). Semen parameters were found to be notably lower in men 40 to 50 years old compared to those younger than 40 years old, particularly for teratospermia ($P = .0001$), and ICSI was performed in all cycles.[477] However, again multivariate analysis was not performed, and donor and recipient ages were not taken into account. In another study of 1392 donor cycles in 1083 recipient pairs, male age was again associated with lower semen volume and sperm motility; however, there was no association with male age and live birth rate even after adjustment for female recipient age.[473]

Taken together, it is clear that further research with very substantial datasets and appropriate statistical analyses is required to resolve whether there is a paternal age-related association with incidence of live birth in IVF.

Most studies of natural conceptions have not found that paternal age is related to chromosomal abnormalities in offspring;[478] however, some believe that there is a paternal contribution.[479] The American College of Medical Genetics does not suggest additional prenatal screening on the basis of paternal age alone.[479] In general, however, advanced paternal age (older than 40 years old) is associated with higher rates of miscarriage,[470,476] some autosomal dominant conditions, autism spectrum disorders, and schizophrenia.[470,480] There is a lack of data investigating whether IVF offspring have an increased risk for any of these paternal age-related disorders compared with that of naturally conceived children. However, as IVF registeries continue to expand, sufficiently robust datasets should eventually be available to enable this gap in our knowledge to be filled.

Oocyte Donation

Early studies of IVF with oocyte donation clearly established the feasibility of the technique, with pregnancy rates being reported in the range of 25% per cycle.[481,482] The technique is now very well established with its utilization

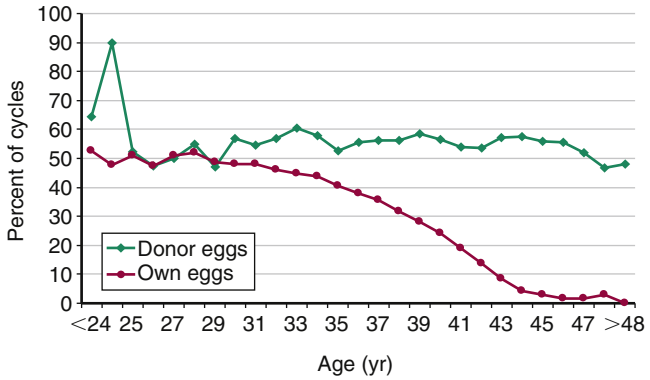


FIGURE 31.26 Percentages of transfers that resulted in live births for 2009 assisted reproductive technology (ART) cycles using fresh embryos from own or donor eggs, by patient's age. *(From CDC Assisted Reproductive Technology [ART] Report 2009. Available at www.cdc.gov/art/ART2009. Accessed Sept. 30, 2012.)*

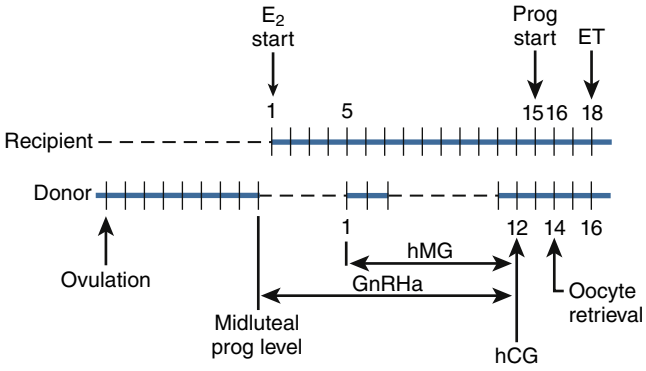


FIGURE 31.27 Schematic of the synchronization of oocyte donor and embryo recipient on a gonadotropin-releasing hormone-agonist analog (GnRHa) with exogenous estrogen (E_2) and progesterone (Prog) protocol. hMG, human menopausal gonadotropin; hCG, human chorionic gonadotropin; ET, embryo transfer. *(From Schmidt-Sarosi CL. In vitro fertilization with donor oocytes. In Keye WR, Chang RJ, Rebar RW, Soules MR [eds]. Infertility: Evaluation and Treatment. Philadelphia, WB Saunders, 1995, p 781.)*

being particularly related to maternal age, although women with premature ovarian failure also benefit from the procedure. In the U.S. in 2009, less than 5% of ART cycles used oocyte donation in women younger than 35 years old, there was a gentle increase in utilization to 12.9% in women at 40 years, but a dramatic age-related upswing to 91.8% in patients older than 48 years old.

An important question in reproduction is, "Which factor has the greater impact on fertility, oocyte age or uterine age?" On the basis of results from IVF with oocyte donation, both factors affect the likelihood of pregnancy and its outcome, but oocyte age appears to have a far greater impact on fecundability than uterine age.[483] As seen in Figure 31.26, while there is a dramatic and unrelenting decline in pregnancy rates with increasing age in women using their own oocytes, this decline is not seen in women using donor (and presumably younger) eggs. However, there is evidence that uterine aging may be related to lower implantation and ongoing pregnancy rates. One study analysed 143 cycles of oocyte donors younger than 30 years old. The implantation rate was higher ($P = 0.042$) and the abortion rate was apparently lower in women younger than 34 years old than in recipients ≥40 years or older.[484] In another study of IVF with oocyte donation, pregnancy losses were substantially higher in women older than 40 years old compared with women younger than 40 years old.[485] Some conflicting data nevertheless exist: In 222 cycles of shared egg donation to 444 recipient pairs, recipient age was not found to be associated with pregnancy rates.[486] Furthermore, a recent analysis showed no difference in pregnancy rates in young recipients compared with those in their late 40s.[487]

IVF with oocyte donation requires the coordination of the reproductive tracts of the donor and recipient (Fig. 31.27). An important insight provided by this technique is that for women with ovarian failure, steroid replacement with estradiol and progesterone is adequate to prepare the endometrium for successful pregnancy. No other ovarian hormone is required to achieve a successful pregnancy. Most oocyte donation programs use "fresh" oocytes, and the theoretical problem of possible transmission of infection from oocyte donor to recipient has never been documented, but in the United States is highly regulated under FDA oversight.

An important ethical issue is the age at which women should be advised not to pursue IVF with oocyte donation. Some centers have treated women over 60 years of age; other centers restrict the application of this technique to women younger than 50. In addition, oocyte donation may be associated with some health risks, and so it is recommended that donors do not donate for more than six cycles.[488]

Gestational Carrier Cycles

Another form of third-party reproduction involves the use of a gestational carrier in which a woman other than the infertile woman conceives a pregnancy for her. In this scenario, the infertile woman undergoes controlled ovarian stimulation and egg retrieval and her oocytes are fertilized *in vitro*, but the resulting embryo(s) is implanted into the uterus of a gestational carrier. The carrier's endometrium is prepared and the synchronization of the cycles of the two women is similar to that of the egg donor and recipient.

Indications for the use of a gestational carrier include congenital absence of the uterus, congenital müllerian malformations, hysterectomy, or surgically uncorrectable anatomical abnormalities such as intrauterine adhesions. Another indication involves women who have a serious medical illness that precludes a safe pregnancy.

Although the mechanics of a gestational carrier cycle are relatively straightforward and routine, this type of third-party reproduction involves complex legal, psychological, and sometimes ethical issues. Potential carriers should have undergone prior uncomplicated pregnancies and completed psychological as well as medical screening. A signed contract between the parties is necessary and laws pertaining to the legal status of the baby vary according to state. Pregnancy rates are dependent on the reproductive prognosis of the oocyte source. Despite its many medical and social complexities, gestational carriers offer some infertile women the opportunity to reproduce using their own and their

partner's gametes when they would otherwise not be able to have a genetic child.

In Vitro Oocyte Maturation

An alternate approach to the retrieval of mature oocytes following ovarian stimulation is the aspiration of immature oocytes and their subsequent maturation *in vitro*. In the setting of *in vitro* maturation (IVM), GV-stage oocytes are retrieved when the follicles reach a diameter of 6 to 12 mm, either with or without hCG priming.[489] Although priming with hCG may improve maturation rates following culture, compared with no priming,[489] such an approach should not strictly be referred to as IVM because the oocytes may have begun the maturation process *in vivo* under the influence of the hCG. Retrieved oocytes are typically cultured for 28 to 36 hours to allow time for progression to metaphase II,[490] thereby simulating the timing from the LH surge *in vivo*.[491] A specialized, smaller needle (19 or 20 gauge, rather than the 16 to 17G needle) is generally used to remove the more adherent immature oocyte-cumulus complex from the membrana granulosa cells lining the follicle, and a lower pressure on the aspiration pump is desirable to obtain a higher yield of oocytes.[492]

The efficiency of IVM varies depending upon whether the oocytes arise from polycystic ovaries, naturally cycling ovaries, or ovaries primed with low doses of gonadotropins. There is also considerable heterogeneity in success rates among clinics even when similar procedures are used in comparable patient populations.[489] The majority of studies indicate that while more than 60% of IVM oocytes achieve nuclear maturation by progressing to metaphase II,[489] a low percentage successfully acquire cytoplasmic maturation and exhibit the ability to support pronuclear formation and early embryonic development. Only 40% to 80% of fertilized IVM oocytes progress through early cleavage, and of those that do cleave and that are transferred, implantation rates of less than 15% are seen in the majority of studies.[489] This low efficiency is further diminished for oocytes matured without their cumulus-corona cells.[493] Therefore, attempts to salvage immature oocytes for clinical use after cumulus-corona cell removal in preparation for ICSI is not recommended with the IVM protocols currently available.[494]

Several challenges remain before IVM can be reliably applied in ART. The IVM system requires optimization; the culture medium requires further refinement beyond the reported benefits of supplementation with Activin A,[495] or the epidermal growth factor family members amphiregulin (Areg) and epiregulin (Ereg) EGF-like growth factors.[496] Use of a three-dimensional culture system to support the structural integrity of the oocyte-cumulus complex either in a prematuration phase[497] or during maturation[498,499] requires further investigation. Moreover, the optimal duration of IVM remains to be determined, and the optimal protocols for preparation of a receptive endometrium need to be identified. Research targeting these gaps in our knowledge is worthwhile. IVM is associated with lower costs due to use of less gonadotropin, and the technique eliminates the possibility of OHSS, thereby having particular application in women at increased risk for this syndrome such as those with PCOS. Furthermore, available data, albeit with still relatively small numbers of children born, indicate that obstetric, perinatal, and neonatal outcomes after IVM are comparable to those resulting from IVF and ICSI.[500] Finally, an efficacious IVM protocol is required when oocytes are cryopreserved at the GV stage for subsequent attempts to achieve pregnancy.

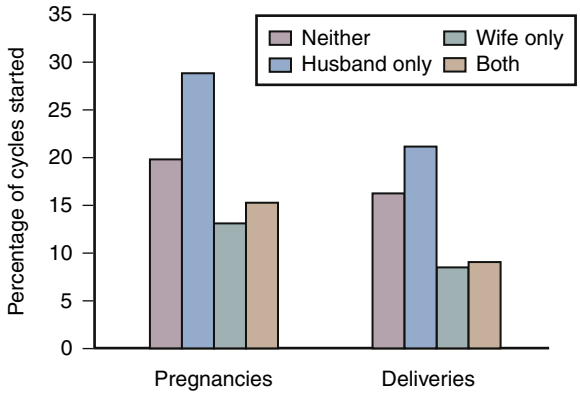


FIGURE 31.28 The pregnancy and delivery rates per cycle intiated after *in vitro* fertilization, stratified by the smoking habits of the male and female partners. Smoking by the female partner substantially decreased the delivery rate per cycle initiated. Smoking by the male partner did not decrease the pregnancy rate. *(From Pattinson HA, Taylor PJ, Pattinson MH. The effect of cigarette smoking on ovarian function and early pregnancy outcome of in vitro fertilization treatment.* Fertil Steril *55:780-784, 1991, with permission of the American Society for Reproductive Medicine.)*

Environmental Exposures and Lifestyle Factors in Assisted Reproductive Techniques

Cigarette Smoking

The effect of cigarette smoking on clinical outcomes has been studied both for IVF and GIFT cycles. In IVF cycles, cigarette smoking is associated with no major change in peak estradiol concentration or number of oocytes retrieved. However, cigarette smoking appears to decrease the pregnancy and live birth rates in IVF if the female partner smokes, but not if the male partner smokes (Fig. 31.28). The effect of smoking on IVF outcome may be dose-dependent. Smoking produces the greatest effects on IVF delivery rates when the female partner smokes more than 20 cigarettes daily (Fig. 31.29).[501]

A clue to the link between cigarette smoking and reduced pregnancy rates in IVF is the observation that smoking accelerates the rate of oocyte loss. Smoking appears to increase prematurely a woman's cycle day 3 FSH concentrations[502,503]; women who smoke have a depleted oocyte pool and may have prematurely aged follicles. However, the antral follicle count may not be affected.[504] Women over 35 years old who smoke during IVF, with or without ICSI have fewer oocytes retrieved and smoking may also affect uterine receptivity. An egg donation model assessing smoking in the recipient found a dose-dependent effect. Recipients smoking more than 10 cigarettes a day had a lower pregnancy rate (52.2% versus 34.1%) than recipients smoking 0 to 10 cigarettes per day.[505] Smoking does not appear to impact fertilization or embryo quality scores in most studies, although the majority of studies are limited by the lack of adjustment for potential confounders.[506]

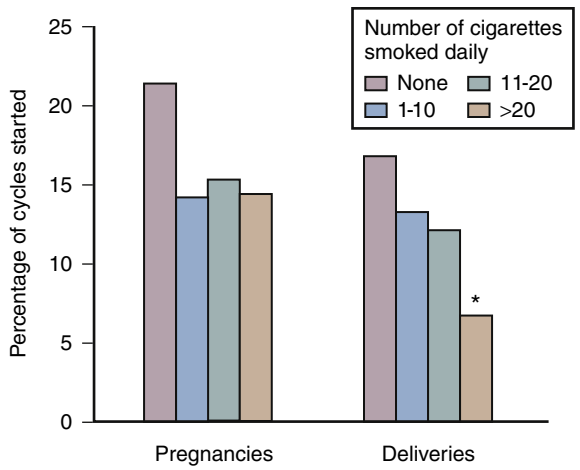


FIGURE 31.29 The *in vitro* fertilization pregnancy and delivery rates per cycle initiated, stratified by the magnitude of the exposure of the female partner to cigarette smoke. When the female partner smoked more than 20 cigarettes per day, there was a substantial decrease in the delivery rate per cycle initiated. *(From Pattinson HA, Taylor PJ, Pattinson MH. The effect of cigarette smoking on ovarian function and early pregnancy outcome of in vitro fertilization treatment.* Fertil Steril *55:780-784, 1991, with permission of the American Society for Reproductive Medicine.)*

However, in a retrospective analysis of a prospective cohort study, cotinine (a nicotine metabolite) was measured in the follicular fluid collected during 3270 IVF treatment cycles from 1909 nonsmoking women between 1994 and 2003. Multivariate analyses found a significant increase in the risk of failed implantation among women exposed to second hand smoke compared with those unexposed (OR 1.52; 95% CI 1.20 to 1.92; risk ratio [RR] 1.17; 95% CI 1.10 to 1.25). There was also a significant decrease in the odds for a live birth among second hand smoke exposed women (OR 0.75; 95% CI, 0.57 to 0.99; RR 0.81; 95% CI 0.66 to 0.99).[507]

Although the effects of smoking on ovarian function may be irreversible, women should discontinue smoking before undergoing an IVF cycle, and should avoid exposure to second hand smoke.

Caffeine Consumption

A prospective study of 221 couples undergoing ART in Southern California reported a reduced pregnancy rate for women consuming 2 to 50 mg caffeine/day (50 mg is roughly the amount of caffeine in half a cup of coffee), compared with women consuming 0 to 2 mg per day during the week of their initial visit (OR 3.1, 95% CI, 1.1 to 9.7). Caffeine intake the week before the procedure or the week after the procedure was not associated with a reduction in IVF success.[508] However, in another study of 618 women undergoing IVF, the average caffeine consumption was 456 mg per day, primarily from coffee, and after multivariate adjustment was associated with a higher risk of miscarriage ($P = 0.007$).[509] Less than 200 mg caffeine intake has not been associated with increases in miscarriage in a prospective cohort study.[510] Although the data are limited, available evidence suggests that it would seem prudent to counsel couples undergoing IVF to limit their caffeine intake to no more than 2 cups per day.

Alcohol Consumption

In one prospective study of 221 women with female factor infertility, one drink per day increased a woman's risk of not becoming pregnant and significantly increased her risk of miscarriage (OR 2.21, 95% CI 1.09 to 4.99).[511] In a large prospective cohort study, 2545 couples contributed 4729 cycles. Drinking was common: 41% of women and 58% of men drank one to six drinks per week. Women drinking ≥4 drinks or more per week were 16% less likely to have a live birth compared with those who drank less than four drinks per week (OR 0.84, CI 0.71 to 0.99). For couples in which both partners drank at least four drinks per week, the odds of live birth were 21% lower compared with couples in which both drank fewer than four drinks per week (OR 0.79; CI 0.66 to 0.96).[512] Based on these collective data, couples undergoing IVF should be counseled that alcohol consumption reduces live birth rates and should be avoided.

Marijuana Use

There are minimal data on marijuana use and IVF, but a single study reported that women who used marijuana more than 90 times in their lifetime had fewer eggs retrieved and one fewer embryo transferred than non-users. Moreover, women smoking marijuana more than 10 times in their lives also had smaller infants at birth compared to nonusers.[513] Clearly, more research is required to investigate the association between marijuana use and IVF success rate.

Obesity

The relationship between body mass index and IVF success rates has recently been reported. In a study of United States IVF clinic data reported to SART, increasing obesity was associated with a significantly lower chance of clinical pregnancy with the use of autologous oocytes ($P < 0.0001$), but no difference with the use of donor oocytes. The live birth rates declined in proportion to the extent of obesity, notably so in young women (younger than 35 years) who generally have higher pregnancy rates.[514,515] In a study from a single center of 1721 women undergoing their first IVF attempt, an inverse U-shaped association between BMI and live birth was observed (Fig. 31.30). These results indicate that women with below normal BMI also have a reduced likelihood of success with IVF success. Although the underlying reason for these observations remains to be clarified, a recent study has suggested that oocyte abnormalites may be partly responsible, at least in morbidly obese women, as reflected by abnormalities in the number of spindles and alignment of chromosomes in those oocytes that failed to fertilize in this population.[516]

Exercise

There has been only one study examining the effect of exercise on IVF outcome. Three large IVF programs in the Boston area prospectively enrolled 2232 patients undergoing their first IVF cycle in a study of outcome predictors. All patients filled out a questionnaire that included the amount, type, and frequency of exercise. Women who reported exercising ≥4 or more hours per week for 1 to 9 years were almost three times more likely to experience cycle cancellation (OR 2.8, 95% CI, 1.5 to 5.3), twice as likely to experience pregnancy loss (OR 2.0, 95% CI, 1.2 to 3.4), and 40% less likely to have a live birth (OR 0.6, 95% CI, 0.4 to 0.8)

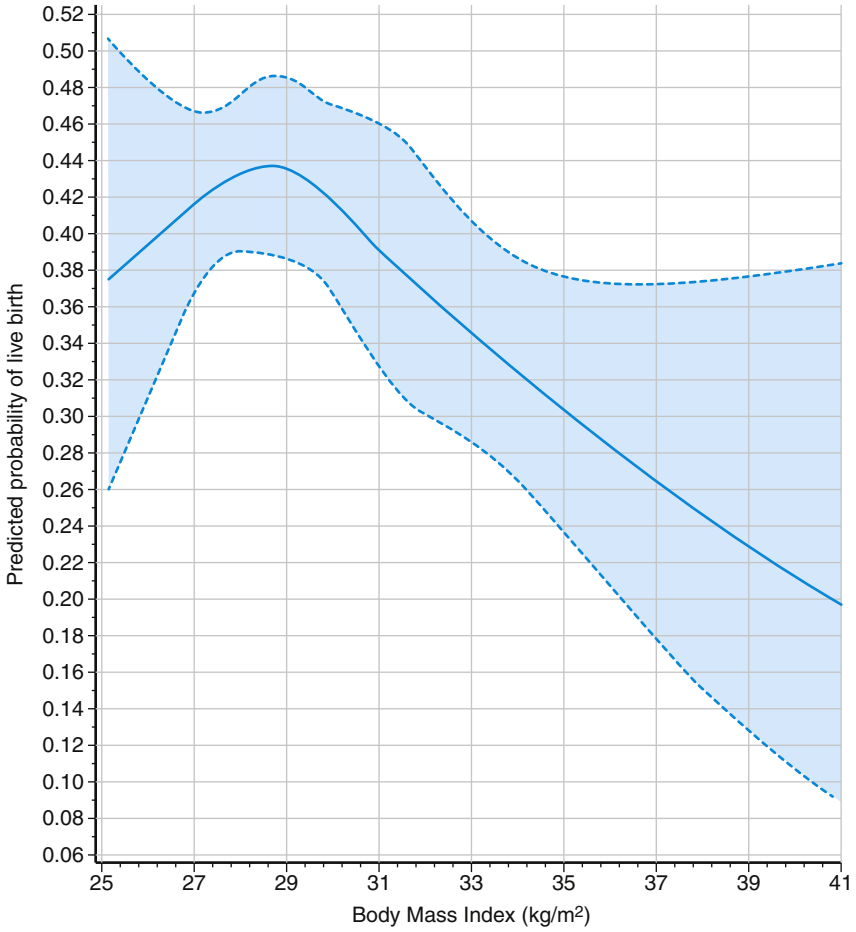


FIGURE 31.30 Cubic regression spline of the predicted probability of live birth by body mass index, adjusted for age, among 1721 women undergoing their first fresh *in vitro* fertilization (IVF) cycle. *Dashed lines* represent 95% confidence intervals. *(Shah DK, Missmer SA, Berry KF, Racowsky C, Ginsburg ES: Effect of obesity on oocyte and embryo quality in women undergoing* in vitro *fertilization,* Obstetrics and Gynecology *118:63–70, 2011.)*

than women who reported not exercising.[517] In this same study, women reporting cardiovascular exercise had a 30% lower chance of a successful live birth than those reporting no cardiovascular exercise. However, when women performed either walking or cardiovascular exercise for more than 9 years, there was no effect on live birth rates compared with those who did not exercise.

Although one should be cautious about drawing conclusions based on single studies, several modifiable life style behaviors seem to influence successful outcomes in assisted reproduction. As with other questions in this field, well-designed studies are sorely needed.

Concluding Remarks

Since the birth of the first IVF baby in 1978, there have been many advances in the field of ART. These advances, both clinical and in the laboratory, have resulted in consistent improvements in success rates over time. However, a deficiency in the field of assisted reproduction is that there are few randomized studies demonstrating the superiority of assisted reproductive techniques over other forms of fertility treatment, such as pelvic reconstructive surgery or empirical ovarian stimulation with gonadotropins, which may be optimal treatments in specific patient populations. Furthermore, many new assisted reproductive technologies are introduced into clinical practice without adequate clinical trials to demonstrate their utility in relation to "standard" IVF treatment procedures. A multitude of factors contribute to the likelihood that a single attempt at IVF will result in live birth. It is critical that rigorous research investigates new technologies prior to their being adopted into routine clinical practice, and that clinicians continually educate themselves about the advances in this rapidly evolving field.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 32

Gamete and Embryo Manipulation

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Since the birth of the first test tube baby in 1978,[1] *in vitro* fertilization (IVF) has become a well-established treatment procedure for certain types of infertility, including long-standing infertility due to tubal disease, endometriosis, unexplained infertility, or infertility involving a male factor. However, soon it became obvious that certain couples with severe male factor infertility could not be helped by conventional IVF. Extremely low sperm counts, impaired motility, and poor morphology represent the main causes of failed fertilization in conventional IVF.

To tackle this problem, several procedures of assisted fertilization based on micromanipulation of oocytes and spermatozoa have been established. These strategies have culminated in intracytoplasmic sperm injection (ICSI), in which a single spermatozoon is directly injected into the ooplasma. In 1992, Palermo and colleagues reported the first human pregnancies and births after replacement of embryos generated by this novel procedure of assisted fertilization.[2] Since then, the number of worldwide centers offering ICSI has increased tremendously, as have the number of treatment cycles per year.[3] Because of the widespread use of ICSI as the ultimate and only option for successful treatment of severe male infertility due to impaired testicular function or obstruction of the excretory ducts, concern about its efficacy and safety is appropriate.

Whereas ICSI represents the major breakthrough for male infertility treatment, preimplantation genetic diagnosis (PGD) would be inconceivable without the establishment of embryo biopsy procedures. Initial attempts to penetrate or open the zona pellucida were undertaken to assist oocyte fertilization[4-6] or increase embryo implantation by facilitating the hatching process.[7,8] This very close access to oocytes or embryos soon resulted in the removal of cellular material (polar bodies or blastomeres, respectively), which allowed genetic testing in this very early developmental stage.[9,10]

As much as PGD relies on embryo biopsy, equally indispensable was the progress in molecular genetics leading to the development of diagnostic procedures at the single-cell level.[11-13] These procedures include gene amplification using polymerase chain reaction (PCR) for monogenic diseases, fluorescence *in situ* hybridization (FISH) for the evaluation of numeric and structural chromosome aberrations and for sex determination in cases of sex-linked diseases and more recently, comprehensive chromosome screening (CCS) methods which allow for the genome-wide analysis of DNA copy number variations.[14] Since the first pregnancy report from the clinical application of PGD,[15] there has been a steady increase in the number of PGD cycles and also in the number of centers performing PGD worldwide.[16]

This chapter surveys the current status of ICSI, emphasizing patient selection, oocyte and sperm handling before microinjection, the procedure and outcome parameters, and clinical application of these technologies. The clinical relevance of assisted hatching before embryo transfer is discussed. Embryo biopsy procedures and single-cell analysis (at the chromosomal level by FISH and CCS; at the monogenic level by PCR) as diagnostic tools for PGD and preimplantation genetic screening (PGS) are described.

Intracytoplasmic Sperm Injection

History of ICSI

Extremely low sperm counts, impaired motility, and abnormal morphological features are the main causes of failed fertilization in conventional IVF. Today, ICSI is the ultimate option to treat these cases of severe male factor infertility (see Chapter 24). One single viable spermatozoon, preferably with good morphological characteristics, is selected by the embryologist and injected into each oocyte available.

The ICSI procedure is based on micromanipulation of oocytes and spermatozoa. Initially, partial zona dissection (PZD) was established to facilitate sperm penetration.[4,6,17,18] The barrier to fertilization represented by the zona pellucida was disrupted mechanically to allow the inseminated sperm cells direct access to the perivitelline space of the oocyte. Subzonal insemination (SUZI) was the next step in micromanipulation techniques.[19-22] SUZI enabled the immediate delivery of several motile sperm cells into the perivitelline space by means of an injection pipette.[23] ICSI is even more invasive because a single spermatozoon is directly injected into the ooplasma, thereby crossing not only the zona pellucida but also the oolemma.

The first successful use of ICSI was to obtain live offspring in rabbits and cattle.[24] The first human pregnancies

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and births resulting from this novel assisted fertilization procedure were reported in 1992.[2] Shortly thereafter, ICSI was found to be superior to SUZI in terms of oocyte fertilization rate,[25-28] number of embryos produced, and embryo implantation rate.[25-28] As a result, ICSI has been used successfully worldwide to treat infertility due to severe oligo-asthenoteratozoospermia or azoospermia caused by impaired testicular function or obstructed excretory ducts.[29,30]

Since the first publication describing the ICSI procedure,[2,22,25,27,28] minor modifications have contributed to reduced rates of oocyte degeneration, oocyte activation (one-pronuclear [1-PN]), and abnormal fertilization (three-pronuclear [3-PN]).[31] Hyaluronidase may be responsible for oocyte activation; therefore, the concentration used during oocyte denudation and the exposure time of oocytes to the enzyme has been reduced.[32] The timing of denudation relative to oocyte pickup (immediately or 4 hours later) does not influence the ICSI results.[33] The orientation of the polar body during injection does, however, influence embryo quality.[34] Motile sperm cells are selected and immobilized before injection.[35] Cytoplasm aspiration to ensure oolemma rupture is critical to the success of the ICSI procedure, because the method of rupture has been correlated with oocyte degeneration.[34] Furthermore, the morphological features of the injected spermatozoon are related to the fertilization outcome of the procedure as well as to the pregnancy outcome.[36] The outcome of the ICSI procedure may benefit from the introduction of some new technical improvements. Noninvasive spindle imaging in human oocytes on the basis of its birefringence with a computer-assisted polarization microscopy system (PolScope™, Cambridge Research & Instrumentation, Woburn, MA) may serve as an accurate indicator of oocyte maturity, quality, and developmental potential.[37-44] High-magnification ICSI, on the other hand, may provide a better morphological selection of spermatozoa to be used for fertilization.[45-50]

Indications for ICSI

Before the era of ICSI, attempts were made to modify and refine conventional IVF to achieve increased rates of conception in cases of male infertility. Today, ICSI has clearly overshadowed the use of modified IVF procedures (including high insemination concentration) for the treatment of severe male factor infertility. ICSI requires only one spermatozoon with a functional genome and centrosome for the fertilization of each oocyte. Indications for ICSI are not restricted to morphologically impaired spermatozoa and include low sperm count and impaired kinetic quality of the sperm cells. ICSI can also be used with spermatozoa from the epididymis or testis when there is an obstruction in the excretory ducts. Azoospermia caused by testicular failure can be treated by ICSI if enough spermatozoa can be retrieved in testicular tissue samples. Box 32.1 gives an overview of the current indications for ICSI.

The ICSI procedure with ejaculated spermatozoa can be used successfully in patients with fertilization failures after conventional IVF, and also in patients with too few morphologically normal and progressive motile spermatozoa present in the ejaculate (less than 500,000). High fertilization and pregnancy rates can be obtained when a motile spermatozoon is injected.[28] Injection of only immotile or probably nonvital spermatozoa results in lower fertilization rates.[51] When only nonvital sperm cells are present in the ejaculate, the use of testicular sperm is indicated.[52] Other semen parameters, such as concentration, morphological features (except for globozoospermia),[53] and high titers of antisperm antibodies,[54] do not influence success rates.[53] Successful ICSI has also been described in patients with acrosomeless spermatozoa.[55,56]

Any form of infertility due to obstruction of the excretory ducts can be treated by ICSI with spermatozoa microsurgically recovered from either the epididymis[57-59] or the testis.[60-62] Obstructive azoospermia can result from congenital bilateral absence of the vas deferens, failed vasectomy reversal, or vasoepididymostomy. When no motile spermatozoa can be retrieved from the epididymis due to epididymal fibrosis, testicular spermatozoa can be isolated from a testicular biopsy specimen.

Testicular biopsy has also proven useful in cases of nonobstructive azoospermia.[63-66] In patients with severely impaired testicular function as a result of incomplete germ cell aplasia (Sertoli cell–only syndrome), hypospermatogenesis, or incomplete maturation arrest; spermatozoa may be recovered, sometimes only after multiple biopsy specimens are obtained. Cryopreservation of supernumerary spermatozoa recovered from the epididymis[67] or the testis[68] is an important issue because microinjection of cryothawed sperm cells can avoid repeated surgery in future ICSI cycles. The ICSI procedure cannot be carried out in approximately 3% of scheduled cycles. Most cancellations occur because no cumulus–oocyte complexes or metaphase II (MII) oocytes are available or because no spermatozoa are found in testicular biopsy specimens of patients with nonobstructive azoospermia.

Box 32.1 Current Indications for Intracytoplasmic Sperm Injection

EJACULATED SPERMATOZOA
- Oligozoospermia
- Asthenozoospermia (caveat for 100% immotile spermatozoa)
- Teratozoospermia (≤4% normal morphology using strict criteria—caveat for globozoospermia)
- High titers of antisperm antibodies
- Repeated fertilization failure after conventional *in vitro* fertilization and embryo transfer
- Autoconserved frozen sperm from patients with cancer in remission
- Ejaculatory disorders (e.g., electroejaculation, retrograde ejaculation)

EPIDIDYMAL SPERMATOZOA
- Congenital bilateral absence of the vas deferens
- Young syndrome
- Failed vasoepididymostomy
- Failed vasovasostomy
- Obstruction of both ejaculatory ducts

TESTICULAR SPERMATOZOA
- All indications for epididymal sperm
- Failure of epididymal sperm recovery because of fibrosis
- Azoospermia caused by testicular failure (maturation arrest, germ cell aplasia)
- Necrozoospermia

Oocyte Collection

A successful ICSI program depends on ovarian stimulation, which is essentially similar to methods used for conventional IVF (see Chapters 30 and 31). Current ovarian stimulation regimens use a combination of gonadotropin-releasing hormone (GnRH) agonists or antagonists, human menopausal gonadotropin, or recombinant follicle-stimulating hormone (FSH), and human chorionic gonadotropin (hCG). This treatment allows the retrieval of a high number of cumulus–oocyte complexes.[69,70] Administration of GnRH agonists allows pituitary down-regulation to occur before the initiation of exogenous FSH. A gonadotropin preparation or recombinant FSH is administered to stimulate development of multiple follicles. Ovulation is usually induced with hCG (10,000 IU). This drug is administered when the serum estradiol level exceeds 1000 pg/mL and when at least two follicles ≥18 mm in mean diameter plus two additional follicles ≥12 mm in one diameter are observed on ultrasound examination. The optimal time for ultrasound-guided transvaginal oocyte aspiration is 36 hours after hCG administration.[71] On average, 11 cumulus–oocyte complexes per cycle can be retrieved.[72] After cumulus and corona cell removal, approximately nine metaphase II oocytes per cycle are available for microinjection.[72]

Although hCG may be used for luteal-phase supplementation, exogenous progesterone (administered intravaginally or intramuscularly) is frequently applied in an attempt to avoid the risk of hCG stimulation of the remaining growing follicles. The clinical introduction of GnRH antagonists[73-75] allowed a powerful and immediate suppression of pituitary gonadotropin release and a rapid recovery of normal secretion of endogenous luteinizing hormone and FSH.[76] By making optimal use of endogenous FSH, the amount of exogenous FSH required for follicular growth could be substantially reduced. A rapid recovery of pituitary luteinizing hormone and FSH release after cessation of GnRH antagonist administration might eliminate the need for additional luteal-phase support.

Handling of Oocytes

It is essential that preparation of retrieved oocytes for ICSI must be carried out under tightly controlled conditions with a constant pH of 7.3 and stable temperature at 37° C. Fluctuations in temperature have been shown to be detrimental to the oocyte microtubular system, including reductions in spindle size, disorganization of microtubules within the spindle, and in some cases, even a complete absence of microtubules.[77,78] Interference with spindle organization can prevent chromosome disassociation, resulting in aneuploidy. For procedures which require extended periods outside of a low CO_2 incubator such as ICSI, it is important to maintain the appropriate pH using buffered culture media such as 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES). The correct temperature can be maintained by properly equilibrating all media and by performing all manipulations on a heated stage. Also, covering all working media with equilibrated parafilm and mineral oil will prevent evaporation and minimize both temperature and pH shifts.[79]

Unlike conventional IVF, in which intact mature cumulus-oocyte complexes are inseminated, fertilization with micromanipulation requires denudation of oocytes (i.e., removal of the surrounding cumulus and corona cells). This strategy allows not only precise injection of the oocytes, but also assessment of their maturity, which is of critical importance for ICSI. Cumulus and corona cells are removed with a combination of enzymatic and mechanical procedures.[32]

Hyaluronidase-purified preparations of bovine origin digest the hyaluronic acid interspersed between the cumulus cells, liberating the oocyte for maturity grading and microinjection. Both the enzyme concentration and the duration of exposure to the enzyme should be limited because they can result in parthenogenetic activation of the oocytes.[80] Because of its purity, recombinant human hyaluronidase, ICSI Cumulase® (Origio), may be used at higher concentrations; as a result, the incubation time becomes less critical.[81] For these reasons, this product may reduce, if not eliminate, the risk of damage or trauma to the oocytes by the mechanical denudation step. Additionally, the risk of animal pathogen contamination is alleviated. Microscopic observations of the denuded oocytes include assessment of the zona pellucida and oocyte and determination of the presence or absence of a germinal vesicle (GV) or first polar body. In 95% of cases, the retrieved cumulus–oocyte complexes usually contain an intact oocyte. The remaining 5% are empty zonae, cracked zonae, or morphologically abnormal oocytes.

Figure 32.1 shows three stages of oocyte maturation. On average, 3.9% of the intact oocytes are at the metaphase I (MI) stage, having undergone breakdown of the germinal vesicle, but not yet extrusion of the first polar body.[72] Approximately 10.3% of the intact oocytes are at the germinal vesicle stage, and approximately 85.8% are in the metaphase II stage, showing the presence of the first polar body.[72] ICSI is carried out only on metaphase II oocytes,

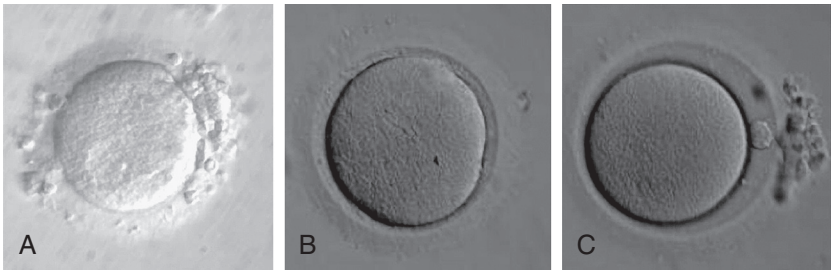


FIGURE 32.1 Oocyte maturity after cumulus and corona cell removal. **A,** A germinal vesicle–stage oocyte is recognized by the presence of a typical germinal vesicle. **B,** Oocytes that have undergone germinal vesicle breakdown but have not yet extruded the first polar body are called "metaphase I oocytes." **C,** A typical metaphase II oocyte shows a first polar body, which indicates that the oocyte is mature and has reached the haploid state. Only metaphase II oocytes are submitted to intracytoplasmic sperm injection.

because only such oocytes have reached the haploid state and thus can be fertilized normally. It has been reported that 74% of the MI oocytes completed meiosis within 20 hours after retrieval.[82] Another study reported that 27% of MI oocytes extruded their polar bodies within 4 hours of the egg retrieval.[83] Oocytes from this study were injected on the day of the egg retrieval in parallel to the oocytes retrieved at MII. This study demonstrated that MI oocytes that completed their maturation *in vitro* had a lower fertilization rate compared to those aspirated at MII. However, there were no differences observed in embryo quality between the two groups of oocytes.[83] Additional studies support these observations, showing that *in vitro* matured oocytes can be normally fertilized, however embryos derived from these oocytes rarely result in pregnancies.[84,85] These results suggest that rescue of MI oocytes of patients with few MII oocytes may increase the number of embryos for transfer; however, the chance to improve pregnancy rates by this procedure is minimal. Germinal vesicle-stage oocytes require overnight (30 hours) incubation in order to reach the MII stage. Few pregnancies have been reported from oocytes that were retrieved at the GV stage although standard IVF treatment with controlled ovarian hyperstimulation was performed.[84,86] Because of the poor results, GV oocytes are usually discarded. Denuded and rinsed oocytes are incubated until the time of microinjection.

PolScope™

As previously mentioned, the integrity of the meiotic spindle in MII oocytes is crucial for normal fertilization and subsequent development. Therefore, it is thought that the morphology of the spindle may serve as a marker of oocyte quality. A modification of the polarized light microscope "PolScope™," has been developed as a noninvasive instrument to view the meiotic spindle in living oocytes.[87] The image of the spindle is based on the highly birefringent characteristic of the microtubule filaments under a polarization microscope. The presence of a birefringent spindle in human oocytes can predict not only a higher fertilization rate, but also greater embryo developmental competence.[40,43,88] Meiotic spindles can be detected in up to 91% of human metaphase II oocytes at the time of ICSI.[42] Because most oocytes possess spindles, the mere presence of a spindle is of limited value. High degrees of misalignment between the meiotic spindle and the first polar body have been described,[42] probably due to polar body displacement during manipulations for cumulus corona removal.[41] The relative position of the spindle within the oocyte, however, has little influence on the developmental potential of the resulting embryos.[40,42] Whether quantitative spindle analysis (estimating the density of tubules within the spindle) offers added value needs to be seen.[38] So far, spindle imaging, in addition to the appearance of the first polar body, is an accurate indicator of oocyte maturity[37] and can help to determine the timing of ICSI.[39,89] Additionally, meiotic spindle dynamics[89] and possible meiotic spindle destruction due to cryopreservation can be monitored.[41]

Sperm Collection

For microinjection, spermatozoa from three different origins are processed: ejaculated sperm and surgically retrieved sperm from either the epididymis or the testis. For all three categories, ICSI, in combination with sperm cryopreservation, is currently used successfully.[67,90,91] All patients selected for ICSI with ejaculated semen undergo a preliminary semen assessment before treatment to verify whether enough spermatozoa (preferably motile) are present to perform ICSI.

Routinely, sperm samples for ICSI are processed by density-gradient centrifugation (using silane-coated silica particle colloid solutions),[92] enriching the number of motile and morphologically normal sperm cells needed for assisted reproduction. Only in cases of extreme oligozoospermia (i.e., when gradient centrifugation results in an insufficient yield of sperm cells for ICSI) is simple washing of the sperm sample performed to reduce the loss of sperm cells for injection. Immediate injection of the oocytes is then indicated because sperm cells lose their initial motility and often die when the sample is simply washed. This consequence can be attributed to the presence of reactive oxygen species and other damaging substances.[93,94]

During microsurgical epididymal sperm aspiration,[62,95] several sperm fractions are collected into separate tubes. Sperm fractions with similar concentration and motility are pooled, and a density-gradient centrifugation is performed. Microdroplets of the resuspended pellet are placed in separate medium droplets adjacent to a central polyvinylpyrrolidone (PVP) droplet in the injection dish. This facilitates the search for and selection of single motile spermatozoa. Spermatozoa are collected using a testicular sperm extraction pipette, which is larger in diameter than an injection pipette (outer diameter 8 to 10 μm instead of 6 to 7 μm). The spermatozoa are then transferred to the PVP droplet and immobilized before injection. Whenever possible, some of the freshly recovered sperm should be frozen for later use in subsequent ICSI cycles, thereby avoiding the need for repeated surgical procedures.[95]

Testicular biopsy specimens, usually obtained with surgical excisional biopsy,[96] are shredded into small pieces with sterile microscope slides[97] or with fine needles on the heated stage of a stereomicroscope. The presence of spermatozoa is assessed with an inverted microscope, which determines whether the surgical procedure can be stopped or whether extra biopsy specimens must be taken. The pieces of biopsy tissue are removed, and the medium is centrifuged at 300 × *g* for 5 minutes. The pellet is then resuspended for the ICSI procedure. Single motile spermatozoa are collected in a manner similar to that used for epididymal sperm, using separate medium droplets that contain fractions of the testicular sperm suspension. If no sperm cells can be found, the tissue pieces can be treated with red blood cell lysis buffer[98] or an enzymatic collagen digestion medium.[99] Lysis of excess red blood cells may facilitate the search for sperm cells, and the use of enzymes may result in the recovery of otherwise inaccessible sperm cells that are initially attached to the tissue. It is not always possible to retrieve testicular spermatozoa from biopsy specimens in patients with nonobstructive azoospermia due to germ cell aplasia or maturation arrest.[100] When testicular spermatozoa are retrieved under local anesthesia with fine-needle aspiration,[101] the aspirated fractions are immediately collected in the injection dish. No further sample processing, except collecting the single motile spermatozoa with a microneedle (testicular sperm extraction pipette), is needed before ICSI.

Sperm Selection

Pentoxifylline

The goal in any ICSI procedure is to use spermatozoa that are viable. Sperm motility is the best indicator that both the functional (protective) plasma membrane and the metabolical processes are in place. Spermatozoa retrieved from the testis are in a different physiological state than sperm that have been transported through the epididymis. Many times these sperm (fresh or frozen-thawed) have extremely low motility or are immobile making it difficult to identify viable sperm for injection. Sperm motility can be stimulated with a variety of chemicals. One of the most commonly utilized chemicals for stimulating sperm motility is pentoxifylline. Pentoxifylline is a nonspecific inhibitor of phosphodiesterase that has stimulatory effects on sperm motility. The stimulatory effect is attributed to elevated intracellular levels of cyclic adenosine monophosphate (cAMP) via inhibition of its breakdown by cAMP phosphodiesterase. Pentoxifylline is also reported to enhance the acrosome reaction due to the increased levels of cAMP.[59] Overstimulation of sperm with pentoxifylline can induce premature acrosome reaction.[24] Therefore, this chemical should only be used on a limited basis in which no motile sperm can be readily identified.

Hypo-Osmotic Swell Test

In cases where there is an absence of native of stimulated sperm motility, assessment of viability is essential. The hypo-osmotic swell test (HOST) is a simple viability test based on the semi-permeability of the intact and physiologically functional plasma membrane. When sperm are exposed to hypo-osmotic conditions, an influx of water to the sperm tail results in an expansion of the cell volume.[17] Cells that are viable will respond to the osmotic pressure of the external solution by attempting to attain equilibrium with the solution. If the solution is hyper-osmotic, the cell will shrink, if the solution is hypo-osmotic, the cell will swell. When viable sperm are bathed in hypo-osmotic solution, the tails coil and the heads swell within 10 seconds. Nonviable sperm will not undergo these changes. Therefore, this assay is effective in identifying viable non-motile sperm for ICSI use. Exposure of sperm for more than 2 to 5 minutes to a hypo-osmotic solution may be damaging and should be avoided. Once a viable sperm is identified, wash the sperm 2 to 3 times in your handling solution and then move them into the PVP solution before they are used for ICSI. Recently, Stanger and colleagues showed a significant correlation between the HOST score and the degree of DNA fragmentation. They observed the lowest level of DNA fragmentation in the sperm's presenting pattern or grade D or E tail (World Health Organization [WHO] criteria) folding during a HOST.[102] Given the simplicity and low cost of this technique, Stanger and colleagues proposed that this test may provide a method of standardizing the selection of sperm for ICSI.[102]

Morphologically Selected Sperm Injection

Conventional ICSI involves sperm selection at an optical magnification of approximately 400X.[36] However, spermatozoa appearing morphologically normal at this magnification may carry various structural abnormalities at the subcellular level, which then remain undetected by the embryologist. Bartoov and colleagues developed a method to evaluate sperm in real-time at high magnification called *motile sperm organelle morphology examination* (MSOME).[46,47] MSOME is performed on an inverted microscope equipped with Normarski interference contrast optics, which allows observation at high magnification (greater than 6000X).[47] This method led to the development of the intracytoplasmic morphologically selected sperm injection (IMSI) procedure, which is based on sperm normality as defined by MSOME classification. The aim of this technology is to improve ICSI outcomes by selecting against sperm that have abnormalities that are associated with DNA damage.[46,103-110] Numerous studies have demonstrated that IMSI significantly improves fertilization rates,[111] embryo quality,[46,48,50,110] blastocyst development,[112] implantation, and pregnancy after cleavage stage embryo transfers[46,48,49,103,113-115] or at the blastocyst stage.[112] In addition, IMSI has been shown to reduce miscarriage rates.[46,48,49,103,114-117] In small prospective randomized trials in unselected infertile populations, IMSI did not provide significant improvements in clinical outcomes when compared with routine ICSI;[113,118] however, severe male factor patients significantly improved embryo development and implantation with IMSI.[111,113,117,118] Large-scale prospective randomized trials are needed to determine the effects of sperm selection using IMSI.

Hyaluronic Acid–Mediated Sperm Selection

In contrast to visually selected spermatozoa, hyaluronic acid–mediated sperm selection is a novel and efficient technique that may alleviate potential problems related to ICSI fertilization with sperm of diminished maturity.[119-121] Hyaluronic acid (HA) is a high molecular weight glycosaminoglycan and is the main component of the extracellular matrix of the cumulus oophorous. Spermatozoa that are able to permanently bind to HA *in vitro* are mature and have completed the spermiogenetic process of plasma membrane remodeling, cytoplasmic extrusion, and nuclear maturity.[122] Hyaluronan bound (HB) sperm exhibit decreased levels of cytoplasmic inclusions, residual histones, and chromosomal aneuploidy,[121] and an increased expression of the heat shock-related 70 kDa protein 2 (HspA2) chaperone protein.[122] Bound sperm have also been shown to have a high density of HA receptors.[123] In contrast, immature spermatozoa with deficient plasma membrane remodeling are not able to bind to HA as there is a deficiency in the zona and HA binding sites.[123] These unbound and immature sperm have a high retention of creatine kinase, are associated with meiotic defects and possibly higher rates of aneuploidy.[123] Sperm bound to HA have also been shown to have lower levels of DNA fragmentation and have a higher percentage of nuclear normalcy compared to those selected with traditional ICSI techniques.[124] Overall, HB-sperm demonstrate increased developmental maturity and enhanced levels of functional competence over their nonbinding counterparts.

Several studies have demonstrated that the injection of HA-sperm improves fertilization rates[125] embryo quality and implantation capacity,[124,126] while others found no differences in fertilization, pregnancy, and implantation rates.[127] What is clear is that HA-sperm injection has not been shown to cause detrimental effects on ICSI outcome parameters.[128] Large multicenter, prospective, randomized studies are needed to determine if the perceived benefits of

Table 32.1 Methods for Selecting the Best Sperm for ICSI: Advantages and Disadvantages

Sperm Selection	Advantages	Disadvantages
Standard ICSI Selection	Simple and inexpensive Fastest method of sperm selection; reduces manipulation time of oocytes and sperm	Selection of sperm is based on motility and morphology, does not exclude spermatozoa with DNA damage
Morphologically Selected Sperm Injection	May increase implantation and pregnancy rates	Sperm selection can be time consuming Equipment cost can be prohibitive Sperm selection should take place at room temperature to prevent DNA damage
HOST	Simple and inexpensive test Differentiates viable and nonviable sperm May be able to differentiate degree of DNA fragmentation	Time consuming Over exposure of sperm to hypo-osmotic conditions can affect viability Can be difficult to use on frozen/thawed samples
Hyaluronic Acid Binding	Assists in selecting mature sperm with normal morphology Selects sperm with increased DNA integrity May reduce rates of aneuploidy	Difficulty micro-manipulating sperm without using a viscous media Sperm selection can be time consuming Relatively expensive
Sperm Head Birefringence	Selects mature sperm that have undergone the acrosome reaction Helps select sperm with lower DNA fragmentation and have a higher degree of nuclear normalcy May improve Day 3 embryo quality and increase implantation rates	Equipment cost can be prohibitive Sperm selection can be time consuming

selecting "physiological" sperm improve clinical results and if HA should be considered a useful tool for sperm selection (Table 32.1).

ICSI Procedure

For the ICSI procedure itself, an inverted microscope equipped with micromanipulators and microinjectors should be available.[31] Magnification capability of 200X and 400X is a prerequisite for precise procedures such as ICSI. A heating stage on the inverted microscope maintains the temperature at 37° C. Ambient temperature control is of vital importance for the survival of oocytes, which are very sensitive to a decrease in temperature that can cause irreversible damage to the meiotic spindle.[77] Micromanipulators allow three-dimensional manipulation (coarse and fine movements) of the holding and injection pipette on the left-hand and right-hand sides, respectively. Microinjectors are used to either fix or release the oocyte with the holding pipette, or to aspirate and inject a spermatozoon with the injection pipette. The injectors can be filled with air or mineral oil. A micrometer controls the plunger. The whole setup is placed on a vibration-proof table to avoid possible interfering motion. Several companies supply microtools for holding and injection; however, some centers continue to prepare their own microtools, which demands extra effort, time, and specialized equipment.[31]

The ICSI procedure involves the injection of a single immobilized spermatozoon into the oocyte. The procedure is carried out in a plastic microinjection dish containing microdroplets covered with mineral oil. A fraction (±1 μL) of the sperm suspension is added to the periphery of the central PVP droplet. Separate medium droplets are used in cases of epididymal or testicular sperm. The oocytes, denuded from their surrounding cumulus and corona cells, are placed in several surrounding medium droplets. The viscous character of the PVP solution slows the motility of sperm cells, thereby facilitating manipulation. The PVP solution also allows for better control of the fluid in the injection needle and prevents sperm cells from sticking to the pipette.

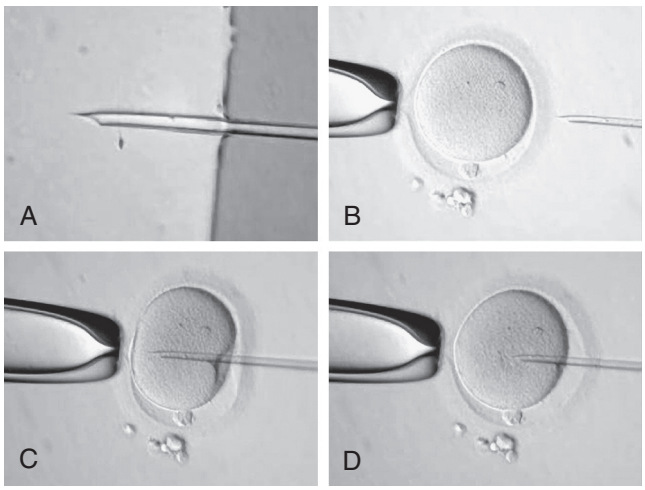


FIGURE 32.2 Intracytoplasmic sperm injection procedure. **A,** A single motile spermatozoon is selected and immobilized by pressing its tail between the microneedle and the bottom of the dish. The sperm cell is then aspirated tail-first into the injection pipette. **B,** Using the holding pipette, the mature oocyte is fixed with the polar body at the 6 o'clock position. The sperm cell is brought to the tip of the injection pipette. **C,** The injection pipette is introduced at the 3 o'clock position and rupture of the oolemma is ascertained by slight suction. Then the sperm cell is delivered into the oocyte with a minimal volume of medium; afterward, the pipette can be carefully withdrawn. **D,** A single sperm cell can be appreciated in the center of the ooplasma.

During ICSI, the following steps can be distinguished: selection and immobilization of a viable sperm cell, correct positioning of the oocyte before injection, and rupture of the oolemma before the release of the sperm cell into the oocyte. Figure 32.2 shows the whole injection procedure. In the injection pipette, which is filled with PVP, a single living, morphologically normal spermatozoon is aspirated. Viability is evidenced by the motility of the sperm cell, even if

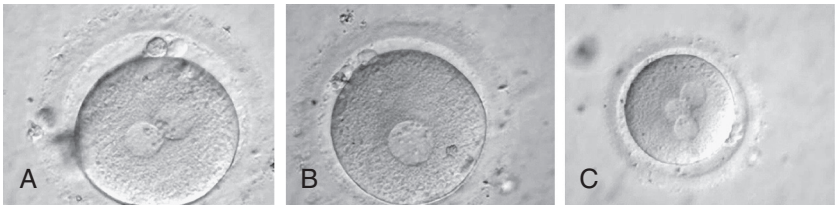


FIGURE 32.3 Fertilization outcome after intracytoplasmic sperm injection. **A,** Oocytes are considered normally fertilized when two individualized or fragmented polar bodies are present, together with two clearly visible pronuclei that contain nucleoli. **B,** Abnormal fertilization may occur as one pronuclear oocyte, probably due to parthenogenic activation. **C,** The occasional finding of three pronuclear oocytes after injection of a single spermatozoon into the ooplasm is probably caused by nonextrusion of the second polar body at the time of fertilization.

it is only a slight twitching of the tail. The sperm cell is then released in a perpendicular position to the injection pipette, which facilitates immobilization. Immobilization of a sperm cell involves rubbing the tail with the pipette against the bottom of the dish, which results in a breakage at one point, preferably below the midpiece. Immobilization of spermatozoa has been proven to be important for oocyte activation, which is achieved by release of sperm cytosolic factors via the ruptured membrane. Increased fertilization rates with ICSI have been reported after aggressive damage to the sperm tail plasma membrane[35,129] especially in the case of testicular or epididymal retrieved sperm. Changes in the sperm plasma membrane and acrosome after immobilization before ICSI have been confirmed by transmission electron microscopy[130] and scanning electron microscopy.[131] Although sperm immobilization is usually performed mechanically with the ICSI needle, laser-induced immobilization has also been described,[132-134] resulting in identical fertilization rates.[132,133]

After immobilization, the sperm cell is again aspirated (now tail-first) to allow the injection of a minimal volume of medium together with the sperm cell. The oocyte is held in position with minimal suction by the holding pipette. The polar body is located at the 6 or 12 o'clock position, which avoids damage to the spindle.[34] Unpublished reports from Palermo's laboratory using Hoechst dye–stained oocytes for microinjection clearly showed no interference with the spindle if oocytes are injected with the polar body at the 6 o'clock position. Although the first polar body does not always coincide with spindle localization, no reports so far describe spindle monitoring during ICSI to reduce spindle damage and increase the chance of fertilization. If both the holding pipette and the oocyte are in perfect focus, the injection needle, containing the immobilized sperm cell near the tip, can be introduced in the equatorial plane of the oocyte at the 3 o'clock position. Permanent focus of the injection pipette tip ensures that the needle remains in the equatorial plane of the oocyte. Passing through the zona pellucida is fairly easy and is achieved by simply advancing the injection pipette. In contrast, the oolemma is not always immediately pierced by simple injection of the needle, and often, minimal suction must be applied. The ooplasm then enters the injection pipette, and sudden acceleration of the flow indicates membrane rupture. Aspiration is immediately stopped, the sperm cell is slowly released into the oocyte with a minimal volume of medium, and the pipette is withdrawn carefully.

Different patterns of oolemma breakage have been described, depending on whether the ooplasm breaks during insertion of the pipette, whether slight or stronger aspiration of the ooplasm is needed, or whether breakage of the oolemma in another place must be attempted.[34,129] Immediate rupture of the oolemma without aspiration has been associated with lower oocyte survival rates.[34,129]

After the injection procedure, oocytes are rinsed and cultured in microdroplets covered with lightweight paraffin oil. The conditions are similar to those used for IVF-inseminated oocytes. The oocytes are kept at 37° C in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2. Injected oocytes are examined for integrity and fertilization approximately 16 to 18 hours after ICSI.[135] An average damage rate of approximately 9% of injected oocytes can be expected, regardless of the origin of the sperm used.[72] Oocytes are considered normally fertilized when two individualized or fragmented polar bodies are present together with two clearly visible pronuclei (2-PN) that contain nucleoli (Fig. 32.3).

The fertilization rate after ICSI is usually expressed per number of injected oocytes and ranges from 57% to 67%, according to the sperm origin.[72] As shown in Figure 32.3, abnormal fertilization may occur, reflected by 1-PN oocytes (approximately 3% of injected oocytes).[72] These oocytes are likely to be parthenogenetically activated as a result of mechanical or chemical factors.[136,137] The occasional finding of 3-PN oocytes (approximately 4%)[72] after injection of a single spermatozoon into the ooplasm is probably caused by failure of extrusion of the second polar body at the time of fertilization.[136] Neither type of embryo resulting from 1-PN or 3-PN oocytes is transferred to patients.

As early as the pronuclear stage, morphological scoring, including alignment and size of pronuclei, alignment and number of nucleoli, and halo effect of the cytoplasm, can serve as a valuable noninvasive selection method in addition to the morphological characteristics on the day of transfer. The morphological features of the pronuclear zygote are related to implantation and pregnancy rates.[138-140] Pronuclear evaluation, therefore, is considered useful in determining the most suitable embryos for transfer, thus achieving an optimal chance of conception.[141,142] Postfertilization, approximately 90% of 2-PN oocytes obtained by ICSI enter cleavage, resulting in multicellular embryos. Cleavage characteristics of the fertilized oocytes are evaluated daily. Early cleavage into the two-cell stage approximately 25 to 27 hours after ICSI seems to be a strong biological indicator of embryonic potential, and may be used as an additional criterion for embryo selection.[143-145]

Normally developing, good-quality embryos reach the four-cell and eight-cell stages, respectively, on day 2 and on the morning of day 3 postmicroinjection (Fig. 32.4). Numbers and sizes of blastomeres and the presence of anucleate cytoplasmic fragments are recorded. The cleaving embryos

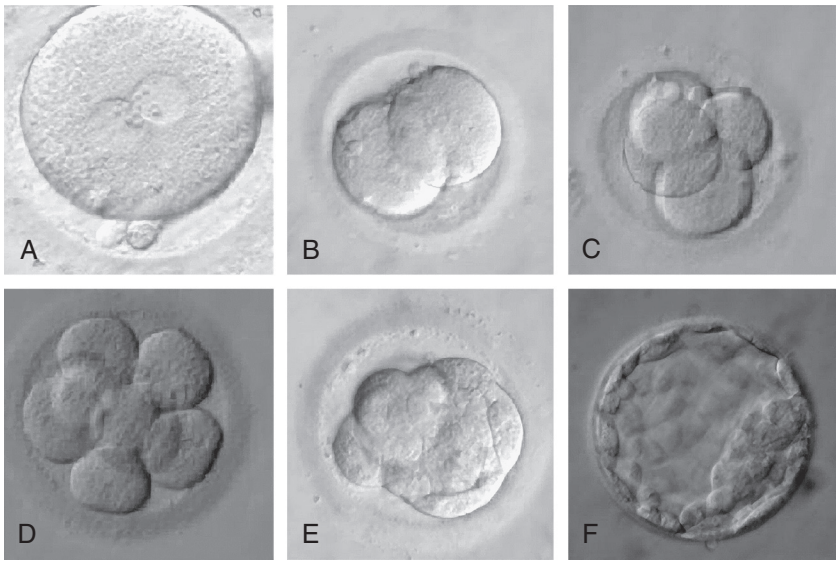


FIGURE 32.4 Embryo cleavage after intracytoplasmic sperm injection. Only embryos resulting from normally fertilized oocytes **(A)** will be transferred to patients. Embryo cleavage is evaluated daily. Two-cell embryos **(B),** four-cell embryos **(C),** and eight-cell embryos **(D)** are usually obtained on day 1 (late afternoon), on day 2, and on the morning of day 3, respectively. The blastomere number is recorded and the embryos are scored according to equality of size of the blastomeres and the presence of anucleate cytoplasmic fragments. On day 4 (sometimes on day 3), a certain degree of compaction can be observed **(E).** For blastocyst **(F)** scoring, the classification system introduced by Gardner and Schoolcraft is used. Embryo transfer is usually done on day 3 (eight-cell stage) or day 5 (blastocyst stage).

are scored according to equality of size of the blastomeres and proportion of anucleate fragments.[146,147] Type A (excellent) embryos do not contain anuclear fragments. Type B (good-quality) embryos have a maximum of 20% of the volume of the embryo filled with anucleate fragments. In type C (fair-quality) embryos, anucleate fragments represent 21% to 50% of the volume of the embryo. Type D (poor-quality) embryos have anucleate fragments present in more than 50% of the volume of the embryo. These embryos cannot be used for transfer to patients. Embryos of types A, B, and C are eligible for transfer. Because embryos with uneven cell cleavage have a lower developmental capacity in comparison with evenly cleaved embryos, emphasis on blastomere size within embryo scoring systems seems appropriate.[148,149] Assessing the degree of compaction is valuable as well because early compaction on day 3 may be associated with increased implantation potential.[150] Multinucleation is a frequently observed phenomenon in cleavage-stage embryos.[151] It may occur as early as the two-cell stage,[152] but has also been observed on days 2 and 3 of preimplantation development.[153,154] Of course, the proportion of multinucleated blastomeres within one embryo is an important parameter. Embryos presenting more than 50% of their blastomeres with multinucleation are not considered for transfer, regardless of the developmental stage at first detection. The presence of one or more multinucleated blastomeres (≤50%) has been associated with impaired cleavage and increased fragmentation,[151] a poor prognosis for blastocyst formation,[154] and reduces the ongoing implantation rate,[151] probably due to chromosomal abnormalities.[155] Excluding these embryos for transfer (if other mononucleated embryos are available) seems prudent practice.

Currently, most centers perform embryo transfers on day 3 after oocyte retrieval. At that time, the embryos are expected to be at the eight-cell stage. Because the embryonic genome is fully activated after the eight-cell stage,[156] it may be beneficial to continue to evaluate embryos at least until after the transition from the maternal to the embryonic genome, making it possible to identify embryos with better developmental potential. The number of embryos transferred depends on the age of the woman and the rank of trial. The only possible strategy to prevent multiple pregnancies in IVF and ICSI is to transfer a single embryo, especially in young women in their first or second attempt.[157-161] In women older than 36 years of age who are undergoing a first or second ICSI attempt, the preference is to transfer two excellent or good-quality embryos. In other cases, three or more embryos may be placed into the uterus. Higher pregnancy rates can be obtained when elective transfer of two or three embryos is possible.[72,162]

Advancements in commercially available culture media allow the culture of human embryos up to the blastocyst stage (day 5 or day 6).[163-166] On day 4 (sometimes on day 3), a certain degree of compaction can be observed. Compaction in the mammalian pre-embryo is a fundamental event that leads to the formation of the trophectoderm, the inner cell mass, and the blastocoele. Full compaction (16-cell to 32-cell stage) is followed by immediate cavitation and blastocoele expansion.[167] For blastocyst scoring, the classification system introduced by Gardner and colleagues can be used.[168] A distinction between early and expanded blastocysts is made, and the latter category is further scored according to the quality of the inner cell mass and the trophectoderm. The possibility of prolonged human embryo culture allows for day 5 or blastocyst transfers. Preferably, expanded blastocysts with a cohesive trophectoderm and a clear inner cell mass are transferred.

Possible advantages of blastocyst transfer are better embryo selection and better synchronization between embryo and endometrium, which may result in higher implantation rates per blastocyst transferred.[169,170] This, in turn, would allow transfer of fewer embryos, thereby decreasing the number of multiple pregnancies.[171-173] However, the superiority of blastocyst transfer over early embryo transfer remains to be proven. A limited number of randomized controlled studies are available. Some show the benefit of blastocyst transfer, whereas others show no advantage of day 5 transfers over day 3 transfers.[170-181] A recent Cochrane review examined the differences in live birth rates between cleavage and blastocyst stage embryo transfer. This extensive review of 23 randomized controlled trials (RCTs) demonstrated that there is a small significant difference in live

birth rates in favor of blastocyst transfer (day 5 or 6) compared to cleavage stage transfer (day 2 or 3).[182] However, it was found that the cumulative clinical pregnancy rates from cleavage (derived from fresh and thawed cycles) resulted in higher clinical pregnancy rates than from blastocyst cycles. The authors point out that this is most likely due to the higher rates of freezing and lower failure to transfer rates in the cleavage-stage transfer protocols.[182]

The cumulative success of a single embryo transfer policy, either on day 3 or on day 5, depends on a good cryopreservation program of cleavage-stage embryos or blastocysts. Supernumerary embryos with less than 20% of anucleate fragments (types A and B) can be cryopreserved on day 2 or day 3 after oocyte retrieval by means of a slow-freezing protocol with dimethylsulfoxide.[183] Alternatively, embryos can be frozen at the blastocyst stage on day 5 or day 6, with glycerol and sucrose used as cryoprotectant agents[184] or vitrified using ethylene glycol.[185-188]

Assisted Hatching

It is generally accepted that human embryos generated by IVF have a low implantation potential: a 10% to 15% rate of implantation per embryo is routinely reported in the literature.[189] Some researchers have suggested that premature transfer of the human embryo into the uterus may account for these low implantation rates. Data on the transfer of human cavitating morulae or blastocysts on day 4 or day 5 of development indicate that such embryos have a higher implantation rate.[169,190] In the case of blastocysts, implantation rates that are twice those of cleavage-stage embryos have been reported.[170] However, many cleavage-stage embryos do not reach the blastocyst stage in *in vitro* culture conditions, and it is not clear how many of these embryos would have implanted had they been transferred at the cleavage stage.[191]

Consequently, a remaining challenge in assisted reproduction is to overcome the obstacle of low implantation rates. Several factors, apart from synchronization between the endometrium and replaced embryos, are responsible. On one hand, endometrial receptivity is a factor in successful embryo implantation,[192] on the other hand, two embryonic factors play a major role in the implantation process. First, implantation failure may result from developmental arrest due to aneuploidy observed in human preimplantation embryos.[193,194] PGS programs, especially in older patients, have been proposed and are undertaken to address this issue (described in more detail later). Second, to implant, the preimplantation embryo must escape from its zona pellucida by means of a process called *hatching*. Following fertilization, the main function of the zona is protection of the embryo and the maintenance of its integrity.[195]

Developing embryos show a gradual thinning of the zona pellucida during culture. The mammalian late blastocyst both expands and thins its zona pellucida through alternating expansion and contraction.[196] Intrinsic processes allowing this thinning before successful hatching include pressure of the blastocyst as it expands against the zona and a lytic mechanism mediated by zona lysins.[197-199] Finally, the zona pellucida ruptures, allowing the embryo to hatch and implant itself into the endometrium.

An important observation leading to the clinical introduction of assisted hatching was the finding that there were higher implantation rates from embryos that were fertilized using microsurgical techniques.[200] In addition, it was observed that cleaved embryos with thinner zonae had higher implantation rates then those with thick zona pellucidas.[201] Many embryos may not succeed in hatching from the zona for different reasons. A naturally thick zona pellucida or hardening of the zona pellucida due to prolonged (suboptimal) *in vitro* culture conditions may interfere with (and prevent) the natural hatching process,[7] leading to implantation failure. Suboptimal culture conditions may cause trophectoderm lysin production to fall below the threshold level needed to promote zona thinning before successful hatching and subsequent implantation.[199] Alternatively, intrinsic lysin production failure unrelated to culture conditions may inhibit normal hatching.[199]

To overcome hatching failure, three different micromanipulation procedures (mechanical, chemical, and laser-induced hatching) have been used to produce holes in the zona pellucida of cleavage-stage embryos or to thin the zona pellucida superficially. Partial zona dissection (PZD) is a form of mechanical hatching that was developed to create an artificial opening of the zona pellucida of early cleaved embryos.[6] This technique is performed by stabilizing the embryo with a holding pipette, while the zona is pierced with a microneedle that is pushed tangentially through the space between the zona pellucida and blastomeres until it pierces the zona again. This spearing leaves a small part of the zona trapped against the microneedle. Rubbing this part of the zona against a holding pipette leaves a narrow incision in the zona. The mechanism of PZD is quick to perform; however, it produces holes of variable sizes that may not always be optimal.[202] A modification of PZD has been described where a second cut is made in the zona below the first cut at a right angle. This leaves a cross-shaped hole on the surface of the zona pellucida (3D-PZD).[203] This technique allows for the creation of a larger opening while maintaining the protection of the zona flaps during embryo transfer.

Chemical-assisted hatching involves the use of acidic Tyrode solution (pH 2.35) to "drill" the zona pellucida.[7] A micro-needle filled with acidic Tyrode solution is used to produce an opening at the 3 o'clock position, facing either empty perivitelline space or extracellular anucleate fragments. The acidic solution is expelled gently over a small area (30 μm), with the tip of the needle held very close to the zona until an opening is obtained. The mean diameter of the gap thus created is 20 ± 6.7 μm (range, 10-36 μm). It should be noted that the acidic Tyrode solution is embryotoxic and may alter the viability of the embryos.

Chemical-assisted hatching may be carried out with extracellular fragment removal.[204,205] However, the real benefit of this procedure must be shown in controlled randomized studies, which are lacking. Cruciate thinning of the zona pellucida using acidic Tyrode solution has been described, but chemical removal of the outside of the zona pellucida of day 3 human embryos has no effect on the implantation rate,[206] whereas the creation of a hole in the zona pellucida has been shown to increase embryo implantation.[7] The limiting factor for successful hatching is, therefore, not the overall thickness, but the resilience of the thin inner layer of the double-layered human zona pellucida.[206]

Table 32.2 Embryo Biopsy Methods: Advantages and Disadvantages

Hatching Methods	Advantages	Disadvantages
Mechanical	There are no effects of chemicals or heat during the drilling procedure Has not been shown to negatively affect fertilization or embryo development Inexpensive	Requires a double tool holder Time consuming Requires high technical skill PZD openings can be narrow and can induce more trauma to blastomeres during biopsy as well as lower hatching rates
Chemical	The size of the hole in the zona pellucida can be increased, which may enhance hatching Relatively inexpensive	Requires a double tool holder Difficult to regulate hole size Acid Tyrodes can be toxic and can change intracellular pH leading to cytoplasmic degeneration
Laser	No need for disposable micropipettes Consistent results and hole sizes Lower degree of blastomere lysis compared with acid Tyrodes Fast and easy to perform	Increased exposure time can damage embryo Equipment cost can be prohibitive Laser drilling creates an aperture in the shape of a groove which can cause constriction of the blastomere and result in lysis

More recently, laser-assisted zona pellucida drilling has been introduced and has been shown to be a suitable alternative to the use of acidic Tyrode's solution for zona drilling.[207,208] Several types of laser sources have been investigated. In a most convenient setup, the laser beam is guided through an optical lens and focused on the biological specimen. There is no need for holding and cutting tools. Lasers with different wavelengths have been proposed to operate in this no touch mode; however, infrared radiation is the most appropriate, especially when mutagenic risks are taken into account. The 1.48-μm diode laser allows rapid, precise, and easily controlled lysis of the zona pellucida.[209-212] The size of the hole can be chosen by varying the irradiation time; typically, a hole with a diameter of 20 μm is produced within 12 to 30 msec irradiation time. Larger diameter holes are obtained by increasing the irradiation time.

Piezo technology, an alternative to laser technology, also produces a precise and controllable defect in the zona, without chemicals that are potentially embryotoxic.[213] The vibration produced by the piezoelectric pulse is used for the assisted hatching. The zona pellucida is carved easily in a limited area that is conical and approximately 30 μm in diameter. Five to eight applications in adjacent areas are needed to treat approximately one third of the visible circumference of the zona pellucida. Thereafter, weaker vibrations are used to create an opening 20 μm in diameter in the thinned zona at the junction between blastomeres (Table 32.2).

Assisted hatching techniques, designed to facilitate embryo escape from the zona, have been used in IVF centers since 1992. The initial indications for assisted hatching were patient age, zona thickness, high basal FSH value, and repeated IVF failure. Several retrospective and prospective studies assessing assisted hatching in these cases have given disparate results. Therefore, the clinical relevance of assisted hatching in cleavage-stage embryos within an assisted reproduction program is heavily debated. Some of the controversy may be due to the different methods used for assisted hatching and, consequently, the irreproducibility of the hole sizes obtained. Patient selection may also be an important origin of conflicting findings. Moreover, many studies are uncontrolled and retrospective. In summarizing the existing randomized studies, several conclusions can be drawn.

Assisted hatching might be beneficial in women with a poor prognosis for IVF or ICSI, particularly those with repeated implantation failure.[214-218] However, there remains conflicting evidence of an age-related benefit of assisted hatching in patients with advanced maternal age.[219,220] In an unselected population of patients, assisted hatching did not increase pregnancy and implantation rates.[221-224] A recent meta-analysis that included randomized controlled trials of assisted hatching versus no assisted hatching and reported live births, clinical pregnancies, or implantation rates, concluded that there is some evidence that assisted hatching may increase the chance of pregnancy beginning in women for whom IVF has been repeatedly unsuccessful, and possibly in older women.[218]

There has been tremendous interest in improving the results of assisted reproduction through transfer of blastocysts after extended culture. Although human blastocysts expand readily *in vitro*, most blastocysts have hatching problems or cannot completely hatch out of the zona, and subsequently degenerate by day 6 or day 7. If the zona is softened enzymatically and zona-free blastocysts can be replaced, better cell–cell interactions and anchoring of the embryo to the endometrium may be expected, hopefully with improved implantation rates, reduced embryonic losses, and fewer embryos replaced.[225] Before transfer, blastocysts are exposed to pronase (10 IU at 37° C) for no longer than 90 seconds, which is the optimal time for softening or complete removal of the zona.[226]

The safety of this procedure was evaluated by light microscopy and transmission electron microscopy, showing that the human embryonic trophectoderm is a very hardy, robust epithelium that withstands pronase treatment.[226] Initial reports are promising, in that improved pregnancy and implantation results were shown with zona-free blastocyst transfers.[225,227-229] In the first randomized controlled study,[230] no statistically significant differences were found in clinical pregnancy, ongoing pregnancy, and implantation rates between zona-intact, blastocyst-stage transfers and zona-manipulated (enzymatic treatment), blastocyst-stage transfers. However, further studies with larger groups of patients are needed to clarify the real effect of this zona manipulation on pregnancy outcome. Whether a possible benefit may be differential for good-quality or poor-quality blastocysts, as reported by Urman and colleagues[231] must also be confirmed.

It is well known that the implantation potential of a frozen/vitrified-thawed/warmed embryo is lower than a fresh embryo. It is thought that the process of cryopreservation

may damage the embryo and cause the zona pellucida to harden, thus preventing the hatching and subsequent implantation from occurring. Several randomized studies investigating the clinical benefit of assisted hatching in cryopreservation cycles have shown both clinical benefits of assisted hatching[232-235] while other groups have shown no effect on implantation rates per embryo transferred.[236-240] A Cochrane database[218] on the effect of assisted hatching (AH) on frozen-thawed embryos including data from four randomized trials showed a significant improvement on the clinical pregnancy rate of frozen embryo transfer cycles; however, there was no evidence that assisted hatching had any effect on live birth rates.[218]

Embryo Biopsy

In 1990, preimplantation genetic diagnosis (PGD) was introduced as a method to genetically screen human embryos during an IVF cycle.[15] These procedures allow us to help couples who are at risk of transmitting inherited diseases to their children and more recently to improve pregnancy rates for certain groups of IVF patients. Genetic analysis of preimplantation developmental stages before transfer into the uterus inevitably involves removal of some cellular material at one of these stages. There are three different sources of genetic material potentially available for PGD: polar bodies from the initial conception, blastomeres from early cleaving embryos, and trophectoderm cells from embryos reaching the blastocyst stage.

Polar Body Biopsy

Polar bodies are byproducts of meiosis and have no biological role in fertilization or in embryo development. Therefore, removal of either the first or second polar body or both for the purpose of genetic testing should not have any deleterious effect on developing embryos. Genetic analysis of the first polar body was first performed by Verlinsky and colleagues[9] and has since been used to successfully screen for maternal mutations in cases of many single-gene defects.[241-246]

Polar body analysis is an indirect method in which the genotype or chromosomal constitution of the oocyte is derived from the complement present in the polar bodies. One challenge associated with polar body chromosome analysis is related to making the decision to discard oocytes with aneuploid polar bodies or based on the aneuploidy of the first polar body.[247] It is thought that both first and second maternal meiotic errors can be excluded only if information on the two polar bodies is obtained.[248] In a recent preclinical study, the ESHRE PGS Task Force aCGH data indicated that 4% of aneuploid polar bodies resulted in euploid zygotes.[249] Recently, Scott and colleagues reported the birth of a chromosomally normal child resulting from an oocyte with reciprocal aneuploid polar bodies.[250] Results from these reports challenge the idea of using polar bodies alone for aneuploid screening. These studies suggest performing aneuploid testing on the developing embryo for more definitive results.

A major limitation of polar body analysis is that only the maternal genetic contribution to the future embryo can be evaluated. This eliminates the possibility of determining the sex of the embryo. In addition, diseases that are detected by assessing changes in gene products are not candidates for this biopsy method. For single-gene disorders, occasional recombination of homologous chromosomes also requires information on both polar bodies. High accuracy in PGD of single-gene disorders can be achieved by application of sequential analysis of the first and second polar bodies in combination with multiplex PCR to detect possible allele dropout and contamination.[251,252] This process is very labor intensive.

The first polar body can be removed from the oocyte on the day of the oocyte collection between 36 and 42 hours after injection of hCG, as long as the oocyte has extruded the first polar body.[9] The second polar body can be removed from the zygote between 18 and 22 hours postinsemination. It is not recommended that both the first and second polar bodies be removed simultaneously as the first polar body may have degenerated by this time, leading to possible diagnostic errors. There are several ways to biopsy polar bodies. The most common method is by mechanical means. A beveled pipette (12 to 15 μm in diameter) can be used for mechanical perforation of the zona pellucida, and once inside the perivitelline space, the polar body can be drawn out by aspiration into the pipette.[243] If the polar body is still attached to the ooplasm, additional incubation may be required to permit complete extrusion.[9] In addition, it has been shown that the use of a laser for biopsy was safe and equivalent regarding the development of blastocysts compared with no biopsy.[253,254] Montag and colleagues also demonstrated equivalent results when comparing laser and standard zona drilling.[255] The use of acid Tyrodes for polar body biopsy should be avoided. Studies using human oocytes showed that, despite fertilization, there is an inhibitory effect on embryo development.[5] This is thought to be primarily due to the direct effect of acid on the oocyte spindle.

Cleavage Stage Biopsy

It seems essential to determine the developmental stage, to allow maximum retrieval of cells at biopsy with minimum reduction in the pregnancy potential of the embryos after the procedure. In humans, one of the best times to perform embryo biopsy is at the eight-cell stage, which is normally on the morning of day 3. At this stage, all of the cells are still totipotent (as confirmed by lineage tracing)[256] and the embryos are not yet compacting. It is believed that allocation to the two lineages of the blastocyst is initiated in the early eight-cell stage. Cells then become polarized at both the membrane and cytoplasmic levels.

It appears that removal of cells after the initiation of polarization does not compromise allocation to the inner cell mass.[257] However, cellular totipotency wanes with further embryonic development and may not be an all-or-none property (i.e., sister blastomeres could express it to different degrees).[258] Blastomeres of cleaving mammalian embryos can replace the loss of one or two blastomeres; this property presumably lasts, whereas blastomeres are allocated to, but not committed to, specific pathways. Apart from the reduction in cellular mass, the development of human embryos to the blastocyst stage *in vitro* is unaffected by biopsy and removal of one cell at the eight-cell stage.[259] This means

that up to one eighth of the embryo can be removed without impairment of its further *in vitro* development. Similar results were obtained in a mouse model.[260]

When Handyside and colleagues[15] reported the first pregnancies from the clinical application of PGD, it became clear that *in vivo* development was not impaired by the biopsy procedure either. In 1994, 32 pregnancies worldwide were reported after all types of preimplantation diagnosis using cleavage-stage biopsy, with 29 infants born and no evidence of adverse effects on development.[261] Moreover, it became evident that pregnancy rates after PGD were comparable to those obtained after standard IVF.[262,263]

Despite the reassuring clinical results so far, however, the discussion remains as to whether the removal of two cells from a seven-cell–stage embryo (or larger) reduces its capacity to implant more than if only one cell were removed.[264] A recent randomized controlled trial showed that removal of two blastomeres significantly decreases the likelihood of blastocyst formation compared with removal of one blastomere, although this did not translate into a significant difference in implantation rate (23.5% versus 17.3% for one-cell and two-cell biopsy, respectively, $P = 0.216$).[265] Taking live births per started cycle as an end point of this study (including 592 biopsy cycles), no significant difference was obtained between one-cell (20.2%) and two-cell biopsy (17.2%, $P = 0.358$). The recommendation is the removal of only one blastomere, provided that diagnostic safety measures are in place to ensure a correct diagnosis on one cell. If these are not available, two cells are needed for diagnosis.

One possible way to estimate the effect of removing blastomeres from cleavage stage embryos can be derived from experience with cryopreservation at the cleavage stage and associated cell loss.[266] It has been shown that the loss of viability after freezing is proportional to the number of blastomeres that lyse during the freezing and thawing process.[267,268] For example, if the expected implantation is 15%, then the loss of one blastomere from an 8-cell embryo would result in an implantation equal to $\frac{7}{8} \times 15 = 13.1\%$. Likewise, the removal of 2 blastomeres from the same embryo would result in an implantation equal to $\frac{6}{8} \times 15 = 11.3\%$.

Currently, cleavage stage embryo biopsy is the most widely practiced form of embryo biopsy worldwide, accounting for around 90% of all reported PGD cycles.[269,270] The latest ESHRE PGD Consortium reported the efficiency of successful embryo biopsy was 98% in over 70,000 cleavage stage embryos in clinical PGD cycles.[269]

Blastocyst Stage Biopsy

The advantage of biopsy at later developmental stages, such as the morula or blastocyst stage, is that relatively more cells can be collected than with biopsy at earlier developmental stages. However, the morula stage is an inappropriate stage for embryo biopsy because of extensive compaction, which occurs at the 16-cell to 32-cell stage in human embryos.[167] The blastocyst stage, on the other hand, offers the advantage that extra embryonic trophectoderm cells can be removed, leaving the inner cell mass (representing the future fetus) fully intact. The ability to biopsy more genetic material has been shown to reduce the risk of amplification failure and allele dropout (ADO).[271] Kokkali and colleagues prospectively compared genotyping success and implantation rates in PGD cycles after the biopsy of cleavage and blastocyst stage embryos.[271] The results of this study show a marked improvement in the successful genotyping of embryos biopsied at the blastocyst stage compared to those biopsied at the cleavage stage (94% versus 75%).[271] This study did not show any differences in the implantation potential of embryos biopsied at either stage.[271] In a more recent paired prospective trial using microarray-based DNA fingerprinting[272] to track the outcome of transferred embryos showed that cleavage-stage biopsy reduces the reproductive potential of embryos whereas biopsy of the trophectoderm does not.[273] In this study there was a significant reduction in implantation rates of biopsied cleavage-stage embryos compared with nonbiopsied controls (31% versus 54%, respectively). In contrast, implantation rates after trophectoderm biopsy were equivalent (52% versus 54%).[273]

A major limitation of PGD at the blastocyst stage is our lack of knowledge of whether the trophectoderm cells are representative of the embryo proper. The degree of blastocyst mosaicism seems lower than in cleavage-stage embryos[274,275]; however, no evidence exists to support the hypothesis of a preferential allocation of euploid cells to the inner cell mass and aneuploid cells to the trophectoderm.[274,276] No single study has compared the genetic constitution of the trophectoderm and that of the inner cell mass of single human blastocysts, which could finally help to clarify the uncertainty about the representation of a trophectoderm cell sample. Another disadvantage of blastocyst biopsy is the time limitation to obtain the diagnostic result if a fresh transfer is attempted. This might necessitate cryopreservation of biopsied blastocysts for transfer in a later unstimulated thaw cycle (Table 32.3).

Embryo Biopsy Procedures

According to the developmental stage, three different biopsy procedures have been well established: polar body removal from oocytes and zygotes, cleavage-stage embryo biopsy, and biopsy at the blastocyst stage (reviewed in De Vos and Van Steirteghem).[277] The biopsy procedure always involves two steps: opening of the zona pellucida and removal of the cellular material. Opening of the zona pellucida can be done in three ways:

1. Mechanically by direct puncture,[243] conventional PZD,[278,279] or three-dimensional PZD[203]
2. Chemically, using acidic Tyrode's solution[280]
3. Laser technology[210,211,281]

Polar body removal has, so far, been primarily linked with mechanical opening of the zona pellucida.[243] Cleavage-stage biopsy mainly involves the use of acidic Tyrode's solution or drilling or laser opening of the zona pellucida[16]; for blastocyst biopsy, both mechanical PZD[282] and laser drilling[283] have been described. Removal of polar bodies and biopsy of blastomeres is performed mainly by aspiration.[243] Extraembryonic trophectoderm cells are often removed by needle dissection at the moment of herniation through the slit created in the zona pellucida.[282,283] Animal models have been used to evaluate the efficacy and safety of most, if not all, of these procedures. Experimental work with human embryos donated for research has confirmed the feasibility and

Table 32.3 Preimplantation Genetic Diagnosis Biopsy at Different Developmental Stages: Advantages and Disadvantages

Developmental Stage	Advantages	Disadvantages
Polar Body	Considered ethically acceptable in many countries The 1st and 2nd polar body are not required for embryo development Increased time to perform diagnosis	Increased risk of oocyte lysis Can only be applied to maternally inherited diseases Requires sequential manipulations Gender determination not possible Increased risk of misdiagnosis compared to cleavage stage embryos
Cleavage Stage	Diagnosis of maternally and paternally inherited diseases Gender determination possible 95% of embryo cohort available for analysis Removal of 1-2 cells still results in viable development	Chromosomal mosaicism compromises accuracy Blastomere selection is critical Single cell sensitive analysis required Possible selected cell allocation to TE/ICM Reduced embryo implantation potential
Blastocyst Stage	Embryo quality is preselected Biopsy of TE cells more ethically acceptable More cells available for biopsy Increased amplification efficiency Decreased chance of misdiagnosis	Fewer embryos to biopsy Embryos must be extended in culture May require multiple manipulations There is a very limited time to perform diagnosis May require embryos to be cryopreserved and transferred at a future cycle

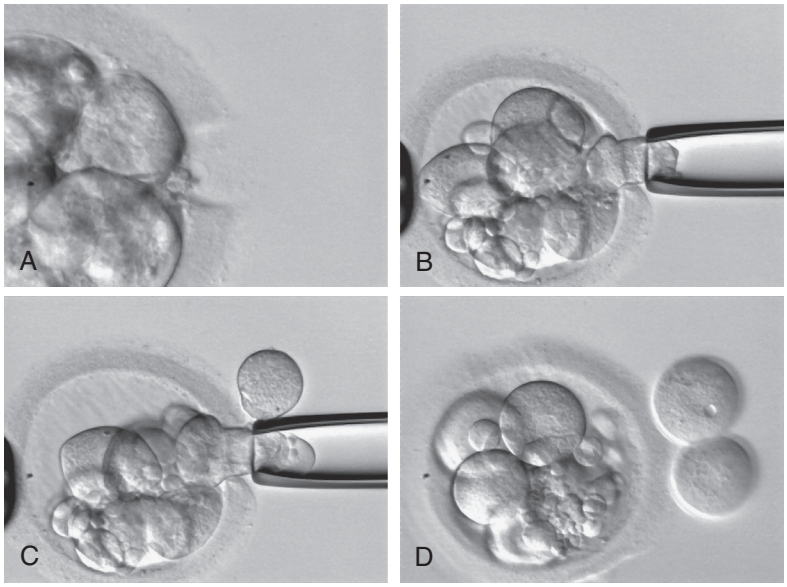


FIGURE 32.5 Cleavage-stage embryo biopsy. **A,** Laser-assisted zona drilling results in an opening in the zona pellucida with an average size of 20 to 30 μm. **B,** A biopsy pipette is introduced through the hole and a first blastomere is aspirated. **C,** Aspiration of a second blastomere can be done in a similar way. **D,** Preferentially, blastomeres with a clearly visible nucleus are aspirated.

efficacy of the procedures; however, safety evaluation has been limited to *in vitro* development.

Cleavage-stage biopsy remains the main approach to the removal of genomic DNA for genetic analysis at preimplantation stages.[15,270,284] Cleavage-stage biopsy is performed in the morning of day 3 after oocyte retrieval (Fig. 32.5). Unfortunately, not all human embryos reach the seven-cell or eight-cell stage by that time. Six-cell–stage embryos may, therefore, be included for one- or two-cell removal, although they are suspected of being of inferior quality because of delayed cleavage.[285]

At the moment of oocyte denudation, care is taken to remove all of the remaining cumulus and corona cells, which can also represent a source of DNA contamination. Chemical zona drilling, followed by blastomere aspiration, is normally done with separate pipettes, using a double-holder setup: one drilling pipette with an inner diameter of approximately 5 to 7 μm and one aspiration or biopsy pipette with an inner diameter of 35 to 40 μm. The target site for drilling is chosen between two blastomeres or in front of anucleate fragments to minimize the deleterious effect of the acidic medium on the cells. Zona drilling for embryo biopsy[280] is not different from the zona drilling technique described earlier for assisted hatching.[7]

Chemical zona drilling is in itself an extremely simple procedure in terms of equipment, but some technical skill may be required when exposing the embryo to an acidified solution. The opening should be of appropriate size, while limiting the extent and duration of exposure of the embryo. The mean diameter of the gap thus created is approximately 20 μm (range, 10 to 36 μm).[7] The rate of dissolution of the zona may vary, and the time taken to complete this process varies accordingly (30 seconds to 2 minutes). Acidic drilling, despite its toxicity and cytoplasmic acidification, is considered safe on the basis of the pregnancies and births reported in the literature.[16]

As an alternative to chemical zona opening, laser-assisted zona pellucida drilling has been introduced for cleavage-stage biopsy[281] (which is similar to the laser drilling technique previously described for assisted hatching). Using the X-Y

microscope stage, the embryo is positioned to place a region of the zona pellucida on the aiming spot. Typically, a trench-like hole with a diameter of 5 to 10 µm is produced in 10 to 15 msec.[211] For embryo biopsy, on average, two to three pulses of 7 msec are applied, resulting in 20 to 30 µm openings. It is important to perforate the zona completely without harming the embryonic cells with the laser. The holes obtained with a laser are more precise than the ones obtained with acidic Tyrode solution. Laser zona opening results in more intact blastomeres compared with the use of acidic Tyrode solution, and because similar pregnancy rates were obtained, it seems advantageous to use a laser for zona drilling.[286]

Blastomere Removal

Blastomere removal involves the introduction of the biopsy pipette into the perivitelline space through the hole in the zona, allowing one or two blastomeres to be removed by gentle aspiration. Cells can be aspirated completely and then removed; alternatively, cells are only partially aspirated and pulled out (given full decompaction and thus no adherence to other blastomeres). Whereas full compaction does not occur before the 16- to 32-cell stage,[167] relatively early assembly of tight junctions occurs between human blastomeres, as shown by ultrastructural studies.[287,288] Because of these membrane adhesions, the biopsy procedure at the seven-cell to eight-cell stage may be difficult to perform because the blastomeres show a strong tendency to adhere to each other. Manipulating the embryos as such may result in a high lysis rate of cells.

A calcium- and magnesium-free medium has been used to loosen the membrane adhesions between blastomeres,[289,290] which allows easier removal of cells and results in less lysis. Embryo biopsy can be performed completely in this medium or, to limit exposure time, embryos can be preincubated for 5 to 10 minutes (normally sufficient for full decompaction) before biopsy in calcium- and magnesium-containing medium. Whether the implantation rate or postimplantation development is affected by the use of calcium- and magnesium-free medium for biopsy remains to be assessed. Therefore, the long-term safety of its clinical application should be further substantiated.

Cleavage stage embryo biopsy offers the advantage of choice of cells to biopsy whereas polar body and blastocyst stage biopsy do not. When selecting the blastomere to biopsy, it is critical to select cells with visible nuclei. Visualization of nuclei can be difficult when cells overlap each other or when the cytoplasm is granular or pitted. However, under high magnification most nuclei should be discernable. Visualization of the nucleus will greatly reduce the risk of biopsying large anucleate fragments. It is also advisable to select the smaller blastomeres for biopsy. The small size indicates that the cell has undergone its mitotic division. Large blastomeres may not have undergone mitotic division or could be in the process of cytokinesis. Biopsying of large cells will greatly reduce the mass of the embryo and reduce to subsequent developmental viability of the embryo. Furthermore, larger cells are at higher risks of being in metaphase which in turn may make the chromosomes more prone to loss during fixation. Finally, multinucleated blastomeres should be avoided during biopsy as they are likely to result in unclear diagnoses.

Trophectoderm Biopsy

With the improvements in embryo culture, blastocyst conversion and high success rates with blastocyst vitrification, an increasing number of labs are performing blastocyst-stage biopsy. The human blastocyst, depending upon developmental stage, can contain more than 100 cells. As such, the biopsy of 6 to 10 cells from the outer layer of the trophectoderm is less likely to have a detrimental effect on the blastocyst's mass of the developing fetus which originates from the inner cell mass (ICM).[282,283,291] The technique of zona slitting (similar to partial zona dissection) can be used to open the zona pellucida with a microneedle.[282] The blastocyst zona can also be opened using a laser.[283] In both cases, care must be taken to avoid touching the trophectoderm cells, and the slit or opening is made opposite the inner cell mass. After definite herniation, trophectoderm cell removal is performed by excision using a glass needle[282,292] or laser energy.[283] Serial biopsy specimens can even be taken from the same blastocyst[293,294] to maximize the number of cells available for PCR or FISH analysis. Several recent studies report promising clinical results with trophectoderm biopsy.[271,295,296]

Preimplantation Genetic Testing

Preimplantation genetic testing (PGT) includes preimplantation genetic diagnosis (PGD) performed for monogenic diseases and translocations, as well as preimplantation genetic screening (PGS) for aneuploid screening. The PGT procedure is a very early form of prenatal diagnosis for patients with a preexisting genetic risk. Oocytes or embryos obtained *in vitro* through assisted reproductive techniques, in particular, ICSI, undergo biopsy and the cells obtained (polar bodies, blastomeres, or trophectoderm cells) are used for genetic diagnosis. In principle, the indications for PGT are similar to those for prenatal diagnosis after chorionic villus sampling or amniocentesis, given that PGT at the single-cell level is technically possible. The main reasons for PGT (in addition to the genetic risk) are previous termination of pregnancy and objection to pregnancy termination. The genetic risk can also be combined with subfertility or infertility.

Preimplantation Genetic Diagnosis

The PGD procedure is used for couples at risk for monogenic disorders. Three disease groups can be distinguished according to the mode of inheritance: X-linked disorders (e.g., Duchenne and Becker muscular dystrophy, fragile X syndrome, hemophilia); autosomal recessive disorders (e.g., cystic fibrosis, thalassemia, spinal muscular atrophy, sickle cell anaemia); and autosomal dominant disorders (e.g., myotonic dystrophy, Huntington disease, Charcot-Marie-Tooth disease, Marfan syndrome).[16] Several laboratories offer patients custom-made PGD tests for monogenic disease, so the exact number of indications for which PGD is offered would be difficult to establish. A prerequisite for PGD is that the mutation is known or otherwise can be analyzed through linked markers. Appropriate primer sets are designed and tested on genomic DNA of the couple and family members. Lymphoblasts of the couple or an affected child allow determination of the amplification efficiency as

well as the contamination and allele dropout rate for each PCR protocol.

More recently, combined PGD and human leukocyte antigen (HLA) matching has been reported,[297] representing a novel way to preselect an unaffected potential donor for an affected sibling requiring stem cell transplantation. Transplantation of hematopoietic stem cells from an HLA-identical sibling donor may be the best treatment option for children with neoplastic and congenital diseases affecting hematopoiesis, such as Fanconi anemia,[298] leukemia, Wiskott-Aldrich syndrome, and β-thalassemia. Cord blood collected at birth is an established source of hematopoietic stem cells. In contrast to individual establishment of protocols for each requesting couple, indirect HLA typing using markers evenly distributed in the HLA polymorphic region is a more elegant and time-saving approach.[299]

The PCR procedure involves exponential amplification of short DNA fragments using two short, single-stranded DNA pieces (primers) that are complementary to the boundaries of the sequence to be amplified. A standard PCR protocol involves 20 to 45 cycles of repeated denaturation (of the double-stranded template DNA) at high temperature, annealing (of the primers to the template DNA) at a lower temperature, and elongation using Taq DNA polymerase. PCR allows analysis of the genetic content of a single cell.[300] In PGD, DNA amplification is used to detect single-gene defects (so-called *monogenic diseases*).

However, PCR protocols are subject to a variety of pitfalls, such as variable amplification efficiency, contamination, and allele dropout.[301] Fluorescent single-cell PCR has not only a higher sensitivity, but also a higher resolution.[12,302] Different measures are taken to avoid or at least detect contamination from genomic DNA (patient or operator) or carry-over contamination (from PCR products amplified earlier).[303] To show contaminating DNA, an increasing number of PCR-based tests now include amplification of hypervariable fragments of DNA in addition to the loci used for diagnosis. These hypervariable polymorphisms are essentially the same as those used for DNA fingerprinting. The presence of more than the expected two alleles indicates contamination (either from an external source or from parental origin). Contamination with parental DNA can occur as a result of inadvertent sampling of a sperm or cumulus cell at the time of embryo biopsy. The management of amplification failure and allele dropout (i.e., nonamplification of one of the two alleles in a single cell) is of extreme importance because it may result in misdiagnosis. Multiplex PCR with linked markers, with or without fluorescent detection,[251,304] is a straightforward strategy. Today, multiplex PCR (i.e., simultaneous amplification of two or more DNA sequences) is used extensively in PGD, especially for more frequently occurring diseases.[305,306] Usually, attempts are made to combine a specific mutation with ideally linked markers or otherwise unlinked markers.[251,306] Allowing simultaneous testing of linked markers alongside the mutation test significantly increases the accuracy of single-cell PCR.[307,308]

The more recent advent of highly effective whole-genome amplification methods, generating abundant assay-ready DNA from single cells for diagnosis of any known single-gene defect, marks a new era for preimplantation genetic testing.[309] The ideal whole-genome amplification technique should have high yield, faithful representation of the original template, and complete coverage of the genome.[310] The use of whole-genome amplification as a universal first step allows subsequent simultaneous analysis of more than one mutation by standard PCR protocols[311-313] or microarrays.[314] The inclusion of several polymorphic markers allows haplotype analysis.[315,316] Preimplantation genetic haplotyping definitely widens the scope and availability of preimplantation testing because one panel of markers can be used for all carriers of the same monogenic disease, bypassing the need for the development of mutation-specific tests.[316]

Preimplantation Genetic Screening

In PGS, embryos are screened for chromosomal abnormalities in patients with impaired fertility, patients of advanced maternal age, patients with several failed IVF cycles (i.e., recurrent implantation failure), patients with recurrent miscarriages, or male patients with nonobstructive azoospermia. Selecting chromosomally normal embryos for replacement should increase implantation rates, reduce spontaneous miscarriage rates, reduce aneuploid conceptions, and improve delivery rates in assisted reproductive technology cycles.[299,317-319] The number of referrals for PGS has only increased since its first clinical application,[320] as evidenced by the European Society of Human Reproduction and Embryology PGD Consortium data collection,[16] but the benefits of PGS are still under debate.

FISH

In FISH, fluorochrome-labeled DNA probes are used that are complementary to DNA sequences specific to individual chromosomes. Simultaneous analysis of several chromosomes is possible, even in interphase cells.[321-323] Polar bodies or blastomeres obtained for biopsy are spread on a glass slide and fixed.[322-324] The labeled probes are added and allowed to hybridize to the chromosomes. Excess probe is removed, and the result can be analyzed using fluorescence microscopy with different filters for different fluorochromes.

At least one FISH probe is now available for every chromosome, although the limited number of fluorochromes available restricts the number of probes that can be simultaneously applied to a single interphase nucleus. Additionally, misdiagnosis due to overlapping signals would become a problem. Commercially available kits containing 9 chromosome-specific probes are often used in a first round; after probe removal, a second round of hybridization for even more chromosomes can be performed.[322]

For Robertsonian translocations (in which a whole chromosome is translocated), simple enumeration of the chromosomes leads to a reliable diagnosis,[325,326] but the situation is more complex for reciprocal translocations (exchange of fragments between chromosomes). A generally adopted practice is the simultaneous use of two differentially labeled subtelomeric probes distal to the breakpoint (one for each chromosome), together with a centromeric probe (to one of the chromosomes involved). This strategy allows for the distinction between normal, balanced genotypes and unbalanced genotypes.[327]

The obvious limitation of FISH is that a restricted number of chromosomes can be tested in a single cell. Aneuploidies at the level of chromosomes that have not been analyzed,

undoubtedly existing, are overlooked.[328] Data from spontaneous abortions suggests that 9 to 12 probe FISH assays detect only 57% to 67% of aneuploid embryos.[329-332] In addition, Munné and colleagues strongly suggests that centers not using probes for chromosomes 15, 16, and 22 have poorer results than those using those probes.[332] If more chromosomes could be analyzed, the value of PGS could likely be increased. Another limitation of FISH is due to the high error rates. PGS error rates using FISH range from 5% to 7% in large studies[333,334] to 50% in smaller studies.[335] Many of these errors are thought to be center- and technique-related and not related to the number of probes. Colls and colleagues demonstrated that there were similar error rates in a 9-probe panel as in a 12-probe panel.[333] These results indicate that the addition of 3 probes to the 9-probe panel does not increase the error rate. Recently, Munné and colleagues published optimized techniques for performing FISH.[332] These techniques have been fully validated, thousands of clinical cycles have been conducted and accuracy rates have been well established.[332]

The initial goal of FISH was to improve pregnancy rates and to reduce miscarriage rates. Despite promising results from studies with matched controls,[318,336] recent randomized prospective clinical trials failed to observe a significantly increased live birth rate after transfer of chromosomally "normal" embryos selected by FISH following PGS.[337-346] As a result of many of these trials, the Practice Committee for the Society for Assisted Reproductive Technology and the Practice Committee of the American Society for Reproductive Medicine issued the following recommendations for PGS[346]:

1. Before PGS is performed, thorough education and counseling must be provided to ensure that patients fully understand the limitations of the technique, the risk of error, and the lack of evidence that PGS improves live-birth rates.
2. Available evidence does not support the use of PGS as currently performed to improve live-birth rates in patients with advanced maternal age.
3. Available evidence does not support the use of PGS as currently performed to improve live-birth rates in patients with previous implantation failure.
4. Because the prevalence of aneuploidy is high in the embryos of patients with recurrent implantation failure, decisions concerning future treatment should not be based on the results of PGS in one or more cycles.
5. Available evidence does not support the use of PGS as currently performed to improve live-birth rates in patients with recurrent pregnancy loss.
6. Available evidence does not support the use of PGS as currently performed to reduce miscarriage rates in patients with recurrent pregnancy loss related to aneuploidy.

This report does not invalidate the concept of aneuploidy screening. However, findings from this report should caution programs from adopting new technologies without adequate preclinical validation and proven clinical benefit.

Comprehensive Chromosome Screening (CCS)

The most successful technique for complete karyotyping of single cells is comparative genomic hybridization (CGH).[347] Differentially labeled tests, DNA (green) and normal reference DNA (red), obtained by whole-genome amplification are hybridized simultaneously to normal metaphase chromosomes. The relative green-to-red ratio for each chromosome is measured using a cooled charge-coupled device camera and image analysis. Deviations from a 1:1 ratio (which in itself lights up as yellow) are indicative of loss (red) or gain (green) of chromosomal material in the tested cell. The obvious advantage of CGH over FISH is that the copy number of all chromosomes can be determined. In addition, CGH provides a more detailed picture of the entire length of each chromosome. Although FISH is routinely used to determine the ploidy of particular chromosomes, all that it really shows is that the target sequence of the probe (often located in the centromere) is present. It does not provide information about the rest of the chromosome. CGH has been successfully applied to single cells[348,349] and interphase blastomeres.[350,351] The early clinical applications[352-354] in view of aneuploidy testing are promising, and CGH showed great potential for translocation carriers as well.[355] One of the biggest drawbacks of CGH has been that it was relatively labor-intensive procedure, and that it took several days to obtain a result. This was longer than 3-day-old embryos could be maintained in culture and required embryos undergoing biopsy to be frozen until results become available. Microarray CGH has been shown to alleviate this time constraint.[247,356-359]

Microarray CGH is made up of DNA sequences specific to human chromosomes spotted onto a glass slide. The principle is the same as metaphase chromosome CGH in that differentially labeled reference DNA and test DNA are hybridized to the slide and differences in the fluorescence ratio are indicative of changes in DNA copy number. Array CGH takes less time, primarily because less than 9 hours is required for hybridization. The benefit of complete karyotyping for PGD is that all CGH studies on blastomeres have identified chromosome errors that would not have been detected if the cells had been analyzed by FISH; however, the procedure remains technically challenging.[347]

The use of single-nucleotide polymorphism (SNP) genotyping arrays has been pioneered by Kearns[360] and Treff and their colleagues, and extensively validated for both whole chromosome and segmental imbalance by the latter group.[361-363] This technology uses complex bioinformatic modeling algorithms to detect whole-chromosome and segmental copy-number changes and to distinguish between meiotic and mitotic errors. The advantage of this technique is that it is possible to analyze hundreds of thousands of loci across the genome using a single array, with an average spacing of 5 kb, enabling high-resolution analysis. At the same time, the genotype information allows the parental origin of any abnormalities to be identified by reference to the genotypes of the two parents.[364] The major drawbacks from this technique is due to the high cost of equipment and the lengthy protocol which can take up to 24 hours for results.[364] More recently, Treff and colleagues have developed and fully validated a real-time PCR based SNP approach which can be completed within 4 hours.[362,365] Preliminary randomized control trial results of 24-chromosome aneuploidy screening with qPCR on trophectoderm biopsies and subsequent fresh euploid blastocyst transfer indicate a significant increase in the success of IVF.[296]

Despite conflicting reports using FISH, aneuploid screening remains an important tool for embryo selection for a large

segment of the infertile population. Recently there have been many advancements in CCS technologies. Many of these screening methods have or are currently undergoing exhaustive preclinical validation to ensure accuracy and to determine positive predictive values prior to their clinical application.[366] Harper and colleagues suggest that these trials should be designed to assess the anticipated benefits (a substantial improvement in live birth rate and reduction in miscarriage rates), in relation to the risk and cost for the couple, while addressing biopsy safety, test accuracy, and diagnostic efficiency, in order to identify appropriate indications for PGS.[367]

Blastocyst Collapse for Cryopreservation

Recent advances in culture systems have made it possible to routinely develop high percentages of human IVF embryos to the blastocyst stage. Transferring viable blastocysts offers high efficiency for maintaining pregnancy rates while minimizing multiple gestations in the human.[177] As culture systems improve and more and more clinics move toward single blastocyst stage transfer, there is an increased need to develop methods of blastocyst cryopreservation that have high rates of survival and has minimal impact on the embryo's viability. Conventional slow freeze methods of freezing applied to human blastocysts had moderate success[368] and the vitrification procedure improved the viability of post-warm blastocysts.[369] The difference in success rates between slow freeze and vitrification methods is likely due to the formation of intracellular ice. An increased rate of cooling and warming with vitrification has helped circumvent this problem, though not eliminate it entirely. Previous reports have shown that warmed blastocyst survival rates were dependant on the developmental stage of the blastocyst and were negatively correlated with the expansion of the blastocoele.[370] Early blastocyst stage embryos grade 1 to 3[371] had a total survival rate of 91%, while more expanded blastocysts grades 4 and 5 had significantly lower survival at 85%.[370] It is thought that the large blastocoele might reduce the success of the vitrification process due to insufficient dehydration and ice crystal formation. To overcome this problem and improve cryotolerance, many programs artificially shrink or collapse the blastocyst.

Collapsing of the blastocoele is utilized prior to cryopreservation, including both slow rate or vitrification procedures, with the intent of removing the blastocoele fluid to prevent harmful ice crystal formation. Though use of nonpermeating and permeating cryoprotectants used in various protocols are meant to remove and replace this blastocoele fluid, efficacy of this exchange may be compromised depending on blastocyst stage, quality of the embryo, type of cryoprotectants, or the length of cryoprotectant exposure time. For example, expanded blastocysts with a high intrablastocoele fluid pressure may not have the same fluid exchange as a hatching or early blastocyst with lower pressure. In these cases especially, collapse of the blastocoele may help standardize cryoprotectant exposure times and ensure less variation in cryoprotectant exchange.

Artificial blastocoele collapse may be performed several different ways. Treatment with hyperosmotic sucrose solutions makes it possible to induce artificial shrinkage without special equipment, instruments, or micromanipulation skills.[372] Collapsing can also be induced by using a holding pipette and an ICSI needle, with the needle being carefully placed between trophectoderm cells to disrupt tight junctions and permit fluid release.[373,374] Slight suction may be required to facilitate blastocoele collapse. Use of this approach entails a lengthy set-up and often necessitates set up of additional culture dishes utilizing a zwitterionic buffer handling media. Blastocysts can also be mechanically collapsed using 29-gauge needles[375] and by rigorously pipetting blastocysts up and down.[376] Alternatively, a pulse of a laser between trophectoderm cells can also facilitate blastocoele collapse and can be performed directly in the embryo culture dish.[374] In both cases, it often takes several minutes for complete collapse to occur. Regardless of the method utilized, embryos should be placed back into the CO_2 incubator until collapse is complete. During the procedure, care should be taken to avoid the ICM and to minimize the amount of trophectoderm cells damaged. Furthermore, use of a laser to facilitate collapse may also entail use of assisted hatching to enlarge the hole made in the zona pellucida. Following blastocoele collapse, there may be cellular debris attached to the inside of the zona pellucida, but the majority of the blastocyst should appear as a centrally located tight cluster of cells. Blastocoele cavities begin to re-expand within 1 hour after the thaw or warming. These observations can be used to assess the survival of cryopreserved blastocysts prior to embryo transfer.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 33

Fertility Preservation

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Infertility and Cancer

Oncofertility lies at the intersection of infertility and cancer. Though both are ancient human concerns, it has only been within the past decade that the two conditions have come together, largely because of significant changes in the perception of and advances in each field. Until very recently, infertility was seen as a social issue rather than a medical condition that warranted intervention, while cancer was thought to lead to unavoidable death. Today, technological and therapeutic advances in oncology and reproductive medicine have changed the lives of patients with these conditions.

The word "cancer" refers to a broad category of abnormal neoplastic diseases, affecting virtually all tissues and organ systems within the human body.[1] Cancer will affect approximately one half of all men and one third of all women in the course of their lifetimes. The 20th century was a time of significant advances in oncological diagnosis and treatment, with improvements in cancer detection and in all oncological treatment modalities (chemotherapy, radiation therapy, and surgery).[1] While cancer remains the second leading cause of death in the United States, in men, cancer death rates declined by 21% between 1990 and 2006, with decreases in rates of death from lung, prostate, and colorectal cancer, specifically.[2] In women, cancer death rates declined by 12% between 1991 and 2006, primarily due to decreased rates of death from breast and colorectal cancer.[2]

The History of Fertility Preservation and Development of Oncofertility

Despite the significant advances made in cancer diagnosis and treatment, a host of undesirable health changes can occur secondary to the cancer itself and/or as side effects of cancer therapy. Among these detrimental effects are alterations in normal reproductive processes in males and females—including fertility, endocrine health, sexuality, or the ability to carry an offspring to term. Injury to the endocrine system, nervous system, vascular system, gonads, and genitalia can all lead to altered reproductive health. As mortality rates from cancer decline, survivorship issues such as post-treatment infertility have become increasingly important.

Fertility management or preservation specifically in the setting of cancer is a recent construct that developed based on the increased survivorship of young cancer patients that has been made possible by extraordinary advances in cancer diagnosis and therapy. For men facing a cancer diagnosis, fertility management primarily involves sperm banking, or sperm cryopreservation. While sperm banking was first described in the 1960s,[3] the first reference to sperm cryopreservation in the cancer setting did not appear until 1980, when 22 men with testicular carcinoma or lymphoma were referred for semen cryopreservation prior to undergoing fertility-threatening cancer therapy.[4] The first case of a successful pregnancy with in vitro fertilization (IVF) using cryopreserved sperm from a man with a testicular malignancy was reported in 1985.[5] Additional successes with IVF using cryopreserved sperm from cancer patients throughout the 1990s were reported.[6,7] Awareness of fertility preservation in men with cancer was hurled into the spotlight in 1996 with the highly publicized use of sperm banking by the cyclist Lance Armstrong—performed prior to undergoing chemotherapy and radiation therapy for testicular cancer—which later allowed him to build a family as a cancer survivor.

While IVF techniques and cryopreservation of embryos had taken hold by the late 1980s, use of these techniques specifically to preserve the fertility of female cancer patients was not reported until 1996, by Brown and colleagues.[8] The authors described a patient with breast carcinoma who underwent natural cycle IVF prior to undergoing chemotherapy. The earliest report of a pregnancy using cryopreserved oocytes for IVF was published by Chen in 1986,[9] though the use of this technique in a cancer patient to achieve pregnancy and live birth was not reported until 2007.[10] Cryopreservation of ovarian tissue was first reported in 1996 by Hovatta and colleagues,[11] and the potential applications of this procedure in fertility preservation for cancer patients was discussed in the same year by Newton and colleagues.[12] Somewhat surprisingly, the first reference to ovarian tissue transplant is in 1895, described in a thesis by Dr. Robert Morris.[13] Yet it wasn't until 2008 that Silber and colleagues transplanted cryopreserved-thawed ovarian tissue to restore fertility in patients with premature ovarian failure,[14] and Andersen and colleagues—using cryopreserved ovarian tissue from patients with malignancy who had undergone gonadotoxic treatments—were able to restore fertility that resulted in successful pregnancies after ovarian tissue transplantation.[15]

In 2006, the field of oncofertility was developed with the express purpose of preserving, expanding, and restoring the reproductive future of individuals—adults as well as children and young adults—whose cancer treatment may compromise their fertility.[16] To ensure that these reproductive needs would be met, the Oncofertility Consortium was established and funded by a National

Institutes of Health Roadmap Grant.[17] In an exemplary model of team science, the Oncofertility Consortium has advanced the field of fertility preservation by coordinating the work of clinicians, basic science researchers, social scientists, ethicists, and humanists so that breakthroughs at the bench would continue to efficiently and safely transform patient care (Fig. 33.1).[17] Over the past decade, the mission of fertility preservation has gained national and international momentum, including partnerships with the European Society of Human Reproduction and Embryology (ESHRE). Moreover, the field of fertility preservation is expanding to include not only patients with cancer but also those with other diseases or conditions or undergoing treatments that may result in compromised reproductive function.

Parallel developments in fertility interventions and advances in cancer diagnostics and therapeutics have led to the convergence of fertility management and oncology and the search for new fertility preservation options for an increasing population of young cancer survivors. Yet it is hoped that with better preventative care, earlier diagnosis of cancer, and targeted cancer therapeutics, fertility preservation in the cancer setting will one day become another footnote in the history of reproductive medicine.

In this chapter, we provide a comprehensive overview of the current state of the field, including how cancer and its treatment impact gonadal and reproductive function; the current fertility preservation strategies for men, women, and children diagnosed with cancer; medical and patient management in the setting of oncofertility; special cases in fertility preservation; legal and ethical issues in the field; and the next frontiers in the basic science that drives oncofertility.

Reproductive Dysfunction as a Result of Cancer or Its Treatment

With greater numbers of patients winning their battle against cancer, there is an increased demand that quality of life for survivors be as close to normal as possible. For young men and women, a high quality of life includes regaining reproductive function and preserving the option to have a family. Yet an unfortunate consequence of the treatments that will preserve these patients' lives is that nearly all aspects of both the male and female reproductive axis can be compromised. In the female, the hypothalamic-pituitary-gonadal (HPG) axis, the ovaries and ovarian follicles, and the uterus are directly affected by cancer treatments; in the male, the testes are at risk, specifically the germinal epithelium and Leydig cells.

Cancer and Reproductive Function

Cancer itself may negatively impact reproductive function. For example, semen quality parameters tend to be poor prior to treatment in men with testicular cancer, leukemia, or lymphoma.[18-20] In females, the association is less clear. In one recent study, the success of ovarian stimulation was compared in women with malignancies prior to cancer treatment to a similar group of women seeking assisted reproduction for male-factor infertility.[21] No differences were found between these two groups of patients in terms of the number of dominant follicles and retrieved oocytes, proportion of mature eggs, or fertilization rates. However, a small meta-analysis that examined all types of malignancies found that there were fewer numbers of oocytes retrieved from women with breast and hormone-dependent cancers.[22]

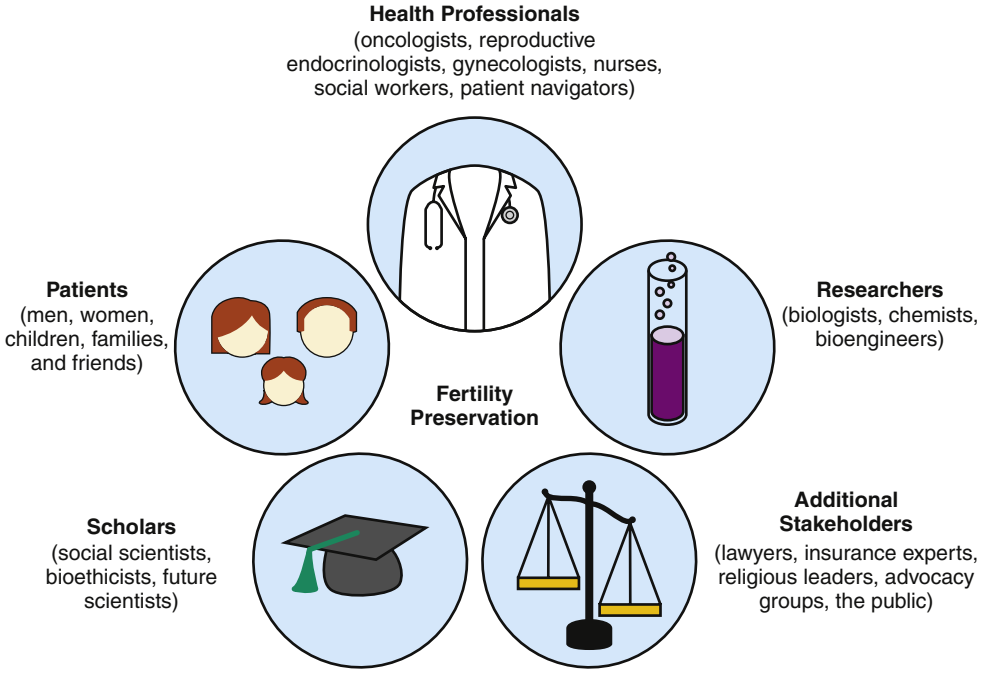


FIGURE 33.1 It has long been known that cancer and iatrogenic insults such as chemotherapy, radiation therapy, and surgery can negatively impact reproductive function; and fertility preservation has a long-standing history in the field of reproductive science. A large, committed, integrated, and multidisciplinary team consisting of health professionals, researchers, scholars, patients, and legal and ethical experts is required to transform the concept of fertility preservation into a clinical reality. With the inception of the Oncofertility Consortium in 2006, the mission of exploring and expanding the reproductive options for men, women, and children with cancer was put into motion with the creation of the required team to address this unmet need nationally and internationally. This movement is now expanding to include patients with any condition or undergoing any treatment that may threaten fertility.

Cancer Treatment and Female Reproductive Function

Several common cancer therapies are known to impair reproductive health. A fertile woman must have: (1) a functioning neuroendocrine system that regulates the menstrual cycle and can maintain a pregnancy; (2) a healthy pool of ovarian follicles that will grow in response to hormonal cues and produce mature and fertilizable gametes; and (3) a receptive uterus that will support embryo implantation and fetal development to term. Cancer treatment can affect each of these (Fig. 33.2). For example, radiotherapy can damage

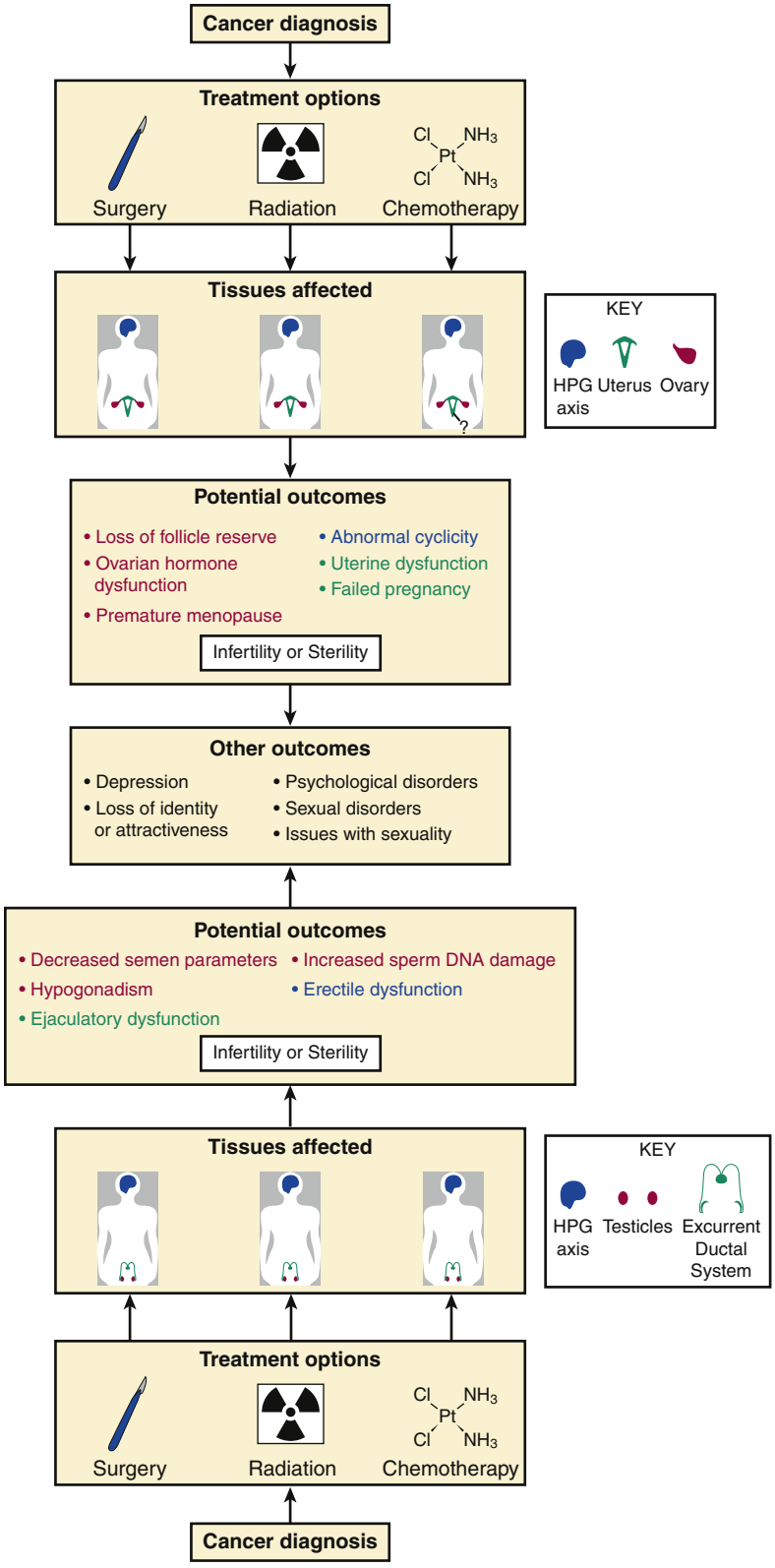


FIGURE 33.2 Major female reproductive systems, including the hypothalamic-pituitary-gonadal (HPG) axis, the uterus, and the ovary, can be negatively impacted by common cancer treatments. In fact, it has been demonstrated that chemotherapy, radiation, and surgery can directly affect all three of these systems with the only question being the effect of chemotherapy on uterine function. Such iatrogenic insults can result in adverse reproductive outcomes ranging from sexual disorders to infertility. Similar to the female, the male gonads and endocrine system are also highly sensitive to common cancer therapies.

growing follicles, which triggers either repair or elimination pathways.[23] In general, actively dividing cells are more susceptible to radiation-induced death, and because oocytes in the young adult are arrested in prophase of meiosis I, they are more resistant to radiation than cells in mitosis. Primordial follicles, which are quiescent, also appear to be more resistant to radiation compared with growing follicles.[23] Nevertheless, the human oocyte is sensitive to radiation therapy. The LD_{50}, or the radiation dose required to destroy 50% of immature human oocytes, is less than 2 Gy.[24] Mathematical modeling predicts that the effective sterilizing dose of radiation is inversely correlated with age: it is 20.3 Gy at birth and 16.5 Gy at 20 years of age.[25]

In addition to being radiosensitive, the ovary is also chemosensitive. Alkylating agents, especially cyclophosphamide and busulphan, are more gonadotoxic compared to other chemotherapeutic agents, including platinum-based drugs, plant alkaloids, and anti-metabolites (Table 33.1).[26,27] Alkylating agents, which cause DNA breaks irrespective of cell cycle stage, are associated with a high risk of primordial follicle death and compromised stromal cell function.[28] Follicle destruction, whether by radiation- or chemotherapy-induced mechanisms, not only leads to gamete loss, but also results in impaired ovarian hormone production and uterine dysfunction. Although follicles may resist cancer therapies, the ovarian reserve may be compromised and depleted early, resulting in premature menopause.

The female neuroendocrine axis (or HPG axis) is also vulnerable to off-target effects of cancer therapies. The HPG axis controls the menstrual cycle and pregnancy by regulating the secretion of hormones, including gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, progesterone, and prolactin. Radiation therapy, particularly targeting the cranium, can cause altered hypothalamic and pituitary function.[23,29,30] There is also evidence that hypothalamic dysfunction can occur after chemotherapy in the absence of cranial irradiation.[31] At the level of the ovary, the negative effects of radiation and chemotherapy on estrogen-producing growing follicles can severely impair the menstrual cycle.

The uterus functions primarily to support embryo implantation as well as fetal growth and development. Although chemotherapy alone does not appear to adversely impact the uterus, radiotherapy can have negative long-term consequences on the ability of the uterus to support a future pregnancy.[32,33] Radiotherapy can reduce uterine volume and elasticity, damage the uterine musculature and endometrium, and decrease the vasculature.[34-36] If women are able to conceive following radiotherapy, they may have an increased risk of adverse pregnancy outcomes, including miscarriage, placental abnormalities, preterm birth, and delivery of infants with low birth weights.[35,37]

Cancer Treatment and Male Reproductive Function

Normal male reproduction is also dependent on a tightly regulated HPG axis. In males, the pulsatile release of GnRH from the hypothalamus acts on cells within the anterior pituitary gland to stimulate the production and release of FSH. FSH is secreted into the bloodstream and binds to FSH receptors on the basolateral aspect of Sertoli cells within the testicle, which stimulates the production of androgen-binding protein by Sertoli cells and the initiation of spermatogenesis. FSH production is modulated by inhibin B, a glycoprotein produced by the Sertoli cells that feeds back to the hypothalamus and pituitary to suppress FSH production and hypothalamic secretion of GnRH. LH is also produced by gonadotroph cells within the anterior pituitary gland; like FSH, the secretion of LH is also regulated by the pulsatile release of GnRH. LH is secreted and stimulates Leydig cells to produce testosterone, and testosterone and FSH act synergistically on the Sertoli cells to initiate and support spermatogenesis. The precise endocrine relationship between the hypothalamus, pituitary gland, and testicles is essential to the normal production of sperm. Disturbance of this balance can have detrimental effects on spermatogenesis, resulting in decreased sperm number and quality.

At the time of cancer diagnosis, patients often present with impairment of the normal HPG axis. These findings are commonly noted in men with testicular tumors that produce alpha-fetoprotein (AFP) and beta-human chorionic gonadotropin (β-hCG). Carroll and colleagues reported in 1987 that two thirds of men with testicular cancer had abnormal FSH, LH, and/or β-hCG levels.[38] Specifically, decreased FSH levels and elevated LH and β-hCG levels were observed in men with poor semen parameters. The study authors asserted that β-hCG might have an inhibitory effect on FSH in some patients, resulting in impaired spermatogenesis. Other etiological mechanisms have been proposed for the endocrine disruption that is sometimes seen in patients with cancer, including central cytokine effects.[39] While this pathway has been suggested to play a role in oncological anorexia-cachexia syndrome, it has not been specifically studied in terms of hormonal reproductive regulation. More work is needed to specifically elucidate the mechanisms by which oncological disease processes disrupt the normal HPG axis.

The testis, like the ovary, is both radiosensitive and chemosensitive. Chemotherapy is widely used in the treatment of cancers affecting males, but its cytotoxic effects on normal tissue can transiently or permanently deplete rapidly dividing cell populations, such as the testicular germinal epithelium. Chemotherapeutic regimens that include alkylating agents are among the most gonadotoxic and often lead to permanent oligospermia or azoospermia. These changes in spermatogenesis are typically seen within 90 to 120 days after administration of the alkylating agent. Platinum-based chemotherapy, such as cisplatin and carboplatin, predominately affect spermatogonia and spermatocytes. While most men treated with platinum regimens will experience an associated decline in semen parameters, the majority (80%) will have return of sperm in the ejaculate within 5 years of completion of chemotherapy.[40] Antimetabolites such as 5-fluorouracil, methotrexate, gemcitabine, and 6-mercaptopurine can also impair spermatogenesis, although the effects are usually transient. Clinicians should be mindful of the fact that, while patients might start on a "fertility friendly" chemotherapeutic regimen, transition to other regimens is not uncommon in the course of cancer

Table 33.1 Infertility Risk Associated with Specific Cancer Treatments and Regimens

High Risk	Intermediate Risk	Low Risk	No Risk	Unknown Risk
Female				
• Whole abdominal or pelvic radiation doses >6 Gy in adult women • Whole abdominal or pelvic radiation doses >10 Gy in postpubertal girls • Total body irradiation (TBI) • Cranial/brain irradiation >40 Gy • CMF, CEF, or CAF x 6 cycles in women >40 years • Cyclophosphamide 5 g/m^2 in women >40 years • Cyclophosphamide 7.5 g/m^2 in girls <20 years • Alkylating chemotherapy (e.g., cyclophosphamide, busulfan, melaphan) conditioning for transplant • Any alkylating agent (e.g., cyclophosphamide, ifosfamide, busulfan, BCNU [carmustine], CCNU [lomustine]) + TBI or pelvic radiation • Protocols containing procarbazine: MOPP, MVPP, COPP, ChlVPP, ChlVPP/EVA, BEACOPP, MOPP/ABVD, COPP/ABVD	• Whole abdominal or pelvic radiation 5 to <10 Gy in postpubertal girls • Spinal radiation doses >25 Gy CMF, CEF, or CAF x 6 cycles in women 30-39 years • AC in women >40 years	• AC in women 30-39 years • CMF, CEF, or CAF x 6 cycles in women <30 years • Nonalkylating chemotherapy: ABVD, CHOP, COP • AC	• Radioactive iodine • MF • Vincristine	• Paclitaxel, docetaxel (taxanes used in AC protocols) • Oxaliplatin • Irinotecan • Bevacizumab • Cetuximab • Trastuzumab • Erlotinib • Imatinib
Male				
• TBI • Testicular radiation dose >2.5 Gy in men • Testicular radiation dose ≥6 Gy in boys • Cranial/brain radiation ≥40 Gy • Protocols containing procarbazine: COPP, MOPP, MVPP, ChlVPP, ChlVPP/EVA, MOPP/ABVD, COPP/ABVD • Alkylating chemotherapy for transplant conditioning (cyclophosphamide, busulfan, melphalan) • Any alkylating agent (e.g., procarbazine, nitrogen mustard, cyclophosphamide) + TBI, pelvic radiation, or testicular radiation • Cyclophosphamide >7.5 g/m^2 • Surgical removal of one or both testicles or the pituitary gland	• Testicular radiation dose 1-6 Gy (due to scatter from abdominal/pelvic radiation) • BEP x 2-4 cycles • Cumulative cisplatin dose <400 mg/m^2 • Cumulative carboplatin dose ≤2g/m^2 • Hormone treatments (prostate cancer) • Surgical procedures within in the pelvis (prostate, bladder, lower large intestine, rectum)	• Testicular radiation dose 0.2-0.7 Gy • Nonalkylating chemotherapy: ABVD, OEPA, NOVP, CHOP, COP	• Testicular radiation dose <0.2 Gy • Radioactive iodine • Interferon-α	• Irinotecan • Bevacizumab • Cetuximab • Trastuzumab • Erlotinib • Imatinib
Pediatric				
• TBI • Whole abdominal or pelvic radiation doses >15 Gy in prepubertal girls or >10 Gy in post-pubertal girls • Testicular radiation dose ≥3 Gy in boys • Cranial/brain irradiation >40 Gy • Spinal irradiation 24-36 Gy • Cyclophosphamide >7.5 g/m^2 in boys • Cyclophosphamide >15-20 g/m^2 in girls • Alkylating chemotherapy (e.g., cyclophosphamide, busulfan, melaphan) conditioning for transplant • Any alkylating agent (e.g., cyclophosphamide, ifosfamide, busulfan, carmustine, lomustine) + TBI, pelvic radiation, or testicular radiation • Protocols containing procarbazine • Surgical removal of one or both testicles	• Whole abdominal or pelvic radiation 10 to <15 Gy in prepubertal girls • Whole abdominal or pelvic radiation 5 to <10 Gy in postpubertal girls • Spinal radiation doses 18-24 Gy • Testicular radiation dose 1-2 Gy (due to scatter from abdominal/pelvic radiation) • Cumulative cisplatin dose of about 500 mg/m^2 (boys only) • Surgical removal of an ovary	• Testicular radiation dose 0.2-0.7 Gy • Nonalkylating chemotherapy	• Testicular radiation dose <0.2 Gy • Radioactive iodine • Methotrexate/5-FU • Vincristine • Interferon-α	• Oxaliplatin • Irinotecan • Bevacizumab • Cetuximab • Trastuzumab • Erlotinib • Imatinib

CMF, cyclophosphamide/methotrexate/fluorouracil; CEF, cyclophosphamide/epirubicin/fluorouracil; CAF, cyclophosphamide/adriamycin (doxorubicin)/fluorouracil; MOPP, mechlorethamine/oncovin; MVPP, mechlorethamine/vinblastine/procarbazine/prednisolone (vincristine)/procarbazine/prednisone; COPP, cyclophosphamide/oncovin/procarbazine/prednisone; ChlVPP, chlorambucil/vinblastine/procarbazine/prednisolone; EVA, etoposide/vinblastine/Adriamycin; BEACOPP, bleomycin/etoposide/adriamycin/cyclophosphamide/oncovin/procarbazine/prednisone; ABVD, adriamycin/bleomycin/vinblastine/dacarbazine; AC, adriamycin/cyclophosphamide; CHOP, cyclophosphamide/hydroxydaunomycin/oncovin/prednisone; COP, cyclophosphamide/oncovin/prednisone; MF, methotrexate/5-fluorouracil; BEP,bleomycin/etoposide/cisplatin; OEPA, oncovin/etoposide/prednisone/adriamycin (doxorubicin); NOVP, novantrone (mitoxantrone)/oncovin/vinblastine/prednisone

From Savemyfertility.org; Fertile Hope, an initiative of LIVE**STRONG**, *Cancer and Fertility: Fast Facts for Reproductive Professionals* (2008); and Meirow D, et al. *Clin Obstet Gynecol.* 2010;53:727–739.

care. The best time for sperm cryopreservation to ensure optimal fertility preservation is prior to the initiation of chemotherapy.

The testis is a highly radiosensitive organ, and radiation-associated declines in semen parameters are commonly seen within 60 to 70 days after radiation treatment. Doses as small as 0.1 Gy can impair spermatogenesis, while doses of just 1.2 Gy have the potential to cause permanent azoospermia.[41-44] Nadir sperm concentration is typically observed 4 to 6 months after completion of radiation treatment, and recovery of spermatogenesis is often noted within 10 to 24 months. Fractionated radiation regimens result in more pronounced spermatogenesis impairment than nonfractionated regimens; the belief is that repeated injury to the germ cells impairs recovery and thus leads to more severe and lasting damage than caused by single, larger doses.

In contrast to germ cells, Leydig cells are relatively radioresistant. While germinal epithelial damage is observed at low doses of radiation, Leydig cell injury is generally not evident until radiation exposure is 20 Gy or more, at which point serum LH elevation and serum testosterone decline are commonly seen.[45]

In addition to the effects of chemotherapy and radiation therapy, surgical procedures can also lead to iatrogenic impairment of male reproductive potential. Orchiectomy leads to loss of testicular mass and thus germ cell mass, diminishing sperm production capability. Patients undergoing radical prostatectomy for prostate cancer, the most common solid cancer in males, sustain disruption of their excurrent ductal system via removal of their prostate gland and seminal vesicles. Men being treated with cystoprostatectomy for bladder cancer are subjected similar excurrent ductal disruption. Retroperitoneal surgery can often result in disruption of the sympathetic nerves that control seminal emission and ejaculation. Retroperitoneal lymph node dissection (RPLND) for men with testicular cancer is one example of this kind of surgery. Template-driven, nerve-sparing approaches are now widely used in the course of RPLND, and these methods represent a technical approach to fertility preservation in males. Finally, pelvic and abdominal exenterative procedures can necessitate resection of gonadal tissue and/or the components of the excurrent ductal system. If the possibility of such a resection arises during surgical planning, preoperative sperm cryopreservation should be carefully considered.

Additional Adverse Reproductive Outcomes

As described above, cancer and its treatments may lead to infertility or sterility due to premature ovarian failure, oligomenorrhea, uterine dysfunction, or altered endocrine function in females and testicular failure, disruption of the excurrent ductal system, and impaired endocrine function in males. However, there are also a number of other side effects of cancer or its treatment that can alter reproductive function. These effects are often overlooked or dismissed but pose a significant challenge to the re-establishment of a high quality of life in cancer survivors (Fig. 33.2). These include increased depression, psychological disorders, and sexuality issues.[46-48] Often patients report loss of identity or attractiveness.

Factors That Contribute to Reproductive Outcomes Following Cancer and Its Treatment

It is quite difficult to predict precisely how a given individual's reproductive potential will be affected by cancer or cancer treatments, as there are many case-dependent variables that contribute to reproductive risk. These factors include but are not limited to a patient's age, genetic background, medical history, and fertility potential prior to cancer diagnosis and treatment. In addition, the radiation and/or chemotherapy treatment regimen used—including dose, duration, frequency, field of treatment, and combination—has a significant bearing on future reproductive function.

Oncofertility Medical Management

Relevant Innate Biological Differences Between Male and Female Reproduction

Fortunately for males and females, the gonads provide a tremendous source of reproductive material for diverse fertility preservation options, ranging from standard to investigational. Innate biological differences in gametogenesis between males and females, especially with respect to age, have largely dictated the types of fertility preservation methods that are currently available or are being developed. In females, it is commonly accepted that oocyte production ceases during mid-gestation, resulting in a fixed number of approximately 1 million follicle-enclosed oocytes at birth that set an individual's reproductive lifespan.[49] The fate of these follicles is to remain quiescent, to activate, or to die. Of those primordial follicles that are activated, only a small fraction will develop to the terminal Graafian follicle stage and produce oocytes that will be ovulated. Once destroyed, there is no mechanism to replace these follicles, as replenishment by ovarian germline stem cells does not appear to be physiologically relevant.[50-54] By puberty, the primordial follicle number decreases to approximately 400,000, and reproductive senescence occurs at the time of menopause, when approximately 1000 primordial follicles remain in the follicle pool.[49] Thus, female oocytes are rare and precious, in addition to requiring relatively invasive techniques to access.

In contrast to oocytes, sperm are continuously generated in the adult testis.[55] The process begins when type A pale spermatogonia differentiate into type B spermatogonia. The type B spermatogonia then differentiate into spermatocytes, which undergo meiosis to form spermatids and ultimately mature haploid spermatozoa. This process results in the production of millions of sperm daily. Because germline stem cells are very active in the male, there is an increased potential for slow recovery of fertility post-treatment if the spermatogonial stem cells (SSCs) are not irreversibly damaged, and the mitotically quiescent type A dark spermatogonia are able to transform into actively dividing and differentiating type A pale spermatogonia.[56] Compared with oocytes, sperm are numerous and can be obtained using relatively simple and noninvasive methods.

Because their gonads are not fully developed, there are much fewer fertility preservation options for prepubertal males and females. Testes of prepubertal males do not contain mature haploid gametes but instead are populated with SSCs that are poised to initiate spermatogenesis and generate sperm

at the onset of puberty. Prepubertal females have follicles that develop in waves until the secondary stage but are then eliminated. It is not until puberty, when cyclic production of gonadotropins promotes the development of follicles to the mature stages, that fertilization-competent eggs are ovulated. Thus, prepubertal populations cannot make use of more standard technologies (e.g., hormone stimulation and IVF) that involve the collection of mature gametes. Instead, current fertility preservation treatments in this population rely primarily on removing and cryopreserving gonadal tissue (see sections on *Special Cases and Investigational Methods & Basic Science*).

Established Fertility Preservation Strategies

National organizations including the American Society of Clinical Oncology (ASCO), the American Society of Reproductive Medicine (ASRM), and the American Academy of Pediatrics (AAP) support the practice of oncofertility. These societies have issued guidelines, listed in Table 33.2, which recommend that oncologists make their patients aware of threats to their fertility, provide them with fertility preservation options, or refer them to reproductive specialists.[57-59] Global statements on oncofertility are forthcoming and will likely parallel those of the U.S. -based physician groups. The Oncofertility Consortium, together with other advocacy groups (Table 33.3), is meeting the urgent need of providing cancer patients with information and developing options to protect their fertility, but its continued success requires that clinicians understand and can discuss currently available fertility preservation technologies with their patients. The following is a brief overview of the standard fertility preservation techniques used in the medical management of both female and male oncofertility patients.

Fertility Preservation for Female Patients

For females, the menu of fertility preservation options is constantly expanding as research breakthroughs are being translated into clinical use (Table 33.4 and Fig. 33.3). Although there are many approaches to fertility preservation, ranging from standard to experimental to theoretical, the unique set of patient- and case-based factors will dictate the best course of action. These factors include a patient's age, ovarian reserve prior to the start of treatment, and type of cancer, as well as the dose, duration, and timing of cancer therapy.[60] It should also be emphasized that female cancer patients who seek fertility preservation are typically young. The average age of patients who have sought fertility preservation methods through the Oncofertility Consortium since 2007 is 27 (unpublished data). Thus, the well-documented decline in egg quality and fertility with advanced maternal age is unlikely to be a compounding factor for these young women.[61-63]

Text continued on p.802

Table 33.2 Fertility Preservation Resources for the Referring Oncology Team and Their Patients

Practice Guidelines

Web Site	Web Address	Patient	Provider
American Society of Clinical Oncology: Clinical Guidelines	http://www.asco.org/ascov2/Practice+&+Guidelines/Guidelines		•
ASRM Ethics Committee: Fertility Preservation and Reproduction in Cancer Patients	http://www.asrm.org/publications/detail.aspx?id=613		•
American Academy of Pediatrics	http://pediatrics.aappublications.org/content/121/5/e1461.full		•

Assisted Reproductive Technology (ART) and Fertility Preservation Resources

Web Site	Web Address	Purpose	Patient	Provider
CDC 2007 ART Report	http://www.cdc.gov/ART/ART2007/ifct.htm	Provides a comparison of ART clinics based on types of ART used, patient diagnoses, success rates that each clinic reported and verified for 2007, and individual program characteristics	•	•
Society for Assisted Reproductive Technologies	http://www.sart.org/	Provides and maintains a standard set of guidelines for ART practices in addition to helping patients locate and contact infertility clinics and view national and individual clinic IVF success rates	•	•
FertiProtekt	http://www.fertiprotekt.de/	Disseminates information on the first European network of providers to offer fertility preservation in Europe and provides data about the costs and effectiveness of fertility preservation		•
Oncofertility Consortium	http://oncofertility.northwestern.edu/	Helps coordinate an interdisciplinary team of medical specialists, basic scientists, and scholars working towards a better quality of life for cancer patients and survivors and provides a listing of top nationwide sites that offer oncofertility	•	•
International Society for Fertility Preservation	http://www.isfp-fertility.org/	Provides scientific information to members and hosts a biennial meeting in fertility preservation		•
Cancer Knowledge Network	http://multimed.current-oncology.com	Provides oncofertility information to Canadians, with links to fertility clinics around the country and current publications and news posts on fertility preservation	•	•

Table 33.3 Online Fertility Preservation Educational and Advocacy Resources for Patients and Providers

Web Site	Web Address	Purpose	Patient	Provider
MyOncofertility	http://www.myoncofertility.org (English) http://es.myoncofertility.org (Spanish)	Provides information for patients detailing fertility preservation options at different time points in the treatment of cancer	•	
Fertile Hope	http://www.fertilehope.org/	Provides reproductive information and support to cancer patients and survivors whose medical treatments present the risk of infertility	•	•
I'm too young for this!	http://i2y.com/	Disseminates age-appropriate information and support to young cancer patients at every stage of their survivorship	•	
Imerman Angels	http://www.imermanangels.org/	Provides personalized connections that enable one-on-one support among cancer fighters, survivors, and caregivers	•	
Center for Young Women's Health (Children's Hospital Boston)	http://www.youngwomenshealth.org/gyn_menu.html#cancer	Helps teen girls, their parents, teachers, and healthcare providers improve their understanding of normal health and development, as well as specific diseases and conditions	•	•
Hope for Two	http://www.pregnantwithcancer.org/	Provides information, support, and hope to women diagnosed with cancer while pregnant	•	
Cancer and Careers	http://www.cancerandcareers.org/	Provides tools and support to women in the workplace who are dealing with a cancer diagnosis	•	•
Lance Armstrong Foundation: Female Infertility	http://www.livestrong.org/Get-Help/Learn-About-Cancer/Cancer-Support-Topics/Physical-Effects-of-Cancer/Female-Infertility	Identifies and addresses the issues faced by cancer survivors by offering support for the physical and emotional effects of cancer	•	
Oncofertility Consortium Blog	http://blog.oncofertility.northwestern.edu/	Provides synopses of recent news articles, new information, and current research surrounding cancer and infertility	•	•
Be Bright Pink	http://www.bebrightpink.org/	Provides education and support tools for high-risk individuals to take control of their breast and ovarian health	•	
PRISM (University of Chicago Medical Center)	http://www.uchospitals.edu/specialties/obgyn/prism	Helps identify, prevent, and treat sexual health problems in female cancer patients and survivors	•	
Cancer Legal Resource Center	https://www.disabilityrightslegalcenter.org/about/cancerlegalresource.cfm	Provides free and confidential information and resources on cancer-related legal issues to cancer survivors, caregivers, healthcare professionals, employers, and others coping with cancer	•	•
SaveMyFertility.org	http://savemyfertility.org/	Serves as a resource for adult cancer patients and the parents of children with cancer who want to learn more about preserving their fertility before and during cancer treatment, and protecting their hormonal health after treatment	•	•
Dormant Buds	http://www.gemmedormienti.it/	An Italian web site that answers questions for patients about the fertility implication of disease treatment through instructional videos and newsletters, and provides professional training by disseminating information about training programs	•	•
Patient Navigator for Fertility Preservation	http://preservefertility.northwestern.edu	Provides a decision tree for patients to determine what fertility options may be appropriate for them	•	
National Cancer Institute	http://www.cancer.gov/cancertopics/aya	Provides information about cancer in young people, how it is diagnosed and treated, where to get treatment, and organizations that serve young people with cancer	•	•
American Cancer Society	http://www.cancer.org/Treatment/TreatmentsandSideEffects/PhysicalSideEffects/FertilityandCancerWhatAreMyOptions/index?sitearea=&level	Provides information on how cancer treatment can affect fertility, ways to preserve fertility before treatment, and fertility options available after treatment	•	•
FERTLINE	http://oncofertility.northwestern.edu/fertline (866) 708-3378	A national phone line that connects patients and providers to one-on-one support to assist them in finding the closest fertility preservation site and receive up-to-date information about their fertility preservation options	•	•

Table 33.4 A Comprehensive Guide to Fertility Preservation Options for Males and Females

Fertility Preservation Option	Male	Female	Suitable for Prepubertal Patients	Potentially Delays Cancer Treatment >2 Weeks	Potentially Delays Cancer Treatment <2 Weeks	Requires Hyperstimulation	Requires Sperm Donor at Time of Procedure	Potentially Preserves or Restores Natural Reproductive and Hormonal Function	Requires Additional ART Procedures to Attempt Pregnancy	Additional Considerations
Standard										
Ovarian transposition/ Oophoropexy		•	•		•			•		Only protects against pelvic irradiation Will not prevent natural ovarian aging from occurring Does not protect the uterus
Gonadal shielding	•	•	•		•			•		Only protects against irradiation
Embryo cryopreservation		•		•		•	•			
Sperm cryopreservation	•			•					•	
Investigational										
Egg cryopreservation		•		•		•			•	Maintains the reproductive autonomy of the patient
Oocyte cryopreservation				•		•			•	Is less effective than egg cryopreservation
Isolation of an oocyte or an egg from a natural cycle		•			•				•	Only results in one oocyte or egg from a natural cycle and takes approximately 2-10 days Is risky for cancer patients who only have one chance for the procedure before treatment
Isolation of oocytes from an ovarian biopsy		•			•				•	Can be performed at any stage of the menstrual cycle
Ovarian tissue cryopreservation followed by transplantation		•	•		•			•		Has the potential to reintroduce cancerous cells Has a limited lifetime
Ovarian hormonal suppression		•			•			•		Is controversial in terms of mechanism of action and efficacy
Testicular sperm extraction (TESE)	•									Rapid intervention when ejaculation not possible

Continued

Table 33.4 A Comprehensive Guide to Fertility Preservation Options for Males and Females—cont'd

Fertility Preservation Option	Male	Female	Suitable for Prepubertal Patients	Potentially Delays Cancer Treatment >2 Weeks	Potentially Delays Cancer Treatment <2 Weeks	Requires Hyperstimulation	Requires Sperm Donor at Time of Procedure	Potentially Preserves or Restores Natural Reproductive and Hormonal Function	Requires Additional ART Procedures to Attempt Pregnancy	Additional Considerations
Testicular fine needle aspiration (TFNA)	•									
Microsurgical epididymal sperm aspiration (MESA)	•									
Percutaneous epididymal sperm aspiration (PESA)	•									
Pre-clinical										
Ovarian tissue cryopreservation followed by *in vitro* follicle growth		•	•		•				•	Avoids the risk of reintroducing cancerous cells Is still being optimized for *in vitro* growth of primordial and primary follicles
Follicle isolation and cryopreservation		•	•		•				•	Avoids the risk of reintroducing cancerous cells Requires *in vitro* follicle growth post-thaw Could be used for transplant
Transplantation of isolated follicles		•	•		•			•		Avoids the risk of reintroducing cancerous cells
Xenotransplantation of ovarian tissue or follicles		•	•		•				•	Avoids the risk of reintroducing cancerous cells
Testicular tissue cryopreservation followed by transplantation	•									No live birth to date
Spermatogonial stem cell cryopreservation followed by transplantation	•									
Xenotransplantation of testicular tissue or spermatogonial stem cells (SSCs)	•									

In vitro spermatogenesis	•			
Use of fertoprotective drugs	•	•	•	
Derivation of oocytes and sperm from stem cells	•	•		
Nonbiological/Third Party				
Sperm, egg, or embryo donor	•	•	•	Can be a very high cost for the patient Will require a surrogate if the patient's uterus has been compromised by cancer treatment
Surrogate		•	•	Bypasses pregnancy complications due to uterine dysfunction Assumes that cryopreserved material is developmentally competent Can also be used if patient's fertility was not compromised by cancer treatment Can be a very high cost for the patient
Adoption	•	•	•	Does not rely on patient's natural fertility Is not guaranteed because adoption agencies may discriminate against cancer survivors

Data from Duncan FE, Jozefik JK, Kim AM, Hirshfeld-Cytron J & Woodruff TK. The gynecologist has a unique role in providing oncofertility care to young cancer patients. *US Obstetrics & Gynaecology* 2011; **6**:24–34 and Stahl PJ, Stember DS & Mulhall, JP. Options for fertility preservation in men and boys with cancer. In *Reproductive Health and Cancer in Adolescents and Young Adults,* Advances in Experimental Medicine and Biology 2012; **733**: 29–39.

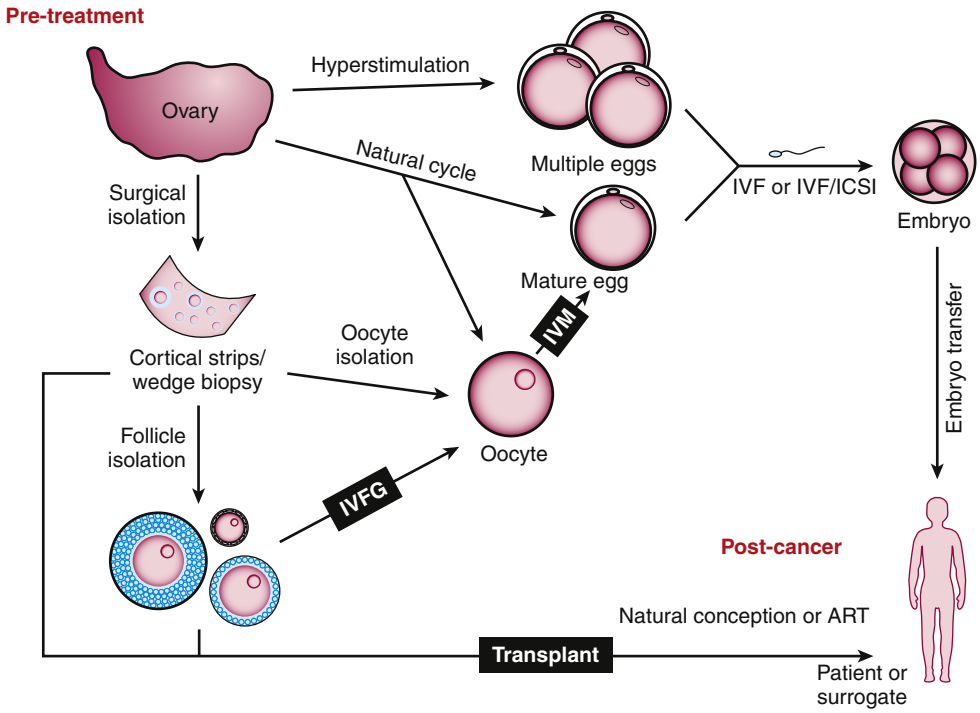


FIGURE 33.3 The ovary contains thousands of oocytes enclosed in follicles, which offers a rich source of gametes for fertility preservation options in females. Pieces of intact ovarian tissue or isolated follicles, immature oocytes, mature eggs, or fertilized embryos obtained prior to the initiation of cancer treatment can be cryopreserved and later thawed and used in standard and investigational technologies to ultimately restore endocrine function and fertility after cancer. The fertility option used is patient-specific and depends on factors such as age, type of cancer, baseline fertility measures, and others. Black boxes highlight investigational techniques that are not yet routine in clinical practice. IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection; IVFG, *in vitro* follicle growth; IVM, *in vitro* maturation.

Embryo Cryopreservation

Despite the broad spectrum of fertility preservation options available to patients, embryo cryopreservation is the only established method recognized by the ASRM.[57] In this method, which is initiated prior to cancer treatment, a woman typically undergoes hormone-induced hyperstimulation to recruit multiple follicles to grow and produce fertilizable eggs (Fig. 33.3). These eggs are then aspirated and fertilized using assisted reproductive technologies (ART) such as IVF or intracytoplasmic sperm injection (ISCI). Embryos are then cryopreserved by slow-freezing or vitrification and stored for the patient's future use.[60,64] Following cancer treatment, these embryos can be thawed and transferred back to the patient's (or a surrogate's) uterus. Cryopreservation of embryos is generally successful. It is estimated that 3.5 million children worldwide have been born to date using ART procedures, and a quarter of these have been generated using cryopreserved and thawed embryos.[65] Furthermore, the obstetrical outcomes of children born after implantation of cryopreserved embryos are comparable to those born after implantation of embryos produced in fresh IVF and ICSI cycles, although long-term follow-up child health studies are still needed.[66]

Despite the success of embryo cryopreservation for fertility preservation, there are several drawbacks of this method in the context of oncofertility. First, this procedure requires time. An ART cycle can take anywhere between 2 to 5 weeks to complete, and this potential delay in cancer treatment may be detrimental for women with aggressive or advanced cancers.[60] In addition, embryo cryopreservation requires ovarian hyperstimulation, which is contraindicated for women with hormone-sensitive cancers.[67,68] This technique also presupposes that a patient is willing to use a sperm donor or has an existing partner who has agreed to create embryos, an issue that is an obstacle for pediatric patients. Finally, a myriad of ethical and potential legal concerns exist surrounding the generation and cryopreservation of embryos that may or may not be used.[69,70]

Oocyte and Ovarian Tissue Cryopreservation and Additional Methods

Beyond embryo cryopreservation, the ASRM maintains that all other fertility preservation methods are experimental and should only be performed in a research setting under Institutional Review Board (IRB) approval.[57] However, the experimental nature of these procedures might not be a barrier to use in specific medical circumstances, such as oncofertility, where they may be a patient's only choice to preserve their future fertility.[71] The ovary contains thousands of follicles in different stages of development, and is a rich source of tissue that can be used in a number of fertility preservation procedures (Fig. 33.3).

For women who want to maintain their reproductive autonomy but do not want to create embryos at the time of cancer diagnosis, oocyte cryopreservation is a potential option (Table 33.4). In this technique, a woman undergoes hormone-induced hyperstimulation to recruit multiple follicles to grow, and the mature eggs are aspirated and cryopreserved for later fertilization.[60,72] Accumulating data suggests this procedure is a viable option, especially in an

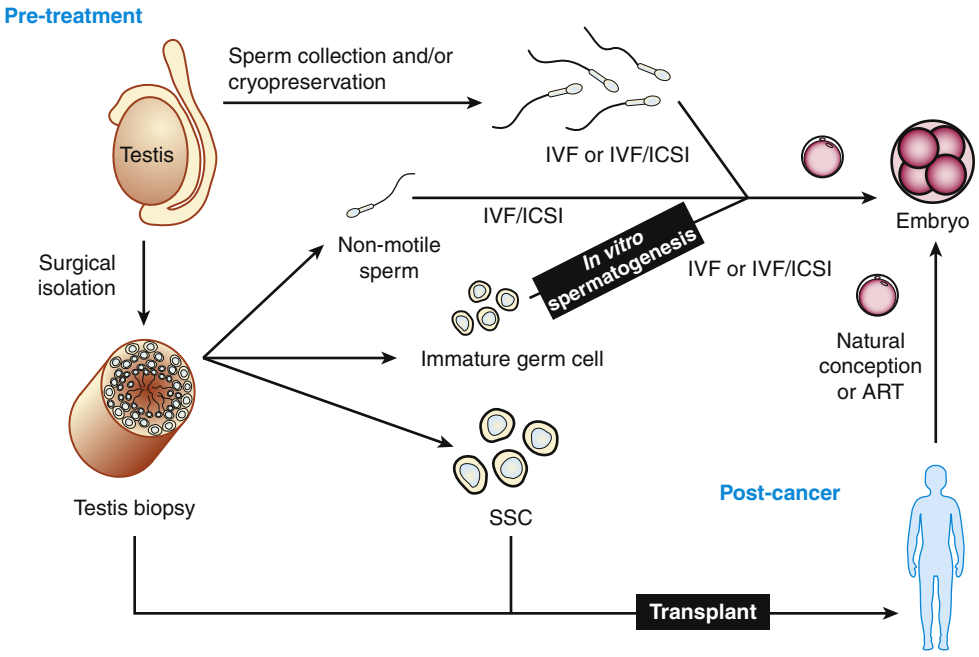


FIGURE 33.4 All fertility preservation options for postpubertal males involve collection of sperm or tissue containing sperm progenitors. Sperm may be used to create embryos via IVF or ICSI, or they may be cryopreserved for later use. Investigational options for prepubertal boys include testicular tissue biopsy and cryopreservation, with transplantation of thawed spermatogonial stem cells (SSCs). Black boxes highlight investigational techniques that are not yet used in clinical practice. IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection.

oncofertility setting.[73] When performed at experienced fertility centers, the live birth rate of IVF using cryopreserved eggs is comparable to that of conventional IVF using fresh eggs,[72] making this a viable option for biological preservation of gametes without the need to have a known or selected sperm donor.

For women who cannot delay their cancer treatment or who cannot be exposed to supraphysiological hormone levels, ovarian tissue cryopreservation for future use is their only option (Table 33.4 and discussed in detail in the *Investigational Methods & Basic Science*). Finally, intraoperative methods, such as ovarian transposition and gonadal shielding, can minimize the impact of radiation on the ovary; however, these techniques do not protect the uterus (Table 33.4). Female patients who have undergone radiotherapy should be counseled adequately about the well-documented negative impact this treatment may have on the uterus and their potential for achieving pregnancy with ART. Patients who have sustained damage to their uterus from radiation therapy may have to use a surrogate, whether they use their own cryopreserved gametes or embryos or decide to use a donor. In addition to these fertility preservation methods, there are also nonbiological strategies to having a family after cancer, such as adoption (Table 33.4 and described in the *Legal and Ethical Issues* section).

Fertility Preservation for Male Patients

Fertility preservation in males is generally more feasible and less invasive than fertility preservation measures available to females. This dichotomy arises due to the fact that most males, despite their oncological diagnosis, are able to provide ejaculated semen samples with viable sperm for cryopreservation. Even very small quantities of sperm can be frozen and used in IVF or ICSI after cancer to achieve biological paternity. Yet while most patients facing a cancer diagnosis can produce an ejaculate suitable for cryopreservation, some patients encounter anejaculation (the inability to ejaculate), aspermia (the lack of an antegrade ejaculate), retrograde ejaculation, necrospermia (only dead sperm present in the ejaculate), or azoospermia. Each of these scenarios can hamper standard male fertility preservation measures. Fortunately, the armamentarium of male reproductive therapeutics that are commonly used to treat such conditions in males without cancer can similarly be applied to oncological patients who experience these problems (Fig. 33.4).

Masturbation and Ejaculation

The ejaculate is the preferred source of sperm for cryopreservation. Men are typically asked to abstain from ejaculation for 2 to 3 days prior to collection of each sample. Patients are discouraged from use of lubricants, jellies, and saliva during the collection process, as these all have spermatotoxic properties. Semen collection can be performed on an inpatient or outpatient basis. Coordination of semen collection in the context of oncological care is essential. Narcotics and sedatives are commonly used in diagnostic and therapeutic procedures for males recently diagnosed with cancer, and these agents can hamper semen collection efforts. Assuring the patient that his privacy will be maintained during semen collection is also critical, as anxiety can impair efforts to produce a semen sample by masturbation.

Penile Vibratory Stimulation (PVS)

Some men experience difficulty reaching climax and are thus unable to produce an ejaculated semen sample by masturbation. Patients at risk for this problem include men on narcotics and sedatives, with peripheral neuropathy, or with significant pelvic, perineal, or genital edema. For these individuals, PVS can help optimize genital tactile stimulatory input and trigger the ejaculatory reflex. To facilitate PVS, a vibratory stimulation device is applied to the ventral surface of the penis, just proximal to the corona. Commercially available vibratory devices commonly feature variable

amplitude and frequency settings, allowing patients to modulate these parameters in order to optimize their stimulatory input. Care must be taken in patients with edema or peripheral neuropathy, as penile skin damage or breakdown can occur in these individuals. Patients are usually able to use these devices successfully alone or in private with their partner. If these efforts are not successful, a healthcare provider can perform the PVS procedure.

Electroejaculation (EEJ)

In some instances, vibratory stimulation does not result in ejaculation. For such cases, another option is EEJ, in which a probe is inserted transrectally to deliver an electrical current to the pelvic structures in a rhythmic fashion. The resultant electrical stimulation of the seminal vesicles and prostate gland usually leads to antegrade seminal expulsion via the urethra. In some cases, the semen may travel in a retrograde fashion back into the bladder. In these instances, sperm wash media can be instilled into the bladder prior to EEJ, and the bladder can be drained after the procedure via catheter to allow isolation of viable sperm. EEJ requires the use of an anesthetic, except in patients with a history of a complete spinal cord injury. Care must be taken during the EEJ procedure to monitor rectal temperature in order to avoid rectal mucosal burns. Anoscopy is usually performed immediately prior to and after EEJ procedures. Monitoring of the rectal mucosa in this fashion is highly recommended. As noted in the above section on PVS, patients at particular risk for anejaculation include those taking narcotics and sedatives, males with peripheral neuropathy, and men with significant pelvic, perineal, or genital edema.

Recovery of Retrograde Sperm Ejaculation From the Urine

As mentioned above, some patients experience the retrograde passage of their ejaculate back into the bladder instead of antegrade out the tip of the penis. Patients at increased risk for retrograde ejaculation include men with autonomic neuropathy, men who have sustained sympathetic nerve injury due to prior surgical procedures such as RPLND or pelvic exenteration, and men with bladder neck incompetence due to a history of bladder neck surgery. Generally, the bladder is a hostile environment for sperm, as urine is highly spermatotoxic. In some cases, sympathomimetic agents such as pseudoephedrine can facilitate conversion from retrograde to antegrade ejaculation. However, in cases where medical therapy is unsuccessful, recovery of viable sperm from the bladder can be achieved by urethral catheterization to drain urine and instill sperm wash media into the bladder prior to ejaculation. After ejaculation, the patient can either urinate out the media, or the urethral catheter can be replaced to allow collection of media from the bladder. The media is then centrifuged, and the sperm are isolated in the resultant pellet. These sperm can then be resuspended in media and cryopreserved.

Surgical Sperm Extraction Procedures

A number of surgical sperm extraction procedures are available to permit sperm retrieval from the testicular tissue or the epididymis. Surgical sperm extraction is generally reserved for those patients with obstruction of their excurrent ductal system, men with ejaculatory dysfunction nonresponsive to PVS and/or EEJ, and men with necrospermia or nonobstructive azoospermia. A number of sperm extraction procedures are available, and surgeon preference often dictates which approach is used.

For men with obstructive azoospermia, a blockage in the sperm delivery system (i.e., epididymis, vas deferens, or ejaculatory duct) prevents passage of sperm in the semen. One example seen occasionally is a man with newly diagnosed cancer who has a history of vasectomy but wishes to preserve future fertility potential. For these men, the ejaculate contains no sperm, so sperm extraction from either the epididymis (Percutaneous Epididymal Sperm Aspiration [PESA] or Microsurgical Epididymal Sperm Aspiration [MESA]) or from the testicle (Microsurgical Testicular Sperm Extraction [Micro-TESE], Testicular Sperm Extraction [TESE], or Testicular Fine Needle Aspiration [TFNA]) are all options. Sperm retrieval rates are generally high in patients with obstruction, but the underlying systemic response to cancer in oncological patients can markedly inhibit sperm production in some cases, as was previously described.

For men with nonobstructive azoospermia, the underlying problem is sperm production. Nonobstructive azoospermia can arise due to endocrine abnormalities such as hypogonadism, which is often associated with oncological diagnoses. As was described earlier in this chapter, low testosterone is frequently seen in cancer patients even before the initiation of cancer therapy. Hypogonadism can also result from narcotic use, which leads to suppression of LH secretion and decreased production of testosterone by Leydig cells. Another common cause of nonobstructive azoospermia is germ cell sloughing. This can occur in men after a febrile systemic response to cancer, as is sometimes seen in lymphoma. These men will often be found to have either nonobstructive azoosperma or necrospermia at the time of attempted sperm cryopreservation. Finally, men with testicular cancer are sometimes found to have nonobstructive azoospermia at the time of semen collection. In some men, impaired spermatogenesis may be associated with a local inflammatory response to the testicular neoplasia, and in others, germ cell absence may have preceded the development of cancer.

For all men with nonobstructive azoospermia, the aim is to isolate and extract sperm from the testicular tissue. "Onco-TESE," described by Schrader and colleagues, involves surgical extraction of sperm from the testicle in male cancer patients with azoospermia.[74] Most clinicians prefer the Micro-TESE technique in such cases, as this microsurgical approach enables the systematic inspection of the seminiferous tubules in search of dilated, opaque tubules that often bear sperm. Clinicians should realize that spermatogenesis is often "patchy" and heterogeneous, and so careful, methodical inspection of the tissue is important technically. As reported by Schrader and colleagues, published sperm retrieval rates for Onco-TESE are approximately 50%.[74]

In addition to sperm cryopreservation, gonadal shielding is also a means to preserve fertility in males who must undergo radiation therapy. Gonadal shielding is a particularly important consideration when pelvic or lower abdominal fields are being treated, as these can result in significant gonadal exposure through radiation scatter.

Gamete and Reproductive Tissue Cryopreservation and Storage

Cryopreservation is a central component of fertility preservation, as reproductive tissues must be harvested before fertility-threatening treatment is initiated and must be stored until treatment is completed.[75] As the array of fertility preservation options expand for both males and females, so does the need to develop long-term storage methods for diverse cell types (oocytes, eggs, spermatogonia, spermatids, spermatozoa, and embryos) and complex tissues containing these cell types (testicular tissue, ovarian tissue, and follicles).[76,77] Although there has been some success with preserving sperm through freeze-drying, most reproductive tissues are stored using cryotechniques.[78-80] In general, these methods require that a tissue is cooled from its physiological temperature to subzero temperatures, is maintained at that temperature for an extended time, and is able to regain cellular viability upon thawing.

The two main types of cryopreservation techniques that are routinely used in ART are slow freezing and vitrification.[75,81] With slow freezing, cells or tissues are exposed to low concentrations of cryoprotectants, which results in a lowering of the intracellular water content during a slow cooling process. The main risk of slow freezing is ice crystal formation during both freezing and thawing.[76] Vitrification was developed to avoid ice crystal formation; in this method, the cells or tissues are equilibrated in high concentrations of cryoprotectants, and then plunged directly in liquid nitrogen. Vitrification is simple, rapid, and economical; however, its main drawback is that it is performed in an open system in which the cells or tissues are in direct contact with liquid nitrogen.[80] Current efforts are focused on developing adequate closed systems to ensure safety. Whether slow freezing or vitrification methods are employed, the greatest risk of cryodamage occurs during either the cooling or the thawing process. Little damage to cells and tissues is thought to occur during extended storage.[75]

In the field of ART, embryo and sperm cryopreservation are considered standard technologies. Preimplantation stage embryos can be cryopreserved at any stage of development (pronuclear, cleavage, or blastocyst). Although the use of embryo vitrification is increasing, to date most embryo cryopreservation cases have used slow-freezing methods. The post-thaw incidence of embryo survival and implantation is 60% to 80% and 15% to 20%, respectively, and there have been approximately half a million babies born worldwide from embryo banking using slow freezing.[75,82,83]

Although the freezing of mature seminal sperm has been routine for decades, it is not a perfect process.[83] In fact, sperm motility and several key sperm structures, including the acrosome, tail, and plasma membrane, are highly sensitive to cryodamage.[84] In the majority of cases, the loss of sperm to cryodamage is negligible since a normal ejaculate contains sufficient sperm to compensate for the loss. However, cryopreservation can become more challenging in cases of oligozoospermia. In these situations, additional procedures such as swim-up tests or centrifugation are typically performed to enrich the population for higher quality sperm.[85]

In contrast to embryos and sperm, unfertilized eggs have been difficult to cryopreserve, with a post-thaw survival rate of only approximately 30% to 50%.[75] This is likely due to the sensitivity of key cellular structures necessary for future fertilization, including cortical granules, the zona pellucida, and the meiotic spindle.[76] Nonetheless, some highly trained laboratories that specialize in egg cryopreservation have reported fertilization and developmental capacity of slow-frozen or vitrified human eggs at a rate that is on par to what has been achieved using fresh eggs, highlighting the potential of this technology.[72,73,86-88] More challenging has been the cryopreservation of immature or germinal vesicle-intact oocytes, since these cells must undergo the additional step of *in vitro* maturation after thawing before they can be fertilized.

Recently, the cryopreservation of ovarian and testicular tissue has become more widespread in the field of fertility preservation, especially in cases involving prepubertal males and females whose gametes are not yet mature.[89,90] Gonadal tissue can be frozen as cell suspensions, tissue pieces, or whole organs. Most tissue has been frozen using slow-freezing protocols, although promising results have also been obtained with vitrification.[91,92] Freezing and thawing tissues versus individual cells is complex as it must be done in such a way that maintains cell-to-cell interactions in a characteristic architecture. This is essential if both endocrine and reproductive function are to be restored when thawed tissues are re-introduced into the patient. Both somatic cells (male: Leydig and Sertoli; female: granulosa, cumulus, and theca) and germ cells (male: spermatogonia and spermatids; female: oocytes) and their arrangement within the tissue must be protected. As discussed in the *Investigational Methods & Basic Science* section, approximately 28 live births have been reported following transplantation of frozen and thawed cortical ovarian tissue.[93,94]

Coupled with the freezing of gonadal tissue is the need to develop and optimize methods for transplantation or isolation of immature gametes from the thawed tissue followed by *in vitro* growth to maturity. In the male, the advent of ICSI has allowed different types of samples to be stored. With ICSI, pregnancies and live births can be achieved with ejaculated, seminal, and testicular spermatozoa, even in cases of low sperm number or quality.[95] In addition, injection of immature round spermatids can be performed clinically.[96]

Because reproductive cells and tissues differ in their size, number, composition, and structure, there is no standard protocol for cryopreservation. The success of this technology is highly site dependent, and patients should be referred to specialized centers with trained staff who can address their specific needs.

Assessing Reproductive Potential Before, During, and After Cancer and Treatment

Assessing a patient's reproductive function in an oncofertility setting is best performed before cancer treatment begins because doing so can help predict how vulnerable a patient may be to cancer therapies and evaluate the most beneficial fertility preservation option. Measuring reproductive function after cancer therapy may indicate whether a potential for restored natural fertility exists or whether the patient must rely on previously performed fertility preservation methods.

In females, ovarian reserve is defined as the functional potential of the ovary and reflects the number and quality of oocytes it contains.[97] Although there is no single predictive

marker of ovarian reserve, several tests have been developed (Table 33.5). Currently, serum anti-müllerian hormone (AMH) levels combined with antral follicle counts (AFC) are the most robust markers for evaluating ovarian reserve.[98-101] AMH is produced by the granulosa cells in developing ovarian follicles,[102,103] and AMH levels do not vary with menstrual cycle day, oral contraceptive use, or pregnancy.[102] Serum AMH levels decrease rapidly after the age of 37, and this drop precedes but is related to the onset of menopause.[104,105] A study performed in regularly menstruating women assessed several hormonal markers of aging and found that serum AMH was the most accurate in predicting the occurrence of the menopausal transition within 4 years.[105]

Serum AMH also correlates with the number of antral follicles, which can be determined by counting the number

Table 33.5 Common Clinical Tests for Assessing Fertility

Reproductive Function Test	Significance
Female	
Anti-müllerian hormone (AMH)	• Blood test performed on any day of the menstrual cycle to measure AMH levels in the bloodstream • AMH is produced by granulosa cells of growing follicles in the ovary • Indicates the size of the pool of growing follicles in the ovary (Lower levels correlate to fewer growing follicles) • This test is best performed in combination with antral follicle counts in the ovary
Day 3 estradiol	• Blood test performed on day 3 of the menstrual cycle to measure estradiol levels in the bloodstream • Estradiol is produced by the granulosa cells of the ovary and the adrenal cortex • High estradiol can suppress FSH or cause problems with ovulation • Always performed in combination with a Day 3 FSH test to reaffirm FSH results
Day 3 follicle-stimulating hormone (FSH)	• Blood test performed on day 3 of the menstrual cycle to measure FSH levels in the body • FSH is synthesized and secreted by the anterior pituitary gland • High FSH correlates to low egg count in the ovary; however, normal FSH does not necessarily correlate to sufficient egg quantity • Performed in combination with a Day 3 Estradiol blood test
Day 3 inhibin B	• Blood test performed on day 3 of the menstrual cycle to measure inhibin B levels in the body • Produced by the granulosa cells in the ovary to inhibit FSH • Lower inhibin B levels may indicate higher FSH levels, which may account for a lower egg count • Used in combination with Day 3 Estradiol and Day 3 FSH test • Inhibin B decreases with age
Antral follicle counts (AFC)	• Transvaginal ultrasound test in which antral follicles approximately 2-10 mm in diameter are counted • Correlates to the number of dormant primordial follicles remaining in the ovary • Best performed in combination with an AMH blood test
Ovarian volume	• Transvaginal ultrasound test to measure the length, width, and depth of both ovaries • Ovarian size decreases with age, so a smaller ovary may indicate a smaller ovarian reserve • Though the difference in ovarian volume may be statistically significant, AFC is a better predictor of ovarian reserve and the clinical value of this test is controversial
Uterine ultrasound	• Ultrasound scan test used to assess uterine size and shape, blood supply, and endometrial thickness • Problems with these uterine characteristics may impact implantation and the ability to conceive • A more invasive follow-up endometrial biopsy may be performed to further assess the quality of the uterus • The clinical value of this test is controversial
Clomiphene citrate challenge test	• Patient is given clomiphene for 5 days to induce ovulation and then a blood test is performed to measure levels of estradiol, FSH, and LH • Causes the release of more than one egg • If FSH levels are elevated, may indicate a diminished ovarian reserve • The clinical value of this test is controversial
Menstruation	• Evaluation of a patient's return to or lack of menstruation post-treatment • May indicate that normal fertility has been restored if a patient begins to cycle post-treatment • A return to menstruation does not guarantee that a person has ovulated or is able to conceive • Does not account for potential early menopause
Male	
Semen analysis	• Parameters measured are ejaculate volume, sperm concentration, sperm motility, and sperm morphology • These can be assessed longitudinally to track testicular function over time and gain insight into reproductive potential
Serum FSH	• Blood test that measures FSH levels to give insight into spermatogenesis • FSH is synthesized and secreted by the anterior pituitary gland • Elevated serum FSH levels (>7.0 mIU/mL) can signal spermatogenic impairment
Sperm DNA damage	• Measured in a variety of ways including the sperm chromatin structure assay, the comet assay, the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, the DFI (DNA fragmentation index) assay, FISH (fluorescence *in situ* hybridization), and the SCD (sperm chromatin dispersion) test • Sperm DNA damage typically increases during the course of cancer treatment but returns to baseline levels over time

From Duncan FE, Jozefik JK, Kim AM, Hirshfeld-Cytron J & Woodruff TK. The gynecologist has a unique role in providing oncofertility care to young cancer patients. *US Obstetrics & Gynaecology* **6**:24–34, 2011 and Meistrich ML. Male gonadal toxicity. *Pediatric Blood & Cancer* **53**:261–266, 2009.

of 2- to 10-mm diameter follicles by transvaginal ultrasound.[105,106] A strong correlation between AMH levels and AFC has been well established in clinical studies.[106-108] The number of small antral follicles is one of the best markers of ovarian age related to the ovarian reserve.[106] It has also been shown that serum AMH levels and AFC, when analyzed together, are indicative of primordial follicle number independent of age.[109] Thus, in combination, serum AMH levels and AFC are strong correlates of ovarian age and the remaining ovarian reserve.

The use of serum AMH levels and AFC to test ovarian reserve has been effectively translated into the oncofertility setting, where various cancer treatments have been shown to compromise the follicle pool.[101,110-112] Clinical studies have shown that serum AMH levels and AFC measured in cancer patients post-treatment were highly correlated to each other and significantly lower than in control patients without cancer.[101,108,113] In a recent longitudinal study, AFC, ovarian volume, and levels of FSH, LH, estrogen, inhibin A and B, activin A, and AMH were compared between regularly menstruating breast cancer patients (ages 22 to 42 years) and patients without cancer.[114] This study demonstrated that, although the breast cancer patients had normal ovarian reserve prior to cancer treatment, there was a significant decrease in AFC and a drastic reduction in AMH levels post-treatment. Being able to correlate markers, such as serum AMH levels and AFC, with a diminished ovarian reserve is critical in the fertility preservation decision-making process.

Performing these tests helps to assess the need for fertility intervention in patients who may be susceptible to early menopause or a decreased ovarian reserve both before and after treatment. Although these tests are an appropriate measure of the remaining developing follicle pool, it is important to remember that they do not guarantee that pregnancy—the ultimate proof of fertility—will occur.[97,99,100] While AMH and AFC are one of the better test combinations for predicting the ovarian reserve, a number of other tests are commonly used to evaluate a woman's fertility status (Table 33.5).[63,100] For example, additional hormonal assays include tests of Day 3 estradiol, FSH, and inhibin B. Dynamic tests such as the clomiphene citrate challenge test can be used to test a patient's ability to ovulate. It is of critical importance to counsel patients that a return to normal menstruation is not necessarily indicative of fertility, because there is still a possibility of anovulation or diminished ovarian reserve.[115,116] In addition to fertility, measures of endocrine function are an important consideration because of the severity of symptoms related to endocrine dysfunction, including precocious puberty, hot flashes, loss of bone density, and loss of sexual function.[117-119]

In males, accurate assessment of reproductive potential throughout the course of cancer therapy is typically easier than in females, given that most males can provide ejaculated semen samples for analysis. Ejaculate volume, sperm concentration, sperm motility, and sperm morphology can be assessed longitudinally to track testicular function and gain insight into reproductive potential. Serum FSH levels provide insight into spermatogenesis, with elevated serum FSH levels (greater than 7.0 mIU/mL) typically signaling spermatogenic impairment. Over the last several years, sperm DNA damage in the wake of cancer therapy has garnered increasing attention. Cancer therapies can indeed result in increased levels of sperm DNA damage, and numerous methods to assess sperm DNA breaks are commercially available. Examples of these tests include the sperm chromatin structure assay, the comet assay, TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, DFI (DNA fragmentation index) assay, FISH (fluorescence *in situ* hybridization), and SCD (sperm chromatin dispersion) test. Meistrich has authored an excellent review that includes a discussion of sperm DNA damage resulting from cancer therapy.[120] While sperm DNA damage and aneuploidy often increase during the course of cancer therapy, most patients experience a return to baseline levels over time. Furthermore, numerous studies have shown no increase in genetic anomalies or birth defects when comparing the offspring of cancer survivors with those of individuals unaffected by cancer.[121-124] As Meistrich notes, most pregnancies considered in the above studies were conceived well after completion of cancer therapy.[123]

Risks and Limitations of Fertility Preservation Techniques

Regardless of which fertility preservation method is employed, patients should be counseled regarding each method's limitations and the risks that may be encountered. ART procedures, which are broadly used in the setting of oncofertility, do not guarantee live births, even in patients without cancer. Currently, the cumulative live-birth rate following IVF is approximately 50%.[61] Recent data describing the outcomes of almost half a million ART cycles are encouraging, and suggest that with favorable patient and embryo characteristics, live birth rates following ART are approaching those of natural fecundity.[125] However, there are potential risks associated with ART procedures. It has been suggested that singletons born following IVF may have an increased risk of low birth weight, prematurity, and perinatal mortality.[126] ART procedures are also associated with a ten-fold increase in the number of multiple births compared with natural conceptions.[126] Furthermore, three imprinting disorders—Beckwith-Wiedemann syndrome, Angelman syndrome, and maternal hypomethylation syndrome—have been linked to ART procedures.[127] Finally, one of the reasons that cancer patients choose not to pursue fertility preservation is their fear of passing their cancer onto their offspring.[128] However, only between 5% to 10% of all cancers are hereditary.[129]

Additionally, cancer survivors may fear pregnancy because of the possibility of cancer recurrence or potential detrimental maternal and fetal health outcomes during the pregnancy. However, multiple studies to date suggest that even for women with hormone-responsive malignancies such as breast cancer, there were no adverse outcomes with regard to cancer recurrence or survival due to pregnancy.[130] It is encouraging that children born to cancer survivors are not at increased risk of genetic abnormalities, birth defects, or cancers compared to those born to parents without a cancer history.[131] There may, however, be an increased risk for miscarriage, low birth weight, and prematurity, largely related to pelvic radiation.[131-133]

Minimizing or avoiding risks of treatment-related infertility exist and counseling should begin before cancer

treatment is initiated if at all possible. There are ways to minimize the risks associated with ART procedures and maximize the likelihood of successful fertility preservation. To increase the chances of a live birth following cancer treatment, patients should be counseled to seek ART and fertility preservation procedures at the most established institutions possible, as not all centers are equivalent (Table 33.2; Assisted Reproductive Technology Resources). For women with hereditary cancers, one way to avoid the risk of potentially transmitting gene mutations is to combine fertility preservation methods with preimplantation genetic testing (PGT). In PGT, genetic testing is performed on a biopsy from a preimplantation stage embryo to ensure that only embryos free of the specific mutation are transferred back into the uterus.[134] PGT has been used to screen for several cancer predisposition gene mutations, including adenomatous polyposis of the colon (APC), neurofibromatosis type 2 (NF2), and inherited breast cancer (BRCA1 and BRCA2).[135]

Investigational Methods and Basic Science

The current standard methods for fertility preservation, which include embryo and sperm cryopreservation, are suitable for only a fraction of cancer patients (i.e., males who can provide a sperm sample and adult women with a partner or sperm donor who have sufficient time and appropriate hormone receptor status). Thus, the research community has been charged with the mission to explore and broaden fertility preservation options to include a greater number of cancer patients, including prepubertal patients. Investigational methods that hold tremendous promise for both males and females include: (1) protecting gonads *in vivo* from iatrogenic insults (fertoprotective adjuvant therapies), (2) developing robust ways to harness the reproductive potential of cells within gonadal tissue (transplantation and *in vitro* gametogenesis), and (3) creating gametes *de novo* through differentiation of embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) (Table 33.4). All of these methods are described below, and are either being tested in preclinical studies or are investigational and should be performed clinically only under IRB approval.

Investigational Methods That Protect Gonadal Tissue *In Vivo*

The current practice of fertility preservation is based on the removal and storage of an individual's reproductive tissues or cells prior to exposure to destructive treatments to preserve fertility. However, another option—"adjuvant" fertoprotection—is an area of active research. With fertoprotective therapy, an individual's existing reproductive tissue remains in the body but is protected from damage caused by cancer treatment using pharmacological interventions. To date, fertoprotection has been attempted in both males and females using two methods. The first involves the use of GnRH analogs to suppress gonadotropins, thereby keeping spermatogenesis and folliculogenesis in a state of suspended animation.[136] This method has been used clinically, and sex-dependent differences in treatment response are evident. In men, only one out of eight clinical trials reported successful protection or restoration of spermatogenesis with GnRH analogs in patients exposed to cytotoxic agents.[136,137] In females, the results remain inconsistent and controversial.[138] Moreover, the mechanism by which GnRH analogs protect fertility is largely unknown.

The second approach to fertoprotective adjuvant therapy is based on preventing gametes from eliciting DNA damage and cell death pathways in response to radiation or chemotherapy. Although promising, this method is still investigational and limited to preclinical studies in animal models. In one study, direct and controlled long-term treatment of nonhuman primate ovaries with the anti-apoptotic factor sphingosine-1-phosphate (S1P) or its long-acting mimetic, FTY720, protected against irradiation-mediated oocyte death.[139] Moreover, adult female primates whose ovaries were exposed to FTY720 prior to irradiation were able to deliver healthy offspring following natural conception. Whether or not these anti-apoptotic agents are also protective against chemotherapy-induced damage in nonhuman primates is unknown. Another potential fertoprotective adjuvant is Gleevec® (imatinib mesylate); in mice, imatinib has been shown to block cisplatin-mediated apoptosis in small ovarian follicles, likely through inhibition of the c-Abl pathway.[140-142] In fact, follicle loss and infertility due to cisplatin exposure was partially prevented if mice were co-treated with imatinib, demonstrating the fertoprotective properties of this approach. Similar fertoprotective strategies may be functional in the male. Treatment of male mice with the immunomodulator AS101 partially protected against cyclophosphamide-induced damage of the testis and sperm.[143] With all these agents, the precise molecular pathways being affected are not known.

Fertoprotection is an attractive option clinically because it has the potential to maintain not only fertility but also endocrine function. However, it is also important to note that all gametes in the body at the time of cancer treatment will be exposed to DNA damaging agents, putting them at risk for defects in genetic integrity that may be passed to the next generation. Therefore, it is equally important that researchers and the pharmaceutical industry continue to focus their efforts on drug development to create "smart" cancer therapies that target the cancer while sparing the reproductive organs.

Investigational Methods That Harness the Reproductive Potential of Cells Within the Testis or Ovary

In males, there are three main investigational methods based on harnessing the potential of diploid gamete precursor cells present in testicular tissue.[144] These methods include: (1) autologous transplantation or xenotransplantation of testicular tissue, (2) transplantation of isolated spermatogonial stem cells (SSCs), and (3) *in vitro* spermatogenesis (Fig. 33.4). Much of the development of these techniques has been performed in animal models with varying degrees of success; of note, none have been proven safe or efficient in humans to date.[145-147] These investigational techniques, their benefits, and their limitations are briefly discussed below, followed by an overview of parallel approaches in females.

Testicular tissue transplantation can be accomplished with the use of either testicular cell suspensions or fragments of testicular tissue. Transplantation can be categorized as either autotransplantation, involving procedures where the gametes are replaced back into the original donor, or xenotransplantation, involving procedures where gametes are placed into an organism from another species, typically an immune-deficient mouse. Schlatt and colleagues have described the successful autotransplantation of primate testicular cell suspensions after testicular irradiation. Their technique involved injection of the cell suspension back into the testicle via the rete testis.[148] The injected germ cell stem cells then repopulated the seminiferous tubules and differentiated in the testicular microenvironment into mature spermatozoa. Although successful autotransplantation has not yet been reported in humans, this technique has been successful in primates, as noted in the above study.

Autologous transplantation has been performed using both cell suspensions and fragments of testicular tissue. When cell suspensions are used, as noted above, the fluid is typically injected into the testicle of the recipient organism via the rete testis. When fragments of testicular tissue are used, they have commonly been surgically placed in a subcutaneous location within an immune-deficient recipient organism. Both approaches have been used in a number of different organisms.[149,150] The benefit of this method is that the stem cell niche is maintained and the SSCs within the tissue can develop and differentiate in their initial intact microenvironment. Although autologous testis grafting has not yet been reported in humans, this technique has been successful in several other species, resulting in restored spermatogenesis and fertility.[148] The drawback to this technology is that there is a risk of reintroducing cancer cells because the testis is an immunologically privileged site. In fact, in a rat model, injection of only 20 leukemic cells into the testis was sufficient to reintroduce disease.[151] Another limitation is that tissue grafting appears to only function optimally when immature testicular tissue is used as the graft because adult tissue undergoes almost complete degeneration after transplantation.

As an alternative approach, xenotransplantation is a procedure in which the testicular tissue is transplanted and allowed to develop *in vivo*, within a niche of a host animal.[145,146,152,153] Mature sperm are then extracted and used in ART. Immunodeficient mice and rats are common hosts. Xenotransplantation has been attempted in several species with varying degrees of success of donor-driven spermatogenesis.[153] Blocks in spermatogenesis are common when the host and donor species are dissimilar, likely because of various biological differences between the two species, such as hormone levels, timing of spermatogenesis, or testis structure. The field of xenotransplantation also faces significant ethical barriers to translation into clinical practice. Although xenotransplantation would eliminate the risk of reseeding cancer cells, there is a risk of transmitting viruses or infectious agents from animals to humans. It is also not known how the genome or epigenetics of human gametes may be impacted following development in another organism. Even if it is not used for clinical purposes, xenotransplantation still provides a useful model to assess the risk of reintroducing malignant cells following transplant, to test the effects of gonadotoxic treatments on human gamete development, to determine the efficacy of cryopreservation techniques, to understand the mechanisms of tissue revascularization after transplant, and to optimize grafting conditions.[154]

A second investigational approach is the transplantation of SSCs isolated either from fresh or cryopreserved testicular tissue.[146,153,155,156] These cells can be transplanted back as a suspension into a testicular environment that is depleted of germ cells. Because of the high colonization potential of SSCs spermatogenesis can be reinitiated in the optimal environment. In this case, the patient's seminiferous epithelium provides the microenvironment for germ cell differentiation. Based on animal studies, the success of SSC transplantation is linearly correlated with the quantity and quality of transplanted cells and on the corresponding quantity and quality of stem cell niches in the recipient testis. For SSC transplantation to be successful, several events must occur. The SSC population must be enriched and purified from other cell populations, including cancer cells. This can be accomplished by cell sorting techniques that make use of SSC-specific markers. The SSC population must also be expanded in culture to increase the number of SSCs available for transplant. Although in humans it has been difficult to identify SSC markers for cell sorting, methods have been developed to expand these cells *in vitro*.[157,158] Once purified and expanded, SSCs need to be injected into the seminiferous tubules or infused into the rete testis. For spermatogenesis to initiate, the SSCs must associate with Sertoli cells and relocate from the lumen to the basement membrane in the seminiferous epithelium. A limitation of this technique is that it requires a suitable stem cell niche, which might be compromised in men with advanced age or who have been exposed to iatrogenic insults. A human clinical trial of germ cell transplantation was initiated in Hodgkin lymphoma patients at Christie hospital in Manchester (UK) in 1999, but no information has been published to date on the fertility outcomes of these patients.[159]

The third approach that holds promise for male fertility preservation is *in vitro* gametogenesis, in which the events required to produce functional spermatozoa are recapitulated entirely outside the body. Such methods are quite challenging because many cell types and signaling pathways must be coordinated over a protracted time frame of several weeks. For more than a century, there have been myriad strategies developed to differentiate male germ cells from multiple species *in vitro*, ranging from tissue and organ culture on permeable supports or encapsulation in hydrogels to Sertoli cell co-culture.[160-163] Although all of these systems have provided key insights into the basic biology of spermatogenesis, they have either failed to recapitulate the whole process or were not able to produce sperm capable of fertilization. Recently, however, significant progress has been reported: fertilization-competent haploid mouse sperm were derived from organ culture on a semi-solid agarose support of testis fragments from 2.5 to 3.5 day old mice.[164] Using this culture method, combined with a gas-liquid interface, round spermatids and sperm were obtained after 23 and 42 days of culture, respectively. Through round spermatid injection (ROSI) or ISCI, live offspring were generated.[164]

Similar to the fertility preservation approaches being investigated for males, transplantation of gonadal cells or tissue (follicles or ovarian cortex) as well as *in vitro* gametogenesis

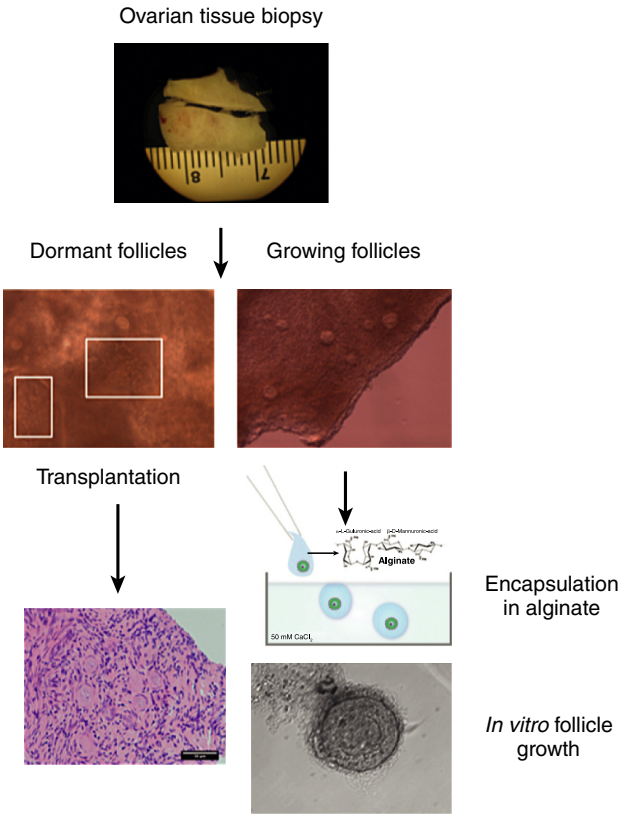


FIGURE 33.5 Ovarian tissue removal (either biopsy or whole organ) followed by cryopreservation is the only current option for prepubertal girls and certain patients who have hormone-responsive cancers or who cannot delay their cancer treatment. The ovarian tissue contains both dormant primordial follicles and growing follicles. The dormant follicles within this tissue will re-establish endocrine function and fertility if the ovarian tissue is transplanted back to the patient. The growing follicles could ultimately be used in methods such as *in vitro* follicle growth, which is currently being developed to obtain mature eggs. This ovarian tissue was obtained from a 15-year-old patient with idiopathic aplastic anemia who had already received immunosuppressive therapy. Her ovary contained visible dormant follicles *(left)* and growing follicles *(right)*.

(ovary and follicle culture) are emerging fertility preservation options for females (Figs. 33.3, 33.5, and 33.6). In fact, in some cases, these technologies are more advanced in females and have already been applied successfully clinically as described below. For certain female populations, removal of ovarian tissue prior to cancer treatment may be the only option for fertility preservation. These populations include prepubertal females, those who do not have time to undergo ART prior to starting cancer treatment, or those who cannot tolerate the supraphysiological hormone exposure required for most ART procedures. Following surgical removal, ovarian tissue is cryopreserved and can be used later for transplantation once the patient has been deemed cancer-free. Although the current approach is to cryopreserve ovarian cortical strips, methods are being developed to freeze whole ovaries for later transplantation through vascular anastomosis.[165,166] The goals of ovarian tissue transplantation are both to restore fertility and endocrine function.

The notion of ovarian transplantation originally gained ground as a viable fertility preservation option when autotransplantation of both fresh and frozen-thawed cortical strips in oophorectomized sheep restored fertility and resulted in the birth of two lambs.[167] In the human, ovarian transplantation was first tested through a series of heterologous transplants, using either fresh cortical tissue or whole ovaries, between nine pairs of monozygotic twins who were discordant for premature ovarian failure.[14] These heterologous transplants of fresh ovarian tissue resulted in 14 pregnancies and 11 live births in 7 of the 9 recipients.[14] In an oncofertility setting, the ovarian tissue is cryopreserved and stored for an indeterminate amount of time prior to transplant.[91] To date, 17 live births have been obtained following autologous transplant of frozen-thawed ovarian tissue, for a total of 28 live births following ovarian tissue grafting.[93,94] Of note, following transplant, these women reported resumption of normal menstrual cycles and several were able to conceive without the use of ART.

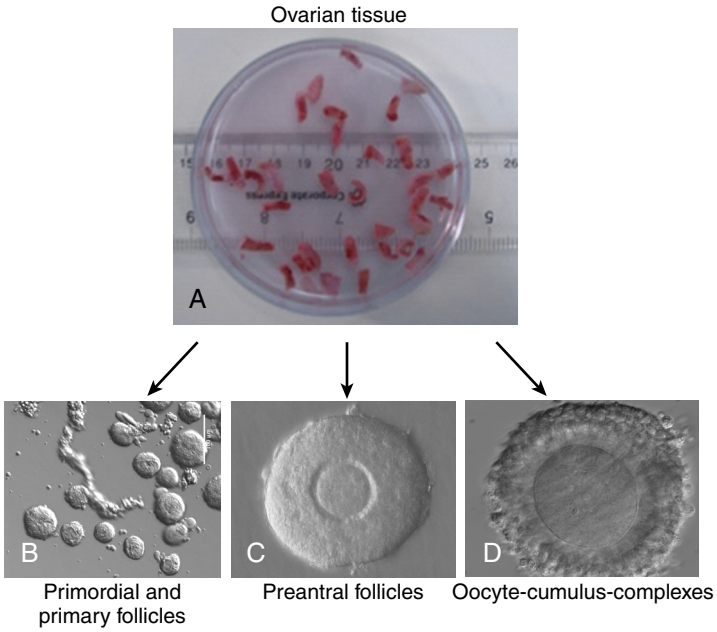


FIGURE 33.6 Human ovarian tissue that is surgically removed for cryopreservation (**A**) contains follicles at early stages of development that can be grown *in vitro* (**B-C**) as well as small antral follicles with oocyte-cumulus-complexes (**D**) that can be used in *in vitro* maturation protocols to obtain mature eggs.

All human live births to date have occurred following orthotopic ovarian transplantation, in which human ovarian tissue is transplanted back to its original location either on the remaining ovary or near the infundibulopelvic ligament or ovarian fossa.[168] An active area of research is heterotopic transplantation, in which the ovarian tissue is grafted subcutaneously at various sites, including the brachioradialis fascia of the forearm or under the rectus sheet in the lower abdomen.[169-171] Heterotopic transplantation is an attractive option because it can be performed in patients who have had pelvic irradiation and may have ovarian scarring, can be performed at the same time the ovarian tissue is removed, and allows for simple monitoring of follicle development.[168] Thus far, heterotopic transplantation in women has had varying degrees of reproductive success, resulting in restored endocrine function, follicle development, gametes capable of fertilization, and preimplantation embryo development.[170-172] Although biochemical pregnancies have been reported from gametes derived from heterotopic transplantation, no live births have occurred in humans.[173] However, there has been a live birth in the nonhuman primate following ectopic transplantation, demonstrating the potential of this technology.[174]

As is true in the field of testicular tissue transplantation, there are several risks and concerns in ovarian tissue transplantation. First, it is difficult to estimate the true efficacy of this technique because, although live births have been reported worldwide following this procedure, it is not known how many total transplants have been attempted that may not have been successful. In addition, the number, quality, and distribution of follicles within the cortical tissue from each patient cannot be predicted, and it is not known how long the transplants will remain functional. Finally, there is a lack of robust methods to detect residual cancer cells in the ovarian tissue, and thus there is an inherent risk of introducing cancer cells upon transplantation.[175-177] Obtaining definitive data about ovarian tissue transplantation will be a slow process because it could be decades before children who have frozen their tissue return to use it.

To overcome some of the risks of ovarian tissue transplantation, methods including xenotransplantation and follicle transplantation are also under investigation. Varying degrees of follicle development have been reported after xenotransplantation of human ovarian tissue. In short-term studies, xenografting human ovarian cortical tissue or isolated follicles for 1 week resulted in significant follicle activation and growth.[178] In a longer-term study, human ovarian tissue xenografts were transplanted under the kidney capsule of mice for 11 and 17 weeks in the presence or absence of FSH.[179] It was determined that follicles could survive and grow during this period and that FSH was required for growth of follicles beyond the secondary stage. Consistent with these findings, when ovarian biopsies from women 26 to 29 years of age were xenografted into mice for 5 months with FSH administered during the final 2 weeks, primordial, primary, secondary, and antral follicles were observed in the harvested tissue.[180] Xenotransplantation of human ovarian tissue into the back muscle of mice has resulted in the formation of large antral follicles and MII eggs.[181] Current studies are focused on determining the optimal sites (intraperitoneal, subcutaneous, intramuscular, beneath the ovarian bursa, or under the kidney capsule) and conditions for xenotransplantation.[182]

Similar to xenotransplantation, transplanting isolated ovarian follicles would reduce the likelihood of reintroducing cancer cells into the patient. Follicles could be isolated from fresh ovarian tissue, cryopreserved, and then thawed at the time of transplant.[183] Alternatively, follicles could be isolated from previously cryopreserved tissue upon thawing.[183] In mice, single-cell suspensions derived from primordial follicles from either fresh or frozen and thawed tissue were aggregated in plasma clots and transplanted into the ovarian bursa.[184,185] These cells reassembled into follicle structures that restored estrous cyclicity and fertility. Whether this approach will also work in nonhuman primates and humans is not known. In addition, although these findings are intriguing, it remains to be determined whether intact isolated follicles are sufficient to restore reproductive function.

Research is currently focused on ways to improve transplantation outcomes by optimizing how the ovarian tissue is cryopreserved and processed prior to transplantation. For example, ovarian tissue cryopreservation using slow-rate freezing is standard in most clinics, but this method is inefficient partly due to significant follicle loss that occurs during the freeze-thaw procedure.[186] Ongoing studies in several species including the bovine, rhesus macaque, and human indicate that vitrification may be a more effective method to cryopreserve both the follicles and the stroma within ovarian tissue.[92,186] Additional challenges with ovarian tissue transplant are slow graft revascularization and ischemia, which can also lead to follicle loss. Methods that decrease inflammation, reduce oxidative damage, inhibit apoptosis, and promote angiogenesis appear to improve the survival and quality of grafts.[187,188] Transplantation of vitrified and thawed mouse ovarian tissue encapsulated in fibrin, which was modified with heparin-binding protein, heparin, and VEGF, resulted in significantly increased blood vessel formation and twice the number of surviving primordial follicles compared to controls in which no biomaterial was used.[187] Increased graft survival was also observed when cryopreserved and thawed human ovarian tissue was pretreated with VEGF-A, vitamin E, and hyaluronan-rich biological glue prior to grafting into immunodeficient mice that had been pretreated with melatonin.[188]

In Vitro Follicle Growth

Though ovarian tissue banking is performed globally, the major hurdle faced by the research community is what to do with the tissue once it is thawed, especially when there is a risk of disseminated disease or genetic predisposition to cancer conversion (e.g., BRCA mutation) with autotransplantation. One approach that is still under development is *in vitro* follicle growth (IVFG), which has resulted in live birth in model organisms. While IVFG has theoretical and practical advantages, it also has technical limitations (Table 33.4), and it is very important that a patient who opts for ovarian tissue banking is aware of these issues.

Many IVFG methods are under development around the globe, reflecting the significant interest within the biomedical community to provide next-generation ART options for cancer patients who have banked their ovarian tissue.[189-214] In all cases, ovarian tissue is recovered from patients and stored for their later use (Figs. 33.5 and 33.6). The majority of follicle types in the removed tissue

are primordial and secondary follicles (Fig. 33.6). Growing follicles can be isolated and cultured on flat plastic, in a hemi-dome, or in a three-dimensional environment (Fig. 33.7). Primate follicles require extended periods of time in culture and the support of dynamic somatic cell-germ cell interactions; this "architectural" requirement led to the development of alginate and other biomaterials that mimic the natural ovarian structure in the late stages of follicle maturation (Figs. 33.5 and 33.7). The alginate hydrogel system has been tested in mouse and has been successfully applied to rhesus monkey, baboon, and human follicles.[215-223] The terminal meiotic maturation of the human oocyte, its fertilization, and its transfer to a human recipient await development. The primary advantage of this system is that follicle growth is carried out completely *in vitro*, producing a mature egg that can be fertilized. Thus, the embryo, which contains no somatic cells from the donor, can be transferred to the patient or to a gestational carrier without the risk of re-introducing cancer to the patient who has just survived her disease. However, this method does not restore endocrine function, and the patient will need endocrine management for pregnancy and the duration of her life.

Investigational Methods to Create Male and Female Gametes *De Novo*

While the derivation of mature sperm and oocytes from stem cells is outside the scope of this chapter, work is progressing on this front and may one day hold promise for young cancer patients who wish to preserve their fertility (Table 33.4).[224]

The Future

As research advances continue to be made, it is likely that many of the investigational procedures highlighted here will be translated clinically if they are not already. Often these procedures require highly trained staff or specialized equipment to perform, and there may be only few centers worldwide with the necessary expertise to do so. Fortunately, recent studies have demonstrated that both male and female mammalian reproductive tissues can be maintained at cold temperatures for extended periods of time, with preserved morphology and function, which would permit transfer of the tissues. For example, in the rhesus macaque, spermatogenic induction to spermatocytes was observed after xenografting of immature testicular tissue that had been kept in ice-cold medium for 24 hours.[225] Ovaries from rhesus macaque kept on ice for between 18 to 24 hours were indistinguishable from fresh ovaries in terms of stroma and follicle morphology and levels of apoptosis.[226] In the human, cortical ovarian tissue that was kept on ice for 4 hours prior to cryopreservation contained follicles that survived following xenografting.[227] Moreover, human ovaries kept on ice for up to 24 hours contain follicles that, when isolated and used for *in vitro* follicle growth, are able to survive, grow, and form antral cavities similarly to those isolated from fresh tissue (unpublished data). The gametes produced by ovarian tissue maintained at cold temperatures for transport purposes

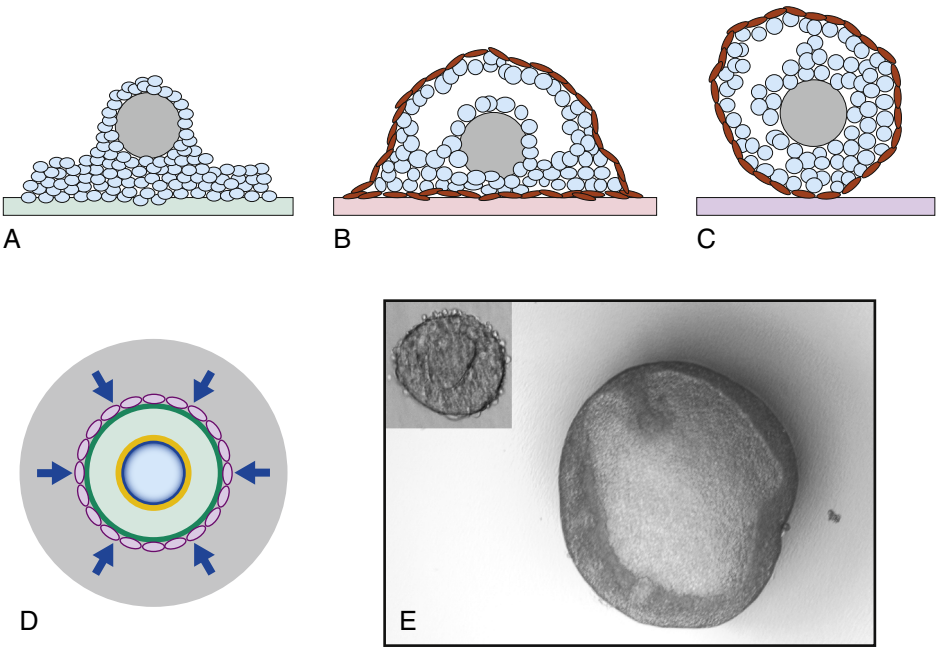


FIGURE 33.7 *In vitro* gametogenesis is a promising fertility preservation option that is actively being developed for both males and females. Several culture systems have been developed over the past decades to support coordinated folliculogenesis and oogenesis outside of the body. These systems include (**A**) growing granulosa-oocyte complexes on collagen transwells or growing isolated follicles on (**B**) adherent or (**C**) nonadherent surfaces. (**D**) Follicles can also be encapsulated and grown in hydrogels such as alginate. This biomaterial allows follicles to maintain their characteristic spherical structure and relative geometry (central oocyte surrounded by granulosa and theca cells) throughout development *in vitro*. Notably, this system is the only one to date that robustly supports the growth and differentiation of ovarian follicles from several large mammalian species, including dog, nonhuman primate, and human (**E**).

appear to be developmentally competent. In fact, a live birth was obtained following autotransplantation of ovarian tissue that had been kept on ice for more than 20 hours prior to cryopreservation.[218,228] Thus, patients seeking these investigational procedures may not have to be limited by their proximity to specialized centers to receive treatment.

The number of clinicians and basic scientists working in the field of oncofertility is growing exponentially. As the field grows, it is imperative that promising work developed in model organisms is first tested using human tissue whenever possible before being translated clinically, as there are known examples where even nonhuman primates may not be adequate models for humans.[218] In addition, researchers working independently on female and male fertility preservation strategies must maintain open lines of communication.

Oncofertility Patient Management

Although they are faced with the stresses inherent in a new cancer diagnosis, adolescent and young adult cancer patients list future fertility as one of their most important concerns. Discussions about fertility preservation are most beneficial when they occur as early as possible after the cancer diagnosis. Unfortunately, few cancer patients have the opportunity to discuss fertility issues with their oncologist or to be referred to a reproductive specialist at or soon after their diagnosis. Both provider- and patient-related issues contribute to the poor communication of fertility concerns and preservation options. Provider barriers include a lack of knowledge of the impact of cancer therapies on fertility and lack of resources for referring patients to reproductive specialists. Increasing providers' awareness of current guidelines regarding fertility preservation for cancer patients, as well as the patient resources that are available, can facilitate timely referral of patients to a consultation with a reproductive specialist. Patients may benefit from educational resources on basic concepts and issues in reproduction and fertility that they can access before their visit to the reproductive specialist. Each of these issues is discussed in more detail in this section.

Fertility Matters to Adolescents and Young Adults Diagnosed with Cancer

After a new cancer diagnosis, patients are focused on understanding prognosis, their impending treatments, the financial burdens, and family and work obligations, yet many young people diagnosed with cancer also have significant concerns about their future fertility. Cancer survivors recall that fertility concerns were influential enough to alter their cancer treatment decisions.[229-231] Fertility concerns can have a substantial impact on patients, both at the time of diagnosis and years later. Several studies have indicated that cancer survivors who are infertile due to their treatments are more likely to have emotional distress.[230,232,233]

Communication Barriers

While it is clear that many patients have questions and concerns about future fertility, it is also clear that these needs are not adequately addressed for a significant number of patients. The time around the initial diagnosis of cancer is stressful for patients, families, and providers, and discussions about the cancer diagnosis, acute risks, prompt treatment, and survival often take priority. Studies have shown that many oncology professionals do not routinely discuss fertility with their reproductive-age patients.[234-237] A 2009 survey found that only 47% of responding oncologists routinely refer patients for fertility preservation counseling, and that female physicians and those who had a favorable attitude about fertility preservation were more likely to refer.[237] Similarly, a 2010 survey of 249 oncologists found that more than half rarely refer patients to a fertility specialist, though 82% have referred patients at some point.[235] When planning cancer treatments, 30% of providers state that they rarely consider a woman's desire for fertility.[235]

There are many factors that may inhibit the oncology team from adequately discussing fertility concerns with patients, including both provider-related issues (e.g., little knowledge about fertility preservation options[238-241] and lack of interprofessional networks that include fertility specialists) and patient-related issues, such as poor survival prognosis, the need for immediate initiation of treatment, the patient's age, pubertal status, parity, marital status, and financial situation.[234,236,239,240] A 2007 qualitative study of 16 oncologists found that physician barriers included lack of knowledge about fertility preservation options, limited awareness of referral information for fertility specialists, the length of time the physician had been practicing (younger providers were more likely to refer patients for a fertility preservation consultation), and the physician's specialty (oncologists who administer chemotherapy were more likely than surgical oncologists to discuss fertility).[239] Patient factors that limited fertility preservation discussions included urgency in need for cancer treatment, perception of fertility preservation as being a low priority, female gender, increased parity, and greater disease progression.

Provider Communication

In 2006, ASCO convened a multidisciplinary panel that drafted and issued guidelines on fertility preservation for cancer patients.[59] These guidelines state "as part of education and informed consent before cancer therapy, oncologists should address the possibility of infertility…and be prepared to discuss possible fertility preservation options or refer appropriate and interested patients to fertility specialists." While clinical judgment should be employed in determining when to raise issues of fertility and fertility preservation with patients, having this discussion at the earliest possible opportunity is encouraged. Other professional societies and organizations, such as ASRM, the American Academy of Pediatrics, and the Oncofertility Consortium, have also developed "best-practice" guidelines and educational resources designed for patients and providers.[17,57,242-244] Improving providers' awareness of these guidelines and resources will improve patient awareness of cancer-related fertility concerns and facilitate timely referral of interested patients to a fertility preservation specialist. Specifically, patient care will likely be improved if earlier referral to a reproductive specialist is implemented.[245]

These provider-focused guidelines and educational tools also include flow diagrams and identify "points of

discussion" between the oncology team and the patient. At a minimum, two essential points that the oncology team must communicate successfully to all reproductive-age cancer patients are that (1) cancer treatments may cause future fertility problems; and (2) patients who express any interest in their future reproductive options can be referred to a reproductive specialist and/or a psychosocial counselor. These two points can be initiated by nonphysician members of the oncology team (such as nurses, a patient navigator, social workers, therapists, etc.).

Once a patient has been referred, the reproductive specialist should also provide a comprehensive consultation to interested patients, detailing risks of their cancer or cancer treatment to fertility, pregnancy after cancer, and all appropriate fertility preservation treatment options. If members of the oncology team feel comfortable discussing fertility in more detail, the ASCO guidelines provide additional "talking points."[59] These points may be discussed early after diagnosis, as patients decide whether to pursue a fertility preservation consultation, or later in the process, if patients request their oncologist's opinion regarding their fertility preservation options. If the oncology team does not initiate the topic of fertility and cancer, ideally, patients can access information on patient-facing web sites, such as MyOncofertilty.org, the Oncofertility Consortium blog, and SaveMyFertility.org (Fig. 33.8A-D), where they can obtain handouts, important contact information, and even download smartphone applications (Fig. 33.8E), that can help start a discussion about fertility with their oncology team (Table 33.3).[246-248] Many of the resources have been translated into Spanish and Italian to ensure broad distribution of information globally (see respective web sites).

Improving the Process

Ideally, the practice of oncofertility can be improved in ways that will decrease decisional conflict, improve patient satisfaction, and allow patients to make well- informed decisions. The first step is to improve patient comprehension about fertility preservation. It has been shown that knowledge and understanding of disease and treatment are closely linked to patient outcomes and quality of life.[249] Potentially, education about fertility preservation could begin at the time that the oncologist offers their patient a referral to a reproductive specialist. Patient knowledge about fertility preservation prior to their consultation with the reproductive specialist is currently very limited, which means that patients are asked to process a great deal of complex medical information during their initial meeting with the fertility preservation specialist. Not surprisingly, patients who sought out web sites such as fertilehope.org prior to their fertility preservation consultation had greater pre-visit knowledge than those who did not seek out this information.[246] One possible approach to improving cancer patients' understanding of fertility preservation issues is for the oncologist to direct patients to educational resources that should be accessed prior to the consultation with the reproductive specialist (Table 33.3); these resources would introduce vocabulary and general concepts in reproduction and fertility that would prepare patients to comprehend and participate in a discussion of the complex concepts and issues of fertility preservation with the reproductive specialist.

The development of innovative communication approaches is essential for further improving patient understanding of their fertility preservation options, especially in the challenging context of a recent cancer diagnosis. Web-based interactive educational tools and decision aids directed at patients may further improve access to fertility preservation information and allow for higher decisional satisfaction for reproductive-age cancer patients. In addition, the inclusion of discussions about fertility concerns as part of the standard of care for all patients will help avoid current biases in referral patterns based on patient characteristics, such as parity and prognosis. Effective oncofertility care is also dependent upon communication across departmental divisions, with the oncology team working closely with reproductive specialists to coordinate care on an individual patient basis. Multidisciplinary clinics have become more common in disciplines such as breast cancer, where surgeons, genetics counselors, medical oncologists, and others come together



FIGURE 33.8 Online resources, such as those developed by the Oncofertility Consortium, offer patients and providers a wealth of information about fertility preservation. Patients can visit various web sites to learn about how cancer may affect their fertility, what current clinical options are available, where to obtain the best care, and how to find support. Providers can keep abreast of basic research advances, find information on how to establish a fertility preservation program, and determine where to refer a patient. **A**, myoncofertility.org; **B**, myoncofertility.org (Spanish); **C**, savemyfertility.org; **D**, fertilitypreservation.northwestern.edu; **E**, oncofertility.northwestern.edu; and **F**, the iSaveFertility smartphone application that provides physicians with pocket guides and fact sheets.

to provide comprehensive care. Oncofertility should strive for a similar multidisciplinary approach to patient care.

An Example of Integrated Care Network: The National Physicians Cooperative

The National Physicians Cooperative is a nationwide network of 56 fertility preservation centers across the United States that consult with approximately 2700 young cancer patients each year (Supplemental Fig. S33.1A, which can be found on the companion Expert Consult Web site at www.expertconsultbook.com). This is an example of integrated care that exists in the U.S. that is also emerging across the globe, including the German group FertiProtekt (Table 33.2). Much like other global programs, the National Physicians Cooperative was established to provide a collaborative forum for the exchange of ideas, clinical research methods, and technologies in order to drive breakthroughs in basic reproductive physiology that may be translated directly to clinical medicine. It also provides a wide range of resources for oncology teams that treat young cancer patients, such as guidance on how to refer patients for fertility preservation, practical advice on how to navigate financial/billing issues, and information on new developments in fertility preservation research and practice that may affect patients. Clinically, these efforts have produced more than 400 annual consultations of young cancer patients with the fertility preservation patient navigator.

The Fertility Preservation Patient Navigator

A patient navigator is an individual who guides patients through and around barriers in the complex healthcare system to help ensure timely diagnosis and treatment. Often nurses or social workers within the health system play a patient navigator role.[247] In fertility preservation, a patient navigator serves as the primary contact for patients and clinicians, facilitating a cohesive relationship between patients and the various health care professionals who are involved in their care. Importantly, the oncofertility patient navigator serves as a bridge between departmental and disciplinary boundaries so that cancer patients are able to receive timely information regarding fertility preservation options and receive coordinated care from both the oncology team and reproductive specialists. The oncofertility patient navigator makes it possible for patients to make well-informed decisions in the window of time prior to starting their cancer treatment.

The navigator's role is to make contact with the patient, collect important clinical information, and set up an appointment for fertility preservation counseling with a reproductive endocrinologist or urologist as appropriate. In addition, there is a 24-hour telephone service called the Oncofertility FERTline, which allows patients and providers to call one number to obtain free, individualized information and referrals for fertility preservation services (Supplemental Fig. S33.1B, which can be found on the companion Expert Consult Web site at www.expertconsultbook.com). Calls from patients and providers throughout the world are triaged and navigated to the physician service in the most geographically favorable location.

With a good referral system and an effective navigator in place, oncology professionals do not need to become experts in fertility. The oncofertility patient navigator can help patients move between the complex specialties of oncology, urology, and reproductive endocrinology, as well as help make a complex topic more understandable and accessible to both clinicians and patients. The navigator can ensure that patients don't get lost between specialties.

In addition to facilitating referrals for fertility preservation consultations, navigators help educate patients about available fertility preservation options and can direct patients to the online educational resources described above (Figs. 33.8A-D). As more educational materials become available, the oncofertility patient navigator is able to sort through what information would be most helpful for a given practice or provider, deliver appropriate materials to patients, inform program developers about gaps in current educational materials, and help drive the next generation of web, print, and smartphone materials.

The oncofertility patient navigator can also provide important financial information and advice regarding fertility preservation procedures. Costs for fertility preservation services vary widely throughout the globe and are often not covered by insurance or country/state healthcare systems. A well-informed patient navigator who stays in touch with patients can ensure that expectations regarding coverage and reimbursement are managed up front and that follow-up letters to insurers are provided. Cost is a big issue for young patients, and fertility specialists and their office staff may not have the capacity to work closely on the special needs of cancer patients. Dedicated oncofertility patient navigators can provide the level of personalized care and interaction that these patients require. They may not be able to alter the payer decision, but they will provide the patient with the knowledge that everything that could be done was done to ease their financial burden. Navigators can also point patients to available programs that may defray the costs of fertility preservation. In these arrangements, fertility centers agree to offer cancer patients heavily discounted IVF cycles and pharmaceutical companies provide patients fertility medications at no cost. This can result in thousands of dollars of savings and put fertility preservation within a patient's financial reach. Unlike ordinary IVF patients who have time to save money for an eventual IVF procedure, the cost management for young cancer patients who are often at the earliest stages of their earning potential represents a barrier and an issue that the overall field must continue to consider on a country-by-country, payer-by-payer basis.

Decision Aids and Patient and Provider Information

Oncofertility strives to give cancer patients options when they are confronting potential infertility as a consequence of cancer treatment. Ideally, fertility preservation decisions must be made before treatment begins, adding stress to the decision-making process. Decisions aids that help navigate both patients and providers through all plausible relationships, alternatives, and outcomes for a given decision provide an ideal platform to convey the importance of each decision within the overall spectrum of care (Figs. 33.9 and 33.10).[248-249]

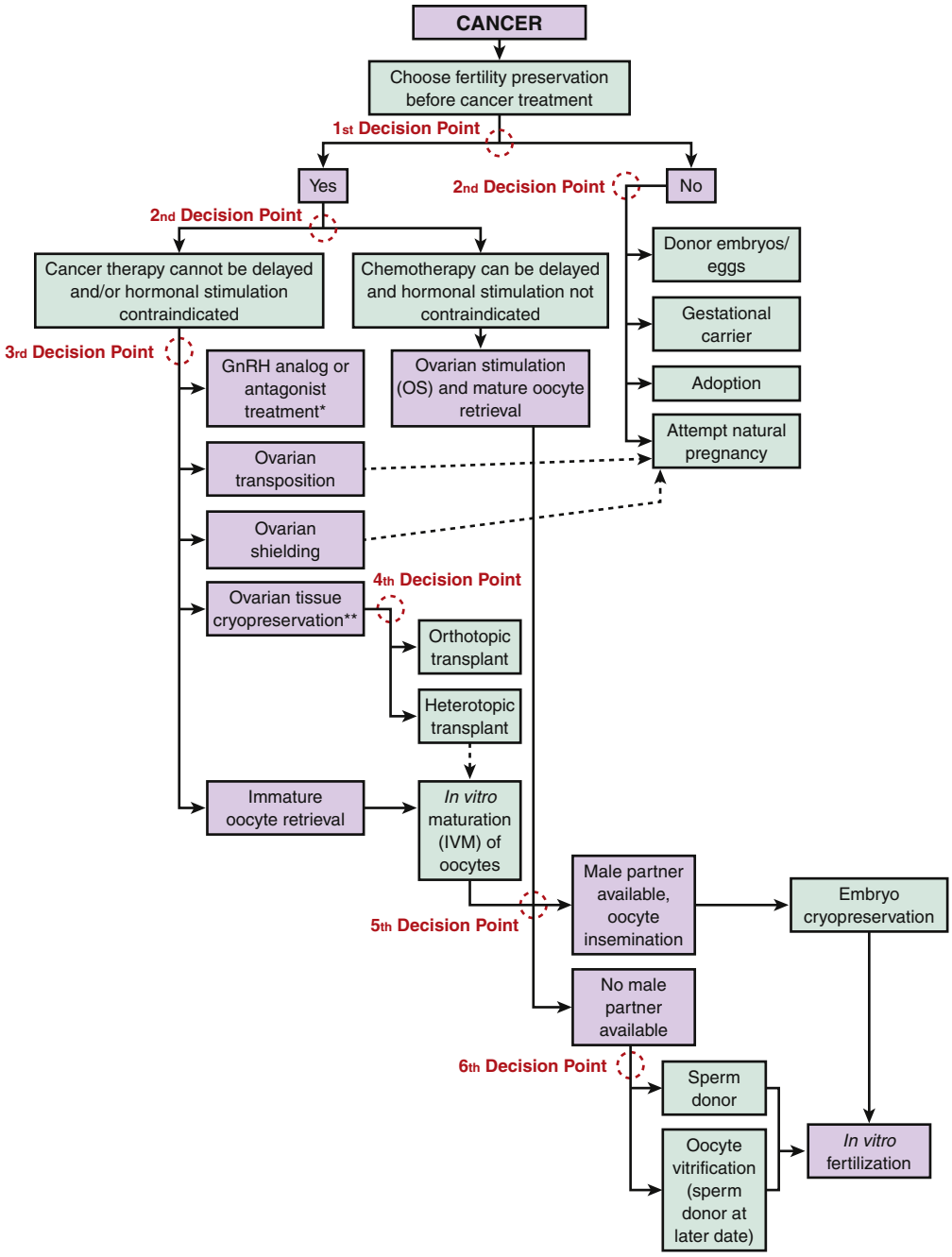


FIGURE 33.9 Decision trees can be useful tools for guiding cancer patients through the complex array of fertility preservation options. This is an example of a decision tree that could be used for females who are seeking fertility information either before or after cancer. Critical decision points are highlighted in red, and *asterisks* mark investigational methods. The female decision tree is more complex than that of males (Fig. 33.10). If using such decision aids, physicians should periodically reassess these trees to ensure that options are current given the rapid pace at which the field is evolving.

Advocacy

It is well known that cancer advocacy groups help educate patients, but many also provide patients and survivors with financial assistance, such as for costly fertility preservation procedures prior to cancer treatment and infertility treatments after cancer. There are several programs in the U.S. that are fairly prominent and are examples of the kinds of programs that are needed throughout the world. The Sharing Hope program of the Lance Armstrong Foundation donates fertility medications to females and assists with on-site or mail-based sperm banking for males. One year of cryopreservation storage fees and reproductive endocrinology costs may also be discounted through this program. Financial programs such as Verna's Purse also help survivors pay for storage fees for existing cryopreserved gametes or tissues. Additional programs may help patients navigate reproductive options after cancer, such as surrogacy. Fertile Action negotiates waived costs for some prescriptions prior to egg and embryo banking and awards grants to provide

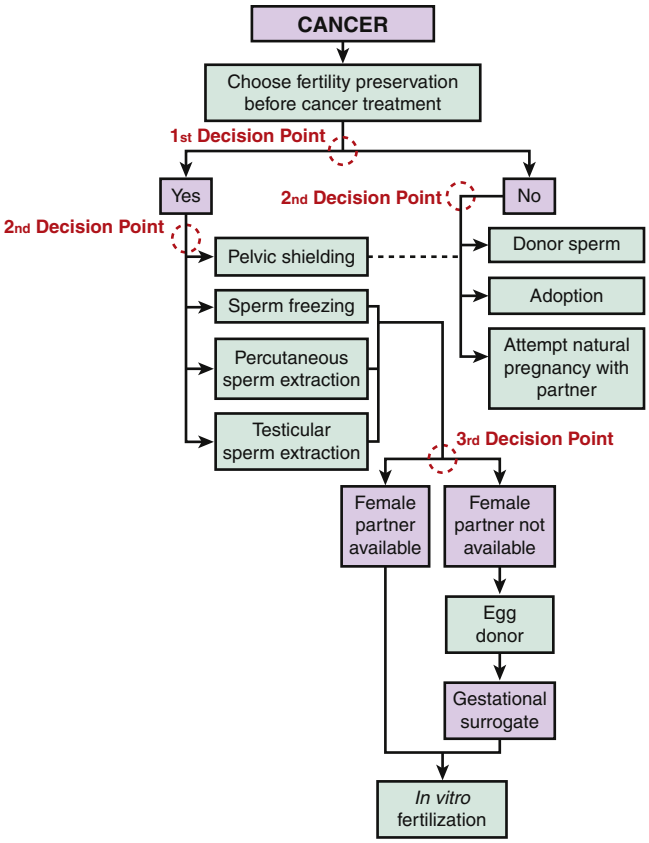


FIGURE 33.10 A decision tree that could be used for males who are seeking fertility information either before or after cancer. Critical decision points are highlighted in red. If using such decision aids, physicians should periodically reassess these trees to ensure that options are current given the rapid pace at which the field is evolving.

financial support during the surrogacy process. Young adult cancer survivors may also apply to the SAM Fund for grants to cover such services.

Establishing an Oncofertility Program

The primary goal of a clinical oncofertility program is to help young cancer patients and their physicians consider the impact of their cancer treatment on future fertility, discuss fertility preservation options soon after diagnosis but before treatment begins, and ensure that cancer patients are given the best opportunity to achieve their fertility goals. Oncofertility programs must provide physicians and patients with timely and comprehensive fertility preservation information, as well as efficiently coordinate the care of cancer patients between healthcare providers in oncology and reproductive endocrinology. A successful oncofertility program requires the establishment of a multidisciplinary team that includes medical specialists (e.g., oncologists, hematologists, rheumatologists, reproductive endocrinologists, urologists, surgeons, anesthesiologists, and pathologists) and their staff, genetic counselors, mental health counselors, the embryology lab team, and the scientific team (Box 33.1). A dedicated patient navigator is an invaluable part of the oncofertility team who not only helps physicians optimally integrate fertility preservation into their patients' cancer treatment plans, but also helps patients become better informed as they move from cancer diagnosis to discussions of future fertility.

Box 33.1 Key Oncofertility Healthcare Team Members

- Medical oncologists and hematologists
- Surgical oncologists
- Radiation oncologists
- Urologists
- Primary care physicians
- Reproductive endocrinologists and infertility specialists
- Bone marrow and stem cell transplant specialists
- Physicians' nurses and staff for all of the above specialties
- Social workers
- Mental health staff
- Patient navigators
- Financial counselors
- Genetic counselors
- Survivorship staff
- Cryopreservation storage representatives
- Institutional review board for investigational procedures

Various healthcare providers, the available facilities and staff, and associated organizations (e.g., cryopreservation facilities, financial aid sources) must work together to best meet the fertility needs of each cancer patient. To do this, oncofertility team members must understand the importance of quick, early fertility preservation referrals prior to cancer treatment; the unique fertility needs of young oncology patients; and how to refer a patient between members of the oncofertility team. Establishing and maintaining multiple lines of communication between various healthcare providers, the patient navigator, and patients is essential to the success of an oncofertility program.[59,70,71,238] An individual patient's oncofertility team is chosen based on patient demographics, cancer diagnosis, the planned cancer treatment, location, available providers, and available facilities and agencies. The National Physicians Cooperative and the FERTline are valuable resources for those who wish to establish a new oncofertility program at their institution, providing advice on everything from staffing to processes to documentation.

Special Cases in Fertility Preservation

The types of patients who seek fertility preservation are diverse, and each patient requires highly personalized care that can be provided by a multidisciplinary oncofertility team, and coordinated by an oncofertility patient navigator. Yet some oncofertility cases are particularly challenging and warrant a more detailed discussion. These include fertility preservation for pediatric and obstetrical patients as well as those who may have nononcological conditions that impact reproductive function.

Prepubertal and Adolescent Populations

Although reproductive-age men and women facing a cancer diagnosis have the option of banking sperm, eggs, or embryos, fertility preservation options for pediatric cancer patients are limited. Once a boy has reached puberty, he can

choose to bank mature sperm.[250] But for prepubertal boys, only experimental or more invasive methods are available, including immature sperm collection by surgical extraction, electroejaculation performed under sedation, or testicular tissue biopsy that is banked with the possibility for transplantation back to the testis to restore spermatogenesis (Table 33.4 and Fig. 33.4).[251] In all boys undergoing cancer treatment, the risk of infertility can be reduced through testicular shielding during radiation therapy and the use of fertility-sparing surgical techniques.

In postpubertal girls, ovarian hyperstimulation followed by IVF and embryo cryopreservation is the most successful option for preserving fertility;[59] however, the lack of a male partner presents a significant obstacle and the process can be emotionally and physically demanding for adolescent girls and their families. For prepubertal girls, hormone treatment and IVF is simply not an option. Experimental ovarian tissue cryopreservation is available to these patients at certain centers.[252] After cancer treatment, this ovarian tissue can potentially be used in one of several techniques discussed in the *Investigational Methods & Basic Science* section.

Because the fertility preservation technologies that are suitable for prepubertal males and females rely on the removal of gonadal tissue, a highly debated issue that must be carefully considered is how much reproductive tissue should be removed for use in potential downstream fertility preservation applications. A risk-benefit analysis must be undertaken and discussed with the patient and/or the patient's parents. Removal of too much tissue is invasive, reduces the possibility that natural reproductive function will be restored, and can result in several other health problems that are associated with reduced or absent gonadal function. On the other hand, enough tissue must be removed in order for fertility preservation to be feasible. This decision is further complicated by several factors. For many of the investigational technologies described, we do not know the minimum amount of tissue required for successful fertility preservation. This is in large part due to the innate patient-specific heterogeneity of the tissue as well as our inability to quantify the reproductive potential of the tissue (i.e., number and quality of gametes present).[253] In females, for example, follicular density in the ovarian cortex is linearly but inversely correlated to patient age,[254] yet in a single ovary, cortical follicle distribution can be very uneven with follicle density varying by up to two orders of magnitude between pieces of tissue.[254] In males, a biopsy might be sufficient in the prepubertal population because the immature testis is enriched in SSCs.[153] In preliminary xenotransplantation studies, tissue from male fetuses or prepubertal boys showed better graft and germ cell survival compared with adult tissues.[255,256] As investigational technologies to utilize the gametes within cryopreserved tissue are developed and translated into routine clinical use, guidelines will have to establish when it is best to perform tissue biopsy or whole organ removal for fertility preservation.

The ovary and testis are unique in that they are organs that contain gametes, and thus the potential for new life.[257] Thus, the ethical and legal uses of organs and gametes are complex, especially in the case of minors where there is a question of who owns the cryopreserved tissue. Furthermore, consent for all medical procedures, including ovarian or testicular tissue removal for banking, must be obtained; however, the act of consent for experimental procedures is particularly complicated for pediatric patients.[70] The parent or legal guardian has the authority to provide consent, but the procedure, if not medically necessary, must not be harmful and it must benefit the patient. Less invasive methods, such as laparoscopic removal of the gonad, are used to minimize risk to the patient. Moreover, it can be argued that ovarian or testicular tissue cryopreservation, though investigational, may one day benefit the patient in terms of allowing him or her to have genetically related offspring. Nevertheless, in some cases—such as in very young patients—additional surgical procedures may introduce added risk that does not outweigh the potential future benefit.[258] Finally, in addition to obtaining consent, when possible, it is also important to have the assent of the child for removal of ovarian or testicular tissue, particularly for adolescent patients who are able to understand the procedure and its risks but are legally unable to provide consent.[257]

Regardless of fertility preservation status, all adolescent and prepubertal patients who have been exposed to gonadotoxic cancer treatments should be followed yearly to monitor the onset and timing of puberty, menstrual, and pregnancy history and to assess sexual function, and a genitourinary exam should be performed.[129] For those at risk of hypogonadism, laboratory screening should include measurement of FSH, LH, testosterone for boys, and estrogen for girls. In boys, semen analysis may also be performed to assess fertility. In girls who received pelvic radiation, high-level ultrasound can be used to assess the uterus. As girls age into womanhood, their gynecologists are often their primary care physicians, and thus are in the position to provide such follow-up care to young female cancer survivors prior to childbearing. Obstetricians can also continue this care for survivors during the gestational period.

Obstetrical Oncology

The obstetrical care of pregnant women, either cancer survivors or women who are diagnosed with cancer during pregnancy, is challenging. For cancer survivors, clinical care requires knowledge of the effects of past cancer treatment exposure on reproductive function—particularly the effects on the uterus—as well as an understanding of the compounded risks associated with pregnancy and cancer. Past exposure to radiation or certain chemotherapeutic agents can damage cardiovascular and pulmonary capacity, two systems that must accommodate the needs of the growing fetus.[259] For these patients, it is critical that the obstetrician work closely with the oncologist to provide integrated care for each patient, not only to ensure a safe delivery of a healthy baby but also to preserve the health of the mother.[260]

Fertility management in a patient diagnosed with cancer during pregnancy is an additional unique and complex situation, but with the additional component of concern for the fetus. A cancer diagnosis is made in approximately 1 out of every 1000 pregnancies and this is expected to rise due to the increasing trend of delayed childbearing.[261-263] Care of these patients involves a difficult balance between treating the woman for cancer and preserving the well-being of the fetus and the possibility of future pregnancy. Aside from the effects of cancer treatment on fertility, factors these

Box 33.2 Potential Nononcological Needs for Fertility Preservation or Counseling

- Autoimmune diseases
  - Systemic lupus erythematosus, multiple sclerosis, chronic kidney disease
- Postsurgical etiologies
  - Inflammatory bowel disease, ulcerative colitis, familiar adenomatous polyposis, endometriosis
- Transplantation and transfusion related disorders
  - Thalassemia major, sickle cell anemia, aplastic anemia, Fanconi anemia, myoproliferative diseases transplant (renal, liver, pancreas), hemochromatosis
- Genetic diseases
  - Turner syndrome, fragile X syndrome, X chromosome deletions
- Metabolic conditions
  - Galactosemia, diabetes, polycystic ovary syndrome

patients and their physicians must consider are the possible risk of fetal toxicity from cancer treatment, patient prognosis, and ability to care for offspring.

Nononcological Applications

In addition to cancer, several other diseases and conditions and their treatments are associated with reduced reproductive function in women and men (Box 33.2).[264] Rheumatological disease and autoimmune diseases such as systemic lupus erythematosus are treated with highly gonadotoxic agents; these patients may preserve their fertility by banking gametes or embryos prior to treatment or between treatment cycles. Nonmalignant hematological disorders such as thalassemia major, sickle cell anemia, aplastic anemia, Fanconi anemia, and myeloproliferative diseases are treated with bone marrow transplantation or hematopoietic stem cell transplant, and patients are exposed to alkylating chemotherapy and radiation that increases the risk of infertility. In boys, cryptorchidism, even after orchiopexy, can significantly decrease fertility later in life. Testicular tissue biopsy cryopreservation has been performed in boys with cryptorchidism, with spermatogonial survival and normal hormone production achieved after thawing.[265] Men with Klinefelter syndrome (XXY) have normal testes development up to puberty, but then develop hypergonadotrophic hypogonadism with degeneration of the seminiferous tubules and almost total loss of sperm production. Small areas of the tubules may still produce viable sperm that are recoverable by micro-TESE (Table 33.4);[266] the sperm can be banked or ICSI can be performed to produce embryos.[267]

Women with Turner syndrome—who have either partial or complete absence of one X chromosome—are at risk of premature ovarian failure. In mosaic Turner syndrome, an X chromosome is lost during embryonic development, such that some proportion of the body's cells contains two normal X chromosomes. Rare cases of pregnancy have been reported in women with mosaic Turner syndrome, and recent work suggests that women with mosaic Turner syndrome may be candidates for fertility preservation by either tissue cryopreservation (in prepubertal girls) or hormone stimulation followed by oocyte cryopreservation (in postpubertal girls and women).[268] Spontaneous puberty and normal gonadotropin and estrogen levels were found to be predictive for the presence of mature follicles in girls with Turner mosaicism,[269] and several case reports have described fertility preservation efforts in this population.[270-273]

Legal and Ethical Issues Surrounding Fertility Preservation

From legal and ethical perspectives, fertility preservation is an inherently complex field because it involves rapid decision-making at a difficult time; the input of multiple stakeholders; the manipulation of reproductive cells, tissue, and organs; and the potential creation of new life. Below is an overview of key legal and ethical issues that have arisen from the advent of oncofertility.

Adoption

Cancer survivors who are infertile may wish to start a family through adoption and there are different hurdles placed on patients depending on the state or country in which the individual resides. Some adoption agencies may be reluctant to work with prospective parents who have a history of cancer due to concerns about the long-term welfare of the child.[274] In the U.S., adoption policies that take into account the health of adoptive parents vary from state to state, and individual adoption agencies use their own discretion in considering the health status of adoptive parents. In a recent survey of 27 agencies in the U.S., only 1 had a policy in place regarding adoption by cancer survivors, another agency required a "reasonable life expectancy," and a third required a doctor's assessment of health.[274] Though no international agencies regard a history of cancer as a disqualifier for adoption, health assessments may be used to determine whether a potential adoptive parent is physically capable of caring for a child.[274] Navigating adoption agency-specific policies can be difficult and frustrating for cancer survivors, and it is clear that discrimination exists; however, there are a number of "cancer-friendly" adoption agencies in the U.S. that have expressed a willingness to work with prospective parents who have a cancer history (Supplemental Table S33.1, which can be found on the companion Expert Consult Web site at *www.expertconsultbook.com*).

Surrogacy

For women who had a reproductive cancer that required hysterectomy, or for women who were exposed to radiation or chemotherapy that impaired their ability to carry a pregnancy to term, gestational surrogacy may be an option for starting a family after cancer.[275-277] If a patient is able to bank either oocytes or embryos prior to their cancer treatment, the surrogate could carry the patient's genetically related baby to term (Table 33.4 and Fig. 33.3). Similar to the topic of adoption, there are state and country-specific laws that may create barriers to surrogacy that must be considered.

Insurance

In the U.S., the lack of insurance coverage is one reason oncologists do not offer fertility preservation as an option

to their patients.[59] There is controversy regarding whether infertility insurance mandates should also cover fertility preservation for cancer patients. One major issue is that cancer patients are not considered to be infertile at the time that treatment is given. The widely accepted definition of infertility stipulates the inability to become pregnant after a period of time, usually 1 year,[17,278] yet this definition does not apply to cancer patients seeking fertility preservation within the small window of opportunity before gonadotoxic treatment begins. Current coverage of infertility treatments assumes that the condition already exists, rather than that the condition can be prevented. In terms of insurance coverage, fertility preservation would be analogous to other measures taken to prevent iatrogenic conditions;[71,279] for example, storing blood in case of an emergency transfusion. In a recent review of the issue, Basco and colleagues suggested that either the existing definition of infertility be changed to include cancer patients; a new definition be created specifically for cancer survivors; or a new insurance mandate be put in place for fertility preservation services provided to cancer patients.[280] The issue of coverage and reimbursement varies globally and is a topic that must be discussed as a global community in order to improve access for all.

Access to Care

Cancer and infertility do not discriminate based on social class or ethnicity; however, in countries where economic barriers determine healthcare access, fertility preservation is simply out of reach for many patients. In the U.S., only some states have mandates for infertility coverage, and many of these may not cover fertility preservation. In the current economic climate, many states may find that IVF is difficult to finance. Under the Affordable Care Act, insurance coverage will be extended to many more Americans, but again, whether this coverage will include fertility preservation in patients with cancer is not clear with the existing definition of infertility. Furthermore, experimental procedures such as ovarian or testicular tissue cryopreservation are performed only under IRB approval and only at certain centers, which may limit regional access to these options for many patients.

Ownership of Biological Material

The infertility field and the legal systems of many countries have grappled with the issue of embryo ownership, particularly in cases of divorce, death of a partner, and same-sex couples. The same issues will be encountered by cancer survivors, particularly as these patients have a higher risk of death than healthy infertile individuals. In addition, ownership of ovarian or testicular tissues may present special obstacles, as the tissue itself contains the potential for many future offspring. New laws will need to be created to define who can acquire and dispose of these tissues. These laws may be particularly complex with regard to ownership of gonadal tissues removed from pediatric cancer patients.

Should We Be Doing Fertility Preservation?

Provider concerns about cancer progression, life expectancy, and the stress of additional interventions have been cited as issues that limit the clinical provision of fertility preservation.[281,282] Many argue that reproduction is a basic right of the patient, and as long as the potential risks to a patient's offspring are discussed, then fertility preservation should be offered. Others have suggested that it is not in the best interest of potential future children to allow patients with cancer—who may have a shorter lifespan—to reproduce. In his ethical analysis, Robertson found that the stress associated with a loss of a parent is no greater than other forms of stress that will be experienced during a child's life, and therefore should not be a reason to withhold options for fertility preservation from cancer patients.[283]

Interestingly, provider beliefs and actions regarding the provision of fertility interventions to cancer patients show a gender disparity. In a survey of pediatric oncologists, a majority of respondents acknowledged that fertility threats to female patients are a major concern for them (83%) and agreed that all pubertal female cancer patients should be offered fertility consultation (73%).[236] However, only 12% reported that they refer female cancer patients to a fertility specialist prior to cancer treatment more than 50% of the time, compared with a 46% referral rate for males. The barriers to referrals for women included a lack of knowledge about fertility management options or referral centers, the concern that fertility preservation procedures take too much time, biases toward the marital status of the cancer patient, the notion that banking embryos is not appropriate for a cancer patient who might not survive her disease, and concerns about the ability of the patient to survive the disease. Note that these issues are all based on provider bias and not on informed decision-making by the patient. Ongoing work to understand the boundary conditions for patients, partners, parents, the interested public (including policymakers), and providers represents an important next chapter in this field.

Summary and Outlook

Fertility preservation has emerged over the last decade as a topic that young cancer patients want addressed. With guidelines from medical societies instructing physicians to act on behalf of their patients' future fertility, the emergence of new options that provide hope to a greater number of patients, and new medical management plans that connect physician services across disciplinary boundaries, the field of oncofertility can address these patients' needs. The global oncofertility community faces immediate challenges of access and affordability, which must be addressed in order to reduce or eliminate disparities based on finances or geo-political concerns. Future work to eliminate the off-target effects of cancer treatment will one day reduce the need for this field, which would be the best outcome for all.

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The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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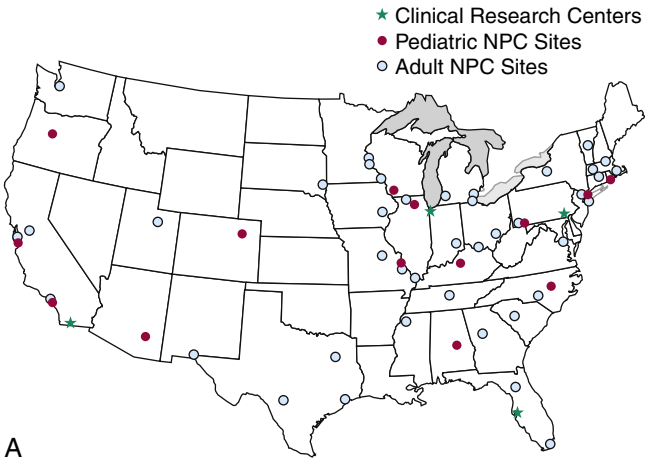
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A



B

FIGURE S33-1 Improving patient access to fertility preservation information in a rapid and efficient manner is essential to ensuring future quality-of-life for patients and their families. To facilitate this communication, the Oncofertility Consortium has created and disseminated several physician-focused tools including **A**, the National Physicians Cooperative (NPC), a nationwide network of clinical sites (research, pediatric, and adult) that are committed to providing patients with fertility preservation information and treatment; and **B**, a hotline where both providers and patients can receive the most up-to-date information and referrals regarding fertility preservation.

Table S33.1 "Cancer-Friendly" Adoption Agencies

Adoption Agency	Web Address	Domestic	International
Abrazo Adoption Associates	www.abrazo.org	•	
Adoption & Beyond	www.adoption-beyond.org	•	•
Adoption Advocates International	www.adoptionadvocates.org	•	•
Adoption Bridges of Kentucky	www.adoptionbridgesofkentucky.org	•	
Adoption Information Services	www.adoptioninfoservices.org	Referral agency	
Adoption Resources	www.adoptionresources.org	•	
Adoptions Together	www.adoptionstogether.org	•	•
Bethany Christian Services	www.bethany.org	•	•
Children's Connections, Inc. (also offers embryo donation)	www.childrensconnections.org	•	•
Family Adoption Services	www.familyadoptionservices.org	•	
Forever Families Through Adoption	www.foreverfamiliesthroughadoption.org	•	
Independent Adoption Center	www.adoptionhelp.org	•	

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CHAPTER 34

Laboratory Assessment

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Frank Z. Stanczyk
Rogerio A. Lobo

This chapter reviews the assessment of hormonal status in the practice of reproductive endocrinology. It is acknowledged that the patient will often provide useful biological information, such as changes induced by hypoestrogenism, skin changes with androgen excess, and galactorrhea in hyperprolactinemia. The clinically relevant symptoms and signs associated with various disorders are discussed in other chapters in this text. Here we will describe various types of hormonal assays and dynamic tests used in the evaluation of reproductive disorders, as well as diagnostic radiographic techniques. In this new edition, we have updated different types of measurements, particularly for steroids and have discussed in more detail aspects of metabolism. We have revised various algorithms used for diagnosis, with an emphasis on what factors need to be measured.

Immunoassays

The predominant assays used to measure steroid, peptide, and protein hormones in serum, plasma, and urine samples during the past 40 years have been immunoassays. These assays have been widely used in both clinical diagnostic and research settings.

As the term "immunoassay" implies, this method of measuring hormones involves an antigen-antibody reaction, where the antigen is the hormone to be measured and the antibody, which is prepared against the hormone, binds to the hormone. The hormone can be a steroid, protein, and peptide. There are two types of hormone immunoassay methods: one uses excess hormone and a limited amount of antibody, whereas the other uses excess antibody. For quantification purposes, immunoassay methods require a labeled marker which can be the radioactive [usually iodine-125(^{125}I)] or nonradioactive (chemiluminescent, fluorescent, or enzymatic) form of the hormone being measured, or a suitable chemical derivative of the hormone.

General Principles of Immunoassays

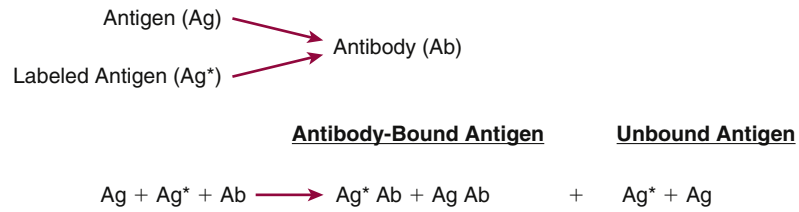
Principle of Antigen-Excess Hormone Immunoassays

The principle of an antigen-excess type of hormone immunoassay involves competition between the hormone being measured and the labeled form of the hormone for a limited amount of the antibody prepared against the hormone. When all three components are combined in a test tube, the net result is a mixture of labeled and unlabeled hormone bound to the antibody, and unbound labeled and unlabeled hormone (Fig. 34.1). From the practical standpoint in the assay, the bound hormone is separated from unbound hormone and the activity of the bound fraction is quantified (e.g., by determining the counts per minute of radioactive iodine in a gamma counter if a ^{125}I-labeled hormone is used as the marker). Separation of hormone-bound and unbound antibody is achieved by one of a variety of different methods, including use of a second antibody (prepared against the first antibody) when an iodinated hormone is used as the labeled antigen, or by magnetic particles when a nonradioactive hormone is used. In this manner, by using different concentrations of a pure hormone (standard), one can generate a standard curve of the hormone. As the concentration of the standard is increased, the antibody-bound labeled marker is displaced; the higher the amount of standard that is added, the lower the amount of marker will be obtained. Thus, in an antigen-excess immunoassay, the standard curve shows an inverse relationship between the different amounts of antibody-bound labeled antigen (hormone) and the different concentrations of the standard (Fig. 34.2). A standard curve is essential in any immunoassay method used to measure the levels of a hormone in a biological fluid.

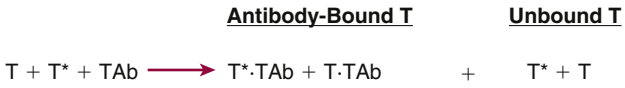
Measurement of a hormone in serum, plasma, or urine by an antigen-excess immunoassay is obtained by first determining the amount of the hormone that is antibody-bound in the sample. The concentration of the hormone is then extrapolated off the standard curve, as shown in Figure 34.2. An appropriate computer program can be used to generate the data rapidly.

Principle of Antibody-Excess Hormone Immunoassays

In contrast to antigen-excess immunoassays, which utilize a limited amount of antibody resulting in competition between labeled and unlabeled antigens, there is no competition between these antigens in antibody-excess immunoassays (often referred to as immunometric assays). Generally, two different antibodies that recognize two different parts of the antigen are used in antibody excess immunoassays, forming a "sandwich," with the antigen in the middle. Thus, this assay method is useful for large molecules such as protein and peptide hormones. Quantitation is achieved



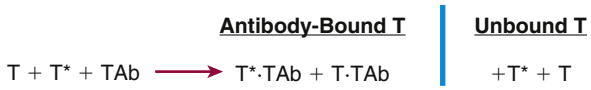
Example: Testosterone (T)



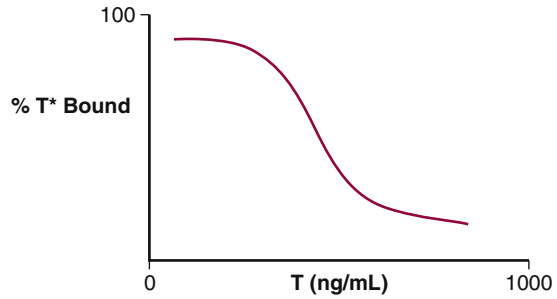
*radioactive or nonradioactive (chemiluminescent, fluorescent or enzymatic) tag

FIGURE 34.1 Measurement of hormones by antigen excess RIA: Theoretical considerations. *(Modified from Stanczyk FZ, Glob. libr.* women's med. *[ISSN:1756-2228] 2009; DOI 10.343/GLOWM.10278.)*

• Separation of Antibody-Bound Testosterone (T) from Unbound T



• Preparation of Standard Curve (Using Different Concentrations of T Standard)



• Measurement of Steroid (e.g., T) in a Sample

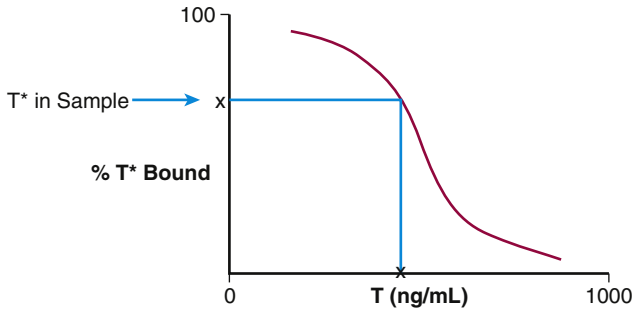


FIGURE 34.2 Measurement of hormones by antigen excess RIA: Practical considerations. *(Modified from Stanczyk FZ, Glob. libr.* women's med. *[ISSN: 1756-2228] 2009; DOI 10.343/GLOWM.10278).*

by labeling one of the antibodies with a radioactive or nonradioactive marker. One of the most widely used antibody-excess immunoassays is the enzyme-linked immunosorbent assay (ELISA) in which quantitation is achieved by attaching an enzyme to one of the antibodies and measuring the optical density of a product formed by reaction of the enzyme with a specific substrate (Fig. 34.3). When one of the antibodies is labeled with a radioactive tracer, this type of assay is referred to as an immunoradiometric assay (IRMA), and when the labeling involves a chemiluminescent or fluorescent tag, it is referred to as an immunochemiluminometric assay (ICMA) or immunofluorometric assay (IFMA), respectively. In contrast to the inverse relationship between antibody-bound labeled antigen and concentration of antigen in the standard curve in an antigen-excess immunoassay, there is a direct relationship between the labeled antibody and antigen concentration in an antibody-excess immunoassay (Fig. 34.4).

Major Components of Immunoassay Systems

There are three major components of immunoassay methods: the antibody, unlabeled antigen (standard or substance to be measured in a biological fluid sample), and labeled

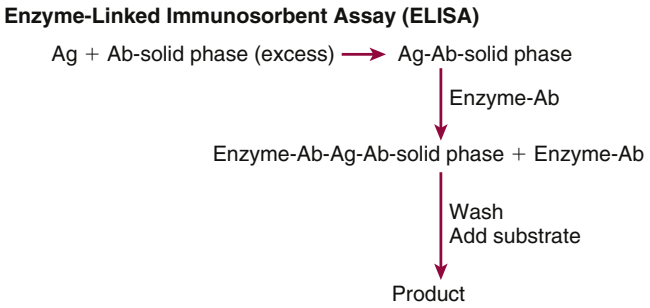


FIGURE 34.3 Principle of the antibody excess immunoassay: Theoretical consideration shown for the ELISA. *(Modified from Stanczyk FZ, Glob. libr.* women's med. *[ISSN:1756-2228] 2009; DOI 10.343/GLOWM.10278.)*

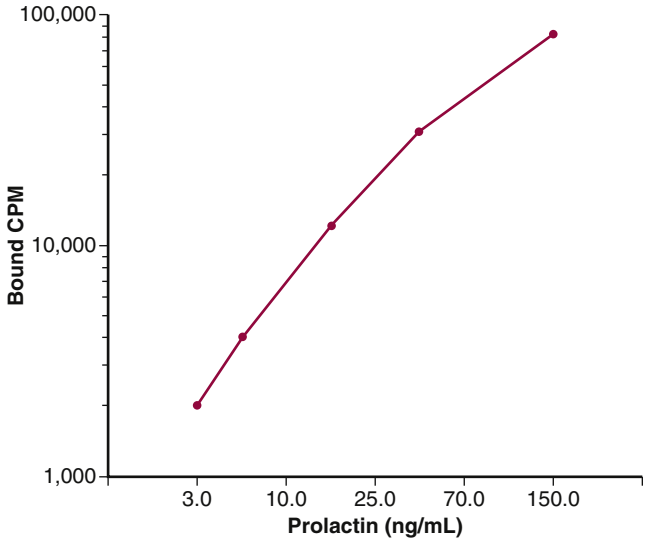


FIGURE 34.4 Typical standard curve obtained by abnormal antibody excess immunoassay; the example shown is for an IRMA. *(From Nakamura RM, Stanczyk FZ. Immunoassays. In Lobo RA, Mishell DR Jr., Paulson RJ, Shoupe D (eds).* Mishell's Textbook of Infertility, Contraception and Reproductive Endocrinology, *4th ed. Oxford, Blackwell, 1997.)*

antigen. These are essential in every immunoassay and are discussed below.

Antibody

The antibody is perhaps the most important component of an immunoassay method. If an antibody in an immunoassay recognizes only the compound that it is intended to measure, then it is likely that the assay will be highly accurate.

Antibodies are commonly produced in animals (polyclonal) or via cell culture (monoclonal). The typical protocol for production of polyclonal antibodies is to disperse a small amount of antigen into an adjuvant (e.g., Freund's) and to inject it intradermally at multiple sites into an animal such as the rabbit. After approximately 3 months, a blood (serum) sample is obtained from the animal and the antibody titer is determined. This procedure involves addition of a fixed amount of labeled antigen to serially diluted aliquots of the serum obtained from the animal. The antibody dilutions start at 1:1000 and cover a range of at least two orders of magnitude. After the bound antigen is separated from the unbound antigen, the antibody dilution that binds between 30% and 70% (usually 40%-50%) of the total activity of the labeled antigen is formed and used for the assay.

To obtain a monoclonal antibody, it is first essential to inject the antigen into a mouse to induce an immunological reaction in the spleen. Each immunized spleen cell can produce an antibody with specific characteristics. The most important step in the production of monoclonal antibodies is screening the spleen cells to separate those capable of secreting a single antibody type. When a myeloma cell from the same species of mouse is fused with the selected spleen cell, a "hypridoma" is produced. This hybridoma continuously secretes antibodies with the same characteristics as the selected spleen cell. A hybridoma cell is capable of producing hundreds of specific antibody molecules per second. Thus, a continuous clone line can be maintained in culture, becoming a source for the production of homogeneous monoclonal antibody molecules.

Antigen

Here, the term "antigen" can refer to the substance that is injected into an animal to produce an antibody or to the standard that is used to generate a standard curve in an immunoassay. In addition, the term "antigen" can refer to the substance that is being measured in a biological fluid (e.g., serum).

Antigen Used to Prepare an Antibody

In general, any molecule larger than 10,000 daltons can elicit an antibody response. With molecules in the 1000 to 10,000 dalton range, eliciting an immune response becomes more difficult. Molecules smaller than 1000 daltons are generally nonantigenic and are coupled to a large protein molecule (e.g., albumin); these small molecules are referred to as haptens. Thus, steroids and some peptides, but not proteins, are prepared as hapten-proteins and injected into the animal to produce a polyclonal or monoclonal antibody.

The site of attachment of the protein carrier (e.g., albumin) is important because it determines the specificity of the antibody. In general, specific antibodies are formed when the attachment site does not involve any functional group on the steroid molecule. A commonly used approach is to prepare a carboxyl derivative at the carbon-6 position of the steroid molecule and to couple it to an amino group of the protein carrier.

Antigen Used as Standard

Steroids and peptides are available commercially and in most instances can now be obtained in a relatively pure state. However, it should never be assumed that these products are 100% pure when developing an immunoassay. If necessary, the compounds can be purified (e.g., by use of high performance-liquid chromatography [HPLC]). In contrast, proteins are generally considered to be more difficult to purify.

Labeled Antigen

Use of an optimum labeled antigen is also important in an immunoassay, as this determines how the compound being measured in a biological fluid, such as serum, is quantified in the assay. The labeled antigen must not only be identifiable by some physical or chemical method (e.g., a radioactivity counter or spectrophotometer) but also must bind to the antibody.

The classical labeled antigens for immunoassays have been radioactive antigens. For example, insulin, the first hormone measured for an RIA, was labeled with radioactive iodine (^{131}I). This isotope had a very short half-life (8 days) and was soon replaced with iodine-125 (^{131}I), which has a half-life of 60 days. Peptides have also been labeled with ^{125}I. For steroids, tritium (^{3}H) was the initial isotope used in RIAs, but was subsequently replaced with ^{125}I. It's important to realize that, unlike proteins and peptides, the steroid molecule itself cannot be iodinated. Instead, the steroid molecule is attached chemically to an iodinated carrier molecule such as histamine or tyrosine methyl ester.

In order to eliminate the high cost of storage and disposal of radioactive material and to allow immunoassays to be carried out on an automated platform, nonradioactive labeled antigens began to be used in these assays. The predominant labels that were attached to antigens were chemiluminescent, fluorescent, and enzyme (which required a substrate); these labels were detected by use of a luminometer, fluorometer, and spectrophotometer, respectively. Presently, there is wide use of the chemiluminescent label in immunoassays on automated platforms and the enzyme label in ELISAs carried out manually.

Automated Immunoassays

Over the past 30 years or so, highly sophisticated automated immunoassay systems have been developed. These systems consist of instruments that not only detect spectral properties of nonradioactive ligands (e.g., chemiluminescence) but also are able to analyze multiple analytes as well as to process multiple samples rapidly and efficiently. These immunoassay systems are capable of utilizing both antigen excess and antibody-excess methodology.

Currently a widely used immunoassay system is the Immulite analyzer (Siemens Healthcare Diagnostics, Deerfield, Illinois). The Immulite system employs enzyme-amplified chemiluminescent technology. The mechanism involves hydrolysis of a stable chemiluminescent substrate, through the action of the enzyme alkaline phosphatase, resulting in an unstable anion that gives rise to sustained emission of light. The emitted light is quantified by use of a luminometer in the instrument.

As an example, the Immulite LH assay utilizes a solid-phase, two-site ICMA. The solid phase 3 consists of a polystyrene bead coated with a monoclonal antibody against LH. The bead is sealed into an Immulite test unit, to which LH standard or serum sample, together with a polyclonal antibody conjugated to alkaline phosphatase, are added simultaneously. During an incubation period, LH is bound to the monoclonal antibody coating the bead and the polyclonal antibody-enzyme conjugate, forming a "sandwich complex." After unbound conjugated antibody is removed by a wash, the amount of complex, which is directly proportional to the LH standard or LH in the sample, is quantified by use of the chemiluminescent substrate described above.

Steroid Hormone Immunoassays

The first RIA was developed in 1969 by Guy Abraham[1] and that was for measurement of estradiol (E_2) in serum or plasma. The E_2 RIA method involved separation of E_2 from interfering metabolites by organic solvent extraction and Celite or Sephadex column chromatography, prior to its quantitation by RIA. The purification steps were added to remove most of the estrogen metabolites. E_2 is readily converted to estrone (E_1) and both E_2 and E_1 are converted to a total of approximately 90 metabolites. The extraction step removes the conjugated steroids, whereas the chromatographic step separates E_2 from potential interfering unconjugated metabolites. The E_2 RIA included a specific antiserum against RIA in conjunction with tritiated E_2, and separation of the antibody-bound and unbound E_2 fractions using charcoal. This method, which is often referred to as a conventional RIA, was shown to be sensitive, specific, precise, and accurate. Soon afterwards it was applied successfully to other sex steroid hormones, such as testosterone and progesterone.

During the decade of the 1970s, RIAs were developed for a variety of natural and synthetic steroid hormones. A notable change in the RIA method during those years was replacement of tritium with iodine in the labeled antigen to improve assay sensitivity. Because of their relatively low cost, RIAs became widely used in diagnostic and research laboratories.

Impact of the Conventional Steroid RIA Method

The immediate impact of the conventional steroid RIA method was that it allowed measurement of an immensely wide range of compounds of clinical and biological importance, and it opened new horizons in endocrinology. The long-term impact of the steroid RIA method was that its use in numerous studies enriched the field of endocrinology with new knowledge, and its use in diagnostic testing provided physicians with valuable information for diagnosing and treating countless patients. The steroid RIA methodology also allowed substantial research into the physiological and pathophysiological roles of steroid hormones in applications such as sexual differentiation, puberty, neuroendocrinology, the menstrual cycle, pregnancy, menopause, and male endocrinology. In addition, the RIA method opened the door for epidemiological studies that permitted us to better understand the role of steroid hormones in the etiology of numerous diseases, notably the hormone-dependent breast and prostate cancers.

Advantages and Disadvantages of Conventional Steroid Radioimmunoassays

Steroid RIA methods with purification steps have the following advantages: First, steroid-binding proteins (e.g., SHBG) are denatured, thereby releasing the steroids (e.g., E_2 and testosterone) that they bind. Second, the purification steps remove numerous potentially interfering metabolites prior to RIA. Third, the RIAs are accurate and reliable when properly validated. Finally, multiple steroids (usually up to five) can be measured in a single aliquot of serum.

Conventional steroid RIAs also have disadvantages. They are cumbersome, time-consuming, costly, and require relatively large sample volumes, especially when the steroid is present in low concentrations. Also, although multiple steroids can be measured in a single aliquot of serum, the measurements have to be done very carefully and are especially time-consuming. In addition, as with all antibody-based assays, since the measurement of the analyte is a

surrogate approach (i.e., radioactivity is measured rather than the actual analyte itself), there is always the possibility of antibody cross-reactivity giving an erroneous result. Furthermore, the presence of auto-antibodies within patients can further affect an assay, leading to falsely high or low values depending on the type of antibody interaction that occurs. Despite these concerns, a well-validated RIA preceded by organic solvent extraction and chromatography is typically an accurate and precise assay for the majority of applications.

Direct Steroid Immunoassays and Their Advantages and Disadvantages

Due to the time-consuming limitations of conventional RIAs, in the late 1970s the radioligands in RIAs were replaced with nonradioactive ligands (chemiluminescent, fluorescent, or enzymatic) and the organic solvent extraction and chromatography steps used prior to RIA were eliminated, allowing direct immunoassays to be performed on an automated platform; this resulted in rapid measurements of steroid hormones. Conventional RIAs continued to be used, but their use was overwhelmingly surpassed by direct immunoassays, particularly in clinical diagnostic laboratories.

The development of automated platforms gave direct assays, such as chemiluminescent immunoassays, the advantages of being convenient, simple, rapid, and relatively inexpensive, and requiring a lower sample volume (usually 0.1 mL). However, these assays also have serious disadvantages. They often overestimate the measurements due to lack of specificity of the antibody, especially in samples obtained from women treated with exogenous steroid hormones.[2] Also, matrix differences may exist between serum samples (particularly hemolyzed and lipemic samples) and solutions of the standard used to prepare the standard curve in the assay. In addition, steroids such as testosterone and E_2 may not be released efficiently from proteins such as sex hormone-binding globulin to which they bind with high affinity in blood. Furthermore, direct immunoassays generally lack the sensitivity to measure accurately low levels of certain steroid hormones such as E_2 and testosterone. Limitations of direct immunoassays for quantifying E_2 in postmenopausal women and testosterone in both premenopausal and postmenopausal women are now well documented in the literature.[3]

Protein and Peptide Hormone Immunoassays

The first protein hormone RIA was developed for insulin and preceded the first steroid hormone RIA, which was for E_2, by about 10 years. The basis for the first protein RIA method was described in 1959 by Yalow and Berson, who showed that ^{131}I-insulin could be displaced by nonradioactive insulin from insulin-binding protein, and that ^{131}I-insulin was inversely and quantitatively related to the total amount of insulin present. This is the same principle that was described earlier for the steroid hormone RIA, and was subsequently used to develop RIAs for protein hormones other than insulin, as well as for peptide hormones. In 1967, Odell and co-workers reported the first RIAs for luteinizing hormone (LH) and follicle stimulating hormone (FSH). Subsequently, RIAs were developed for a variety of other protein hormones, such as human chorionic gonadotropin (hCG), prolactin and thyroid-stimulating, and for peptide hormones such as gonatropin releasing hormone (GnRH) and adrenocorticotropic hormone (ACTH). The protein and peptide hormone RIAs utilized specific antisera in conjunction with the relevant ^{125}I-proteins and peptides. The change from the ^{131}I label to the ^{125}I label provided a longer-life for the labeled material (8 days versus 60 days, respectively), so that the proteins and peptides did not require iodination as often for the RIAs.

Immunoradiometric Assay (IRMA)

In the late 1960s and early 1970s, the IRMA method was developed for measurement of proteins, which included insulin, FSH, and LH. Subsequently, the IRMA was also used to measure peptides such as GnRH and ACTH. The IRMA differed from the RIA in that the compound to be measured is assayed directly by combination with a specific labeled antibody rather than in competition with a labeled antigen for a limited amount of antibody.

Current two-site immunometric assays depend on the use of two antibodies directed to distinct antigenic epitopes on the protein or peptide being measured. This assay method is ideal for use of monoclonal antibodies, thereby increasing assay specificity. In addition, two-site immunometric assays offer greater sensitivity because they are much less dependent on the affinity of the antibody than RIAs. An appropriate choice of antibodies directed to different antigenic sites can further enhance the affinity and thereby the sensitivity of the assay. Thus, highly sensitive immunometric assays opened up new clinical opportunities (e.g., use of the sensitive TSH assay to diagnose hypothyroidism).

Two-site immunometric assays also have certain limitations. They cannot be used to measure small ligand molecules such as steroids and very small peptides since they display only one antigenic site at a time. Also, immunometric assays are liable to high-dose "hook" effects. This results from very high levels of antigen that saturate the binding sites on the capture antibody and still leave antigen free to bind to the tracer antibody.

Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA differs from the IRMA in that the ELISA utilizes a solid-phase procedure and an enzyme-labeled instead of a radioactively-labeled antibody. The most common format used for the ELISA is the multi-well plate made of polystyrene or polyvinyl. This is convenient to handle since centrifugation is not required and multiple wash steps are readily automated. Antibody is coated on the wall of each well and this antibody binds the corresponding antigen in the test sample. The antigen-antibody complex is quantified by addition of an enzyme-labeled specific antibody. Following addition of the appropriate substrate, the end-point (product) can be "read" spectrophotometrically, using an automated reader.

Standards Used in Protein Immunoassays

Unlike steroids and small peptides (e.g., GnRH), proteins are considerably more difficult to purify. Although some proteins such as insulin are available in a highly purified state for use as standards in immunoassays, others such as

FSH, LH, and hCG have not yet been prepared in sufficient amounts in a highly purified form. Although the latter proteins are available from international agencies, their purity based on biological potency per unit weight, is often less than that of highly purified preparations reported by individual investigators. In order to be able to compare results obtained in different laboratories and at different times in the same laboratory, considerable effort has been made to use a single material as a standard.

Reference materials are available for FSH, LH, and hCAG. They are provided by the World Health Organization (WHO), which obtains them from the National Institute for Biological Standards and Controls in Hertfordshire, England. Two types of reference materials are available, namely, an International Standard (IS) and an International Reference Preparation (IRP). The IS is a material that has a potency established in 10 to 20 expert laboratories throughout the world, whereas the potency of an IRP is established by only several laboratories.

With the advent of RIAs for gonadotropins, a partially purified extract of human pituitary glands (code name LER-907) was made available by the National Institute of Health in the United States. This preparation was provided in large amounts to the WHO, which purified it further in 1976 and called it the First International Reference Preparation of Pituitary FSH and LH (1st IRP-FSH and LH). Subsequently, another partially purified extract of LER-907 was prepared in 1980 to replace the 1st IRP-FSH and LH. It was called the 2nd International Reference Preparation of Pituitary FSH and LH (2nd IRP-FSH and LH) and was assigned the code number 78/549. Highly purified preparations of pituitary FSH and LH have also been prepared. The First International Reference Preparation for Human Pituitary LH (1st IRP-LH, code number 68/40) was available in 1974. Subsequently (in 1988), this material was replaced by the Second International Standard for Pituitary LH (2nd IS-LH; code number 80/552). In 1986, the First International Standard for Pituitary FSH (1st IS-FSH, code number 83/575) was established. Presently, both the 2nd IRP-FSH and LH and 2nd IS-LH are used as standards in gonadotropin immunoassays.

The initial standard used in hCG immunoassays was a partially purified hCG preparation obtained from urine of women in their first trimester of pregnancy and was called the Second International Standard for hCG. Subsequently, a highly purified preparation was developed and was referred to as the Third International Standard. This preparation is presently used as the standard in hCG immunoassays.

Validation of Immunoassays

Before an immunoassay can be used to measure any compound, it must first be validated with respect to sensitivity, specificity, accuracy, and precision. Procedures used to validate immunoassays are described below.

Sensitivity

The sensitivity of an assay is defined as the smallest amount of the substance being measured that can be distinguished from zero. In practice, assay sensitivity depends on the precision of the standard curve, which can be measured by assaying 10 replicates of each concentration of standard and the "zero" standard, which contains no standard. This allows calculation of the mean ± standard deviation (SD) amount of the antibody-bound marker, corresponding to each concentration of standard. The sensitivity of an assay is the lowest standard concentration that yields a mean amount (e.g., counts per minute) of antibody-bound marker differing by two SDs from the mean amount of antibody-bound marker associated with the zero standard.

Assay sensitivity expressed in that manner, in practical terms, refers to the sensitivity of the standard curve. However, it is important to know the assay sensitivity in terms of the lowest amount of analyte that can be measured per milliliter of sample. This can be determined by calculating the product of the lowest standard concentration (based on standard curve sensitivity) and, if applicable, dilution factors, as well as the factor accounting for procedural loss (in assays using an extraction and/or chromatographic step).

The sensitivity of an assay is especially important when very low serum levels of a compound are being analyzed (e.g., measurement E_2 in samples from postmenopausal women or from patients treated with an aromatase inhibitor).

Specificity

Assay specificity refers to the degree of interference from cross-reaction that is encountered from substances other than the target analyte. The specificity of an immunoassay is usually assessed in two different ways. First, the cross-reactivity (expressed as a percentage) of the antibody is determined by comparing the dose-response standard curve of the substance being measured with dose-response curves obtained for compounds that may be present in the same sample as the analyte, and that may bind to the antibody. The percent cross-reaction of an antiserum with a substance is calculated from the mass of standard that yields 50% inhibition of binding of the assay marker to the antibody, divided by the mass of the cross-reacting substance that gives the same percentage of inhibition (50%), and multiplied by 100%.

A second approach for defining immunoassay specificity is to compare the analyte values of a group of samples measured by a specific immunoassay method with those obtained by a classic method, such as mass spectrometry. If this is not possible and the analytes are being measured in an immunoassay without chromatography, the analyte values should be compared with the values obtained by an immunoassay that uses a chromatographic step (e.g., Celite column partition chromatography) to separate the analyte in question from interfering metabolites.

Accuracy

Assay accuracy defines the extent to which a given measurement agrees with the actual value. One commonly used method to establish the accuracy of an immunoassay is based on the finding of linearity (parallelism) between the assay standard curve and serial dilutions (using assay buffer) of several samples containing known high concentrations of the analyte. Another method for establishing immunoassay accuracy is based on the recovery of added ("spiked") standard, at different levels, from patient samples. This method is often used for assays that require an extraction or a chromatographic step (e.g., steroid hormone assays). Both the parallelism and the "spiked" sample methods are limited by the precision of carrying out the dilutions or "spiking."

Precision

The precision of an assay refers to the variability that exists when multiple measurements of the compound are made on the same sample. In practice, both intraassay precision and interassay precision are determined and are usually expressed as the coefficient of variation of replicate measures. The coefficient of variation (expressed as a percentage) is calculated by dividing the standard deviation by the mean of replicate determinations of an analyte, and then multiplying by 100. Intraassay precision is assessed by measuring the analyte in replicate samples (usually 7 to 10) within the same assay. Interassay precision is determined by measuring the analyte in replicate samples (at least five), each of which is included in a different assay. Both intraassay and interassay precision should be determined in three different concentrations of the analyte (high, midrange, and low).

Interferences in Immunoassays

Circulating human antibodies that react with animal proteins (anti-animal antibodies) are often an unrecognized and unsuspected source of interference in immunoassays. This is particularly true with two-site immunoassays. The most common human anti-animal antibody interference is caused by human antimouse antibodies (HAMA). HAMA can cause either positive or negative interference in two-site mouse monoclonal antibody-based immunoassays. Strategies for preventing the development of anti-animal antibodies have been proposed. Awareness of laboratory personnel and clinicians of the problems associated with this type of interference in immunoassay methods is important.

Mass Spectrometry Assays

Measurements of steroid hormones by mass spectrometry (MS) assays actually preceded their measurements by RIA. As early as 1966, the first comprehensive urinary steroid profile was produced by gas chromatography-mass spectrometry (GC-MS). This method combines the resolving power of gas chromatography with the high sensitivity and specificity of the mass spectrometer. Separation of steroids by gas chromatography requires that they be first derivatized to increase their volatility, selectivity, and detectability. The mass spectrometer functions as a unique detector that provides structural information on individual solutes as they elute from the gas chromatography column. The MS technique first involves ionization of the compound being measured at the ionization source, and is followed by separation and detection of the ions in the mass analyzer. A mass spectrum is produced in which the relative abundance of a particular ion is plotted as a function of the mass-to-charge (m/z) ratio, and the concentration of the compound is then obtained.

Due to the complexity of the methodology and associated costs of the instrumentation and reagents, as well as the need for a highly trained individual to carry out the assays, use of GC-MS assays was restricted to a limited number of laboratories, primarily at pharmaceutical companies. Thus, for a period of about 30 years after the development of the first RIA in 1969, conventional RIAs and direct immunoassays were the predominant methods used to quantify steroid hormones in clinical diagnostic and research laboratories due to their relative ease of performance and considerably lower overall costs. However, advances in liquid chromatography technology in the 1980s led to the development of a high performance liquid chromatography-MS (LC-MS) instrument in 1987. In addition, invention of an electrospray source by Nobel laureate, John B. Fenn, in 1990, and the subsequent development of chemical ionization greatly improved routine analysis of steroids. This technology facilitates ionization of compounds present in liquid droplets and sprays the molecules directly into the mass spectrometer from the HPLC unit. The advancements allowed for simple coupling of the liquid chromatography eluent with the mass spectrometer and often negated the need for derivatizing the steroid, thereby reducing the complexity of the assay and shortening the assay run time dramatically. These factors greatly increased the throughput of patient samples, while still providing highly accurate and precise results.

In recent years, there has been a large increase in the use of assays that utilize either liquid chromatography or gas chromatography with tandem MS (LC-MS and MS or GC-MS and MS). Tandem MS consists of two mass spectrometers in series connected by a chamber (collision cell). After chromatography, the sample is processed in the first mass spectrometer to obtain the precursor ion which is then fragmented in the collision cell into product ions. The mass of the product ions is then determined in the detector of the second mass spectrometer. This method has high specificity, sensitivity and throughput.

During the past decade there has been a substantial increase in the use of LC-MS and MS assays, particularly in major diagnostic clinical laboratories, and to a lesser extent in some research laboratories. However, there are still situations where a GC-MS assay provides higher chromatographic resolution and even sensitivity than LC-MS. A particular strength of GC-MS and GC-MS and MS assays is their high applicability to measurement of large numbers of structurally similar compounds. They remain the most powerful assay method for defining defects in steroid hormone metabolism.

Because of the high validity and throughput of steroid hormone MS assays, there is a rapidly growing use of this methodology in both clinical and research laboratories. In larger reference laboratories, these assays have replaced the conventional RIAs, which are cumbersome and time-consuming, and direct immunoassays, which lack specificity and/or sensitivity. The MS technology has been implemented successfully for routine analysis of steroid hormones in major clinical diagnostic laboratories. Although the high-cost of MS instrumentation, related operating costs, and requirement for high technical expertise have prohibited smaller laboratories from using this instrumentation for high-throughput routine testing of steroid hormones, this situation is changing and MS assays are becoming much more widely used.

In addition to use of the MS methodology for routine analysis of steroid hormones, this methodology is now sufficiently rapid and robust for measuring these hormones in large epidemiological studies with high specificity and sensitivity. An important advantage of MS assays is their capability of measuring multiple steroids in a single aliquot of serum or urine. In contrast, generally only up to 5 different steroids

can be measured in a single serum aliquot (usually 1 mL) by conventional RIA. Several years ago, at the National Cancer Institute, MS assays were developed for quantifying as many as 15 different estrogens in only 0.5 of serum or urine.[4] Measurement of metabolite profiles of a steroid hormone has the potential to provide highly valuable information in diagnosing patients and in a variety of studies, particularly epidemiological studies. Quantitative analysis of metabolite profiles of small molecular weight molecules such as steroid hormones is referred to as metabolomics and is a rapidly growing field of research.

Standardization of Steroid Hormone Assays

Standardization in measuring steroid hormones is desirable so that test results are accurate and reliable, independent of the assay used and the time and place of measurement. Although there seems to be general agreement that MS assays will become the "gold standard" for steroid hormone measurements, there are many challenges to overcome before this occurs.[5] It is important to realize that the MS technology faces variability issues similar to those of conventional RIAs and direct immunoassays that need to be addressed. Differences in accuracy among MS assay methods appears to be attributable to variation in calibration of assay standards, whereas differences in assay precision may be explained, at least in part, by variations in sample preparation.

The Center for Disease Control and Prevention (CDC), with broad input from various professional societies, particularly the Endocrine Society, is leading a Hormone Standardization Program with an initial focus on standardizing testosterone measurements.[5] The conceptual approach of this program is built on experiences gained from successful standardization programs that CDC maintains or has supported (e.g., the cholesterol standardization program and national glycohemoglobin standardization program). The testosterone standardization program consists of three basic steps: calibrating individual assays, developing a reference system, and verifying end-user test performance. Although it is evident from previous efforts at the CDC that vast improvements in measurement performance can be achieved through assay standardization, based on their experience it could take years to accomplish such achievements.

Reference Intervals of Steroid, Protein, and Peptide Hormones

An important requirement in clinical diagnostic testing in which measurements of steroid, peptide, or protein hormones are determined by conventional and direct immunoassays or MS assays is reference intervals. These intervals should be derived from well-characterized, adequate-sized populations using standardized procedures such as those formulated by the Clinical and Laboratory Standards Institution. Frequently, only limited information is available about subjects used to establish reference intervals. Also, an often neglected aspect in establishing reference intervals for hormone measurements is biologically influencing factors that may affect these measurements. The major factors include gender, age, body mass index, pubertal stage, menopausal status, phase of menstrual cycle, pregnancy, and diurnal rhythm.

It is especially important to compare reference intervals for steroid hormones measured by MS assays to those obtained by conventional RIAs, since most of our knowledge about the role of steroid hormones in normal women and men, as well as in various endocrine diseases, is based on the data obtained by the latter methodology. Presently it appears that the reference intervals obtained by the two methods do not differ significantly.

Measurement of Specific Reproductive Protein and Peptide Hormones

Gonadotropins

Classic RIAs of gonadotropins have low specificity and sensitivity and often cannot distinguish low levels from low-normal values. This difficulty is compounded by the pulsatile nature of gonadotropin secretion. Thus, with RIAs it is not possible to detect the low levels of gonadotropins that would occur in cases of hypothalamic or pituitary failure or prior to puberty. Immunometric assays are more sensitive and specific and have improved the measurement of gonadotropins.[6] Therefore, IRMAs or ICMAs should be used in the evaluation of patients suspected of having hypogonadotropic hypogonadism and in pediatric patients. In contrast, the ELISA for gonadotropins is considered less sensitive and exhibits higher nonspecific binding.

A problem that may arise in measurements of LH includes significant cross-reactivity with hCG and its pulsatile secretion pattern (pulses approximately every 60 to 90 minutes). The requirement of precision in values would necessitate that samples, taken 15 to 20 minutes apart, be pooled for analysis. In the clinical setting, however, this is not necessary because low levels usually remain low and high values are not usually within the normal range. The concern about pulsatility is more for research purposes and is far less pronounced for FSH samples, where pulses are less frequent, occurring approximately every 3 hours.

Apart from alterations in FSH and LH associated with various disorders and physiological states, FSH levels are increased by levodopa and ketoconazole and decreased by the administration of estrogens and phenothiazines. LH is increased by ketoconazole and decreased by administration of sex steroids, phenothiazines, digoxin, and propranolol.

Blood Levels of LH and FSH in Women and Children

In most clinical diagnostic laboratories, serum FSH and LH measurements are determined by ICMAs. In our laboratory, the FSH reference ranges for the follicular, midcycle, and luteal phases are 2.8 to 14.4 mIU/mL, 5.8 to 21 mIU/mL, and 1.2 to 9.0 mIU/mL, respectively; the corresponding ranges for LH are 1.1 to 11.6 mIU/mL, 17 to 77 mIU/mL, and 1 to 14.7 mIU/mL. For postmenopausal women, the FSH and LH reference ranges are 21.7 to 153 mIU/mL and 11.3 to 39.8 mIU/mL, respectively.

During midcycle, serum LH levels increase fourfold to sixfold, whereas serum FSH levels increase twofold to threefold. The midcycle increase of gonadotropins lasts approximately 2 days.

During the luteal phase, serum LH and FSH levels are similar to or slightly lower than their respective levels during the follicular phase. Therefore, in healthy women, the LH to FSH ratio is approximately 1.0 during the follicular phase, but increases during ovulation.

Before puberty, gonadotropin levels are lower than in adult women, and the LH to FSH ratio is less than 1 because of the relative predominance of FSH secretion. At this age, RIAs have not been helpful in distinguishing patients with low gonadotropin secretion from healthy children; therefore, the third-generation LH and FSH IRMA or ICMA is used. These assays are also useful for measuring changes in LH and FSH during the pubertal progression. From Tanner stages P1 to P5, LH concentrations using the IRMA increase more than thirtyfold, but increase only twofold to fourfold when measured by RIA. Therefore, the third-generation immunometric assays are the preferred techniques when measuring LH in serum samples from prepubertal patients, patients with suspected precocious puberty, and those with suspected hypogonadotropic hypogonadism. It has been reported that, using a third-generation fluorometric immunoassay, values of LH higher than 0.6 mUI/mL are sufficient to suggest the diagnosis of gonadotropin-dependent precocious puberty and may permit this diagnosis to be made in two thirds of such patients.

During the perimenopausal period, serum FSH levels increase substantially, whereas LH levels remain within the normal range. After menopause, levels of both gonadotropins are high, but serum FSH levels are higher than LH levels. In older women, LH levels progressively decrease, approaching premenopausal levels.

High values of LH, with increased LH/FSH ratios, are found in 50% of women with polycystic ovary syndrome (PCOS). In the past, some clinicians based the diagnosis of PCOS on the finding of elevated LH levels and LH to FSH ratios higher than 2 or 3. Although an increased LH level or LH to FSH ratio may be useful for confirming the diagnosis of PCOS, many patients with PCOS have normal serum LH levels and LH to FSH ratios.

High levels of serum LH and FSH are typical of ovarian failure and may be found in patients with gonadal dysgenesis. Rarely, pituitary tumors may produce FSH or LH. Measurement of α-subunits and determination of the gonadotropin responses to thyrotropin-releasing hormone (TRH) may be useful for the diagnosis of gonadotropin adenomas.

Dynamic Tests for Gonadotropin Evaluation

Gonadotropin-Releasing Hormone Test

Synthetic gonadotropin-releasing hormone (GnRH), the hypothalamic decapeptide, has been used to stimulate LH and FSH secretion to identify abnormalities that cannot be diagnosed with baseline determinations. This test is primarily used to differentiate between hypothalamic and pituitary causes of gonadotropin deficiency and to uncover a maturational delay in secretion at the time of puberty. Otherwise, the test largely reflects baseline measurements. For example, in patients with PCOS and women with ovarian failure, GnRH stimulation results in exaggerated increases in LH in the setting of PCOS and in FSH elevations in women with ovarian failure.

When differentiating hypothalamic from pituitary defects in gonadotropin secretion, the test requires initial GnRH priming. The priming may be accomplished by daily intramuscular administration of 100 μg GnRH for 1 week before testing. With this approach, an absent or low response distinguishes the more common patient with a hypothalamic problem from a rare patient with a congenital or acquired pituitary defect of gonadotropin secretion.

The GnRH test is also used to probe the maturational state of the hypothalamic-pituitary-gonadal axis. Pubertal LH and FSH responses are observed in patients with gonadotropin-dependent precocious puberty (GDPP) and this test is needed when no third-generation assays are available.[7] The test may be performed in many ways, but requires at least two baseline samples obtained 15 minutes apart before GnRH administration. Intravenous doses of 25 or 100 μg are then administered, and blood samples are obtained at 20, 30, 60, 90, and 120 minutes. For practical purposes, samples obtained at 30 minutes and 60 minutes are sufficient for clinical interpretation. Using conventional RIA methods, after administration of 100 μg GnRH, an LH peak of more than 15 mIU/mL in girls and greater than 25 mIU/mL in boys is suggestive of GDPP. Using the CMA LH assay, after the same dose of GnRH, the cutoff value is greater than 8.0 mIU/mL. Similar cutoff values are observed when using LH IFMA (greater than 6.9 mIU/mL in girls and greater than 9.6 mIU/mL in boys).[8] Another approach is to use LH responses 2 hours after a long-acting GnRH analog is administered. LH values of greater than 10 mIU/mL (by IFMA) suggest GDPP.[9]

The only validated method to assess hypothalamic GnRH release is by measuring the spontaneous pulsatility of gonadotropins, which is generally reserved for research purposes. Blood samples are obtained at 10- to 15-minute intervals for 6 to 24 hours, depending on whether diurnal changes are being assessed.

Clomiphene Citrate Test

In the past, the ability of the hypothalamic-pituitary axis to respond to negative feedback of estrogen was assessed with clomiphene citrate (Clomiphene). Clomiphene (50 to 100 mg daily) is given orally for 5 days, and LH and FSH levels are measured before and at the end of the test. A normal response is an LH response of greater than 50 mIU/mL. This test lacks sensitivity and specificity and should be abandoned for this purpose.

The clomiphene citrate test is most frequently used in the assessment of ovarian reserve. In the past the clomiphene test was considered to be more valuable than a single measurement of FSH and estradiol on day 3 of the cycle. Clomiphene (100 mg) is administered on days 5 through 9 of the cycle, and the FSH level is measured initially on day 3 and again on day 10. Any value (day 3 or 10) above the threshold for the laboratory is considered a positive test result. Generally, an abnormal FSH level in this setting is a value greater than 10 to 12 mIU/mL; however, it is important that each laboratory define its own abnormal cutoff point because of the considerable variability among the different FSH assays. Some recent data, however, suggest that this test may not be more valuable than baseline FSH assessments.[10]

Human Chorionic Gonadotropin

Many tissues, including the pituitary, produce hCG at very low levels, whereas the placenta produces it in large quantities. Most assays are standardized to measure only the large quantities produced by the placenta or by hCG-secreting tumors. It should be considered that hCG, in effect, refers to four different structural moieties: hCG, which most people refer to, is produced by the villous syncytiotrophoblast; hyperglycosylated hCG is produced by the cytotrophoblast and has more of an aggressive role in implantation and trophoblastic disease; free β-subunit which is often produced in malignancies; and pituitary hCG which is secreted similarly to LH in a pulsatile fashion from the pituitary at low levels, noted most commonly in postmenopausal women.[11] Cross-reactivity between these moieties occurs to various degrees in different clinical assays for hCG, warranting more specific assays when clinically needed. For example the hyperglycosylated form of hCG which usually denotes a more successful pregnancy and implantation early in pregnancy may be reflected in a "routine" hCG clinical assay to varying degress, from 50% to 90%. The currently used Immulite system reflects hyperglycosylated hCG quite well, ~ 90%, while other immunoassays may not.

During pregnancy, hCG levels increase rapidly, doubling approximately every 2 days until 12 weeks. After the third month of pregnancy, hCG levels decrease and plateau at a constant level until term. Measurements of hCG are used widely to confirm pregnancy and monitor trophoblastic function in complications of pregnancy, such as spontaneous abortion and ectopic pregnancy. Measurements of β-hCG during pregnancy, when combined with other tests (nuchal translucency, pregnancy-associated plasma protein A, inhibin A, or estriol), may be useful for screening for trisomies, such as Down's syndrome.

High quantities of hCG are produced in trophoblastic disease and by nongestational trophoblastic tumors and in men by germ cell tumors. In general, all of these tumors may produce the intact hormone, but most produce only β-hCG. Patients with elevated β-hCG levels should be evaluated extensively to exclude an underlying malignant process. Measurement of β-hCG levels by sensitive assays is also useful for monitoring for a recurrence after tumor extirpation and may aid in the diagnosis of the tumor and reflect disease prognosis.

A special issue exists with measurement of low levels of βhCG in normal women. When low levels are found (usually in the range of 5 to 20 mIU/mL), possibilities include pregnancy, an hCG-producing tumor (including choriocarcinoma), normal pituitary secretion (usually in a postmenopausal woman), and interference in the assay (often encountered with "sandwich"-type assays). The first approach is to repeat the value with a different assay system, and to measure hCG in urine. More specific assays such as that of β-subunit may be used and if this explains the source, a malignancy should be sought. By the process of elimination, low levels of hCG which are not explained by differences in assays most likely reflect pituitary secretion, and the proof of this occurrence is by the use of pituitary suppression, as with oral contraceptive pills, which will suppress the hCG levels.

Prolactin

Serum prolactin (PRL) is generally measured by ICMA. The upper limit of the normal range is generally reported as 15 to 20 ng/mL in men and 20 to 25 ng/mL in women; although the true normal levels are usually lower (up to 18 ng/mL in women). Serum PRL values are influenced by estrogens, drugs (e.g., phenothiazines, metoclopramide), stress, food consumption, breast stimulation, and even venipuncture. Because of diurnal changes and transient increases after meals, samples should routinely be obtained at midmorning.

The presence in blood of macroprolactin (monomeric PRL + PRL autoantibody) or dimeric forms of PRL (so-called big-PRL forms) may lead to elevated PRL levels that are not associated with biological activity. In particular, macroprolactinemia may be an important clinical problem resulting in up to 10% of cases of misdiagnosed hyperprolactinemia. When macroprolactinemia is suspected (e.g., in patients with autoimmune diseases), polyethylene glycol precipitation should be carried out before the immunoassay.[12] Recently, new commercial methods for the measurement of PRL that involve ultrafiltration have been introduced and may be useful to detect macroprolactin or big prolactin in clinical practice.[13] In normal women, immunoreactive blood prolactin results mostly from monomeric prolactin (≥95%). Dimeric prolactin forms (so called big-prolactin) and macroprolactin (a prolactin-antibody complex of molecular weight greater than 100 kDa) are generally present in low concentrations (less than 1%). However, macroprolactinemia is present in more than 15% of subjects having increased immunoreactive prolactin and in some patients hyperprolactinemia is the result of elevated macroprolactin levels, misleading the medical approach.

The gold standard method for detecting macroprolactin is gel filtration chromatography, a procedure that allows for quantification of all three variants of PRL. However, this method is labor intensive and most clinical laboratories prefer using precipitation with polyethylene glycol (PEG). A low PRL recovery after PEG treatment indicates the presence of macroprolactin.

While it has been suggested to screen for macroprolactin in all hyperprolactinemic patients, PRL blood levels greater 85 ng/mL are not generally explained by macroprolactinemia and the Pituitary Society has published guidelines indicating that values of blood prolactin ≥150 ng/mL are always suggestive of a pituitary prolactinoma.[14] Therefore, it is reasonable to screen for macroprolactin only in patients having PRL values between 25 and 150 ng/mL. PRL recovery ≤30% following PEG precipitation indicates the presence of macroprolactinemia.[13] Because true hyperprolactinemia and macroprolactinemia may coexist, post PEG PRL values should not exceed 13 ng/mL.

In pregnancy, serum PRL levels begin to increase by 6 weeks of gestation and rise progressively to reach approximately 200 ng/mL at term. In nonlactating women, PRL levels return to normal 2 to 3 weeks postpartum.[15] With menopause, serum PRL declines slightly as a result of the reduction of estrogen.

Serum PRL levels greater than 200 ng/mL are diagnostic of pituitary adenoma, but patients with microadenomas often have levels that are considerably lower. Levels between 50 and 200 ng/mL may be found in patients with

non-PRL-secreting adenomas compressing the pituitary stalk. If moderately elevated PRL levels are found, evaluation of thyroid function is necessary because hypothyroidism results in elevations of serum PRL.

Dynamic Tests for Prolactin Evaluation

Dynamic tests for the evaluation of PRL secretion are seldom used and were popular when brain imaging was less sophisticated. Computed tomography (CT) or magnetic resonance imaging (MRI) is much more valuable for the diagnosis and assessment of a pituitary microadenoma or macroadenoma.

Thyrotropin-releasing hormone, the hypothalamic peptide that stimulates the secretion of thyroid-stimulating hormone (TSH), may be used to evaluate the pituitary secretion of PRL. In general, patients with a prolactinoma do not experience further increases in serum PRL after TRH stimulation. Therefore, this test has been used to differentiate between functional forms of hyperprolactinemia and PRL-secreting pituitary adenomas. However, the utility of the TRH test has been questioned because the PRL response largely depends on basal PRL levels. The test is carried out after an overnight fast, with TRH (200 μg) being administered intravenously. Blood samples for PRL are taken at the initiation of the test and again at 30, 60, and 90 minutes. A normal PRL response is an increase of at least 100% of basal levels. Psychiatric disorders and ingestion of thyroid hormone, corticosteroids, and tranquilizers may affect the PRL response. The TRH test is still used to diagnose pituitary thyroid disorders and for subtle forms of hypothyroidism; for these purposes, TSH levels are measured.

An alternative to the TRH test for the assessment of PRL disorders is the use of metoclopramide (10 mg intravenously). A blood sample is obtained immediately after metoclopramide administration, and again at 30, 60, 90, and 120 minutes. A PRL increment of 100 ng/mL at 60 to 120 minutes is considered normal.

Inhibins and Activin

Activin A and B have significant roles in the ovary. In general, Activin opposes the role of Inhibin and is a major binding protein of follistatin which neutralizes its biological activity. Nevertheless, there is no major role for the measurement of Activin at present in clinical practice. Recent experimental evidence, however points to its potential clinical role in disorders of pregnancy such as preeclampsia, as well as being a marker in endometriosis, although this has been questioned recently.[16]

Serum inhibin A and B levels may be measured by specific ELISA, and reflect the secretion from various populations of granulosa cells. As previously noted, a characteristic of peptide assays is that multiple subunits make assays difficult. This is particularly true in the case of inhibin assays, which have been highly variable and imprecise. Serum values vary in adult women during the menstrual cycle, with inhibin A being lowest during the follicular phase and peaking during the midluteal phase; inhibin B exhibits an opposite pattern (Fig. 34.5). During pregnancy, inhibin A decreases during the second trimester, whereas inhibin B rises from the third

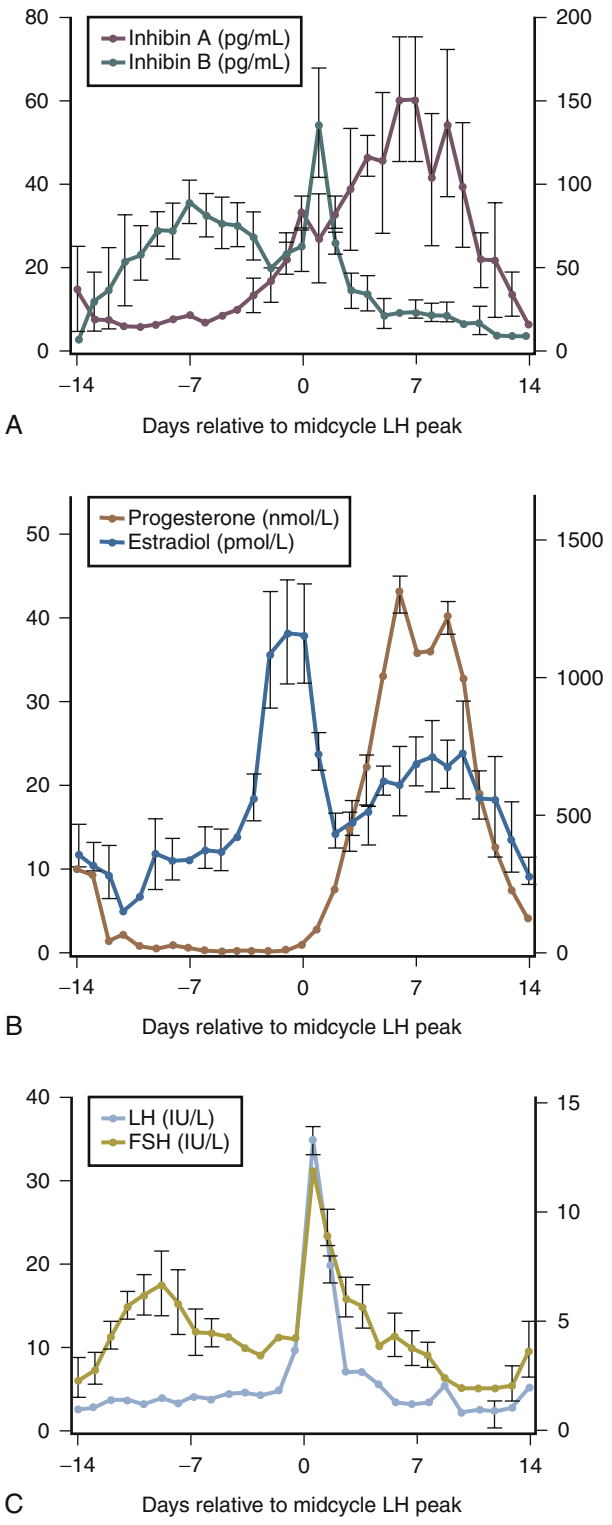


FIGURE 34.5 Plasma concentrations of inhibin A and inhibin B. **A**, estradiol and progesterone **B**, and luteinizing hormone (LH) and follicle-stimulating hormone (FSH) **C**, during the female menstrual cycle. Data displayed with respect to the day of midcycle LH peak. Mean concentrations are shown ± standard error of the mean. *(Adapted from Groome NP, Illingworth PJ, O'Brien M, et al. Measurement of dimeric inhibin B throughout the human menstrual cycle.* J Clin Endocrinol Metab *81:1401–1405, 1996.)*

trimester to peak at term. After menopause, levels of both inhibins decline markedly.

The measurement of inhibin may be useful in several conditions. Some granulosa cell tumors produce high quantities of inhibin B, which may be useful preoperatively and for postoperative surveillance, because it may detect tumor recurrence. Measurement of inhibin B also has been proposed for monitoring certain complications of pregnancy and for evaluating the number of small follicles present in the ovary in the early follicular phase. The measurement may be useful in assessing reproductive aging because a low level of inhibin B on day 3 may indicate a declining ovarian reserve. Lack of standardization of inhibin B measurements has made this difficult.

In men, inhibin B is produced by Sertoli cells and has been considered a good marker of spermatogenesis, beginning to rise after puberty. It has also been used in the differential diagnosis of cryptorchidism versus anorchia where levels are not detectable in anorchia. It may also be helpful in distinguishing between various types of hypogonadism.

Anti-Müllerian Hormone: General Utility and in the Assessment of Ovarian Reserve

Anti-müllerian hormone (AMH) is a homodimeric glycoprotein linked by disulfide bonds with a molecular weight of 140kDa that belongs to the Transforming Growth Factor-B (TGF-B) superfamily. AMH plays an important role in male sex differentiation as its production by the embryonic Sertoli cells induces the regression of müllerian ducts.

In the past, AMH has been measured by two different commercial enzyme-linked immunosorbent (ELISA) assays provided by Diagnostics Systems Laboratory (DSL 10-14400) and by Immunotech. Because of the consolidation of DSL by Beckman Coulter, the Beckman assay is now used (AMH Gen II assay) and values obtained by AMH Gen II assay are 40% higher than previous DSL values.[17]

In women, AMH is produced by the granulosa cells of pre-antral and small antral follicles and its values are virtually undetectable at the birth but increase gradually until puberty and then remain relatively stable during the early reproductive period. In the third decade (30s), AMH levels start declining and become very low during perimenopausal period.[18] The measurement of AMH in blood is mainly used to assess ovarian reserve but may be useful also to diagnose alterations of follicle recruitment and initial growth. In fact, measurement of AMH has been used in evaluation of sexual ambiguity (male neonates have much higher levels than female neonates)[19] and to distinguish between hypogonadism and delayed puberty. Low levels of AMH are found in Turner syndrome and hypogonadotropic hypogonadism.

Serum AMH levels are 2 to 3 fold higher in PCOS, consistent with the presence of an increased number of preantral and small antral follicles.[20] It has been reported that serum AMH values greater than 35 pmol/L (or greater than 5 ng/mL) are a sensitive marker of PCOS [21] and may be useful for the diagnosis of this disorder, mostly when ovarian sonography is not available. In PCOS as in normal women, serum levels of AMH begin to decline in the third decade and the decline becomes more marked after the 40s. Not having an age-specific level of AMH is one of the difficulties in using AMH in isolation for the diagnosis of PCOS. We have observed that women with PCOS who have the lowest

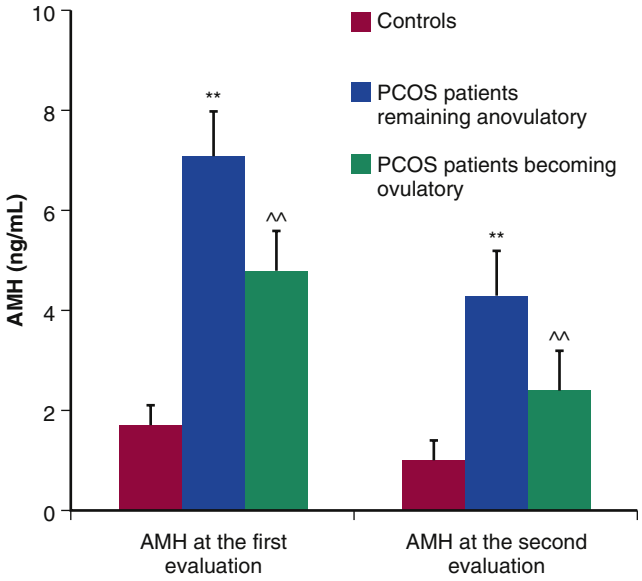


FIGURE 34.6 *P* less than 0.01 versus controls and PCOS becoming ovulatory, *p*, 0.01 versus controls. *(Adapted From Carmina E, Campagne AM, Mansueto P, Vitale G, Kort D, Lobo R. Does the level of serum antimüllerian hormone predict ovulatory function in women with PCOS with aging?* Fertil Steril *98(4):1043-1046, 2012.)*

AMH values have the highest probability of having more normal menstrual cycles with aging[22] (Fig. 34.6).

Assessing ovarian reserve has become an extremely important part of the management of women with reproductive disorders. Whereas ovarian aging becomes accelerated in the late 30s, even younger women, particularly those who have undergone cancer treatment, may have diminished ovarian reserve.[23]

Although measurement of serum FSH and estradiol levels on day 2 to 3 has been used widely, other tests include the clomiphene challenge test (described earlier), counting antral follicles on ultrasound, measuring levels of inhibin B, and measuring anti-müllerian hormone (AMH). Although some have suggested using a combination of these to provide a composite score criterion of ovarian reserve, in our hands, AMH level has proved to be the most useful, as suggested by others (Fig. 34.7).[24] Levels of AMH are cycle-dependent and tend not to fluctuate as much as FSH levels. Receiver operating characteristic curves have been constructed to assess the probability of pregnancy. Our experience suggests that a value of less than 0.4 ng/mL carries a poor prognosis, although lab values vary considerably. Figure 34.8 depicts the age-specific decline in AMH levels associated with aging from a large database of women presenting for fertility treatment.[25] Both Inhibin B and AMH may be helpful in predicting the onset of menopause, but the data for AMH are more robust because of less variability between assays. Several groups have confirmed that once AMH levels become undetectable (usually less than 0.05 ng/mL) menopause will occur within 5 years[26] (Fig. 34.9).

It is important to note that protracted storage of whole blood at room temperature profoundly influences the AMH concentration (incremental increase of 40% over approximately 3.5 days) measured in the subsequently separated serum.[27] However, when samples are collected into maintained tubes with a gel separator, the AMH remains unaffected for many days at room temperature.

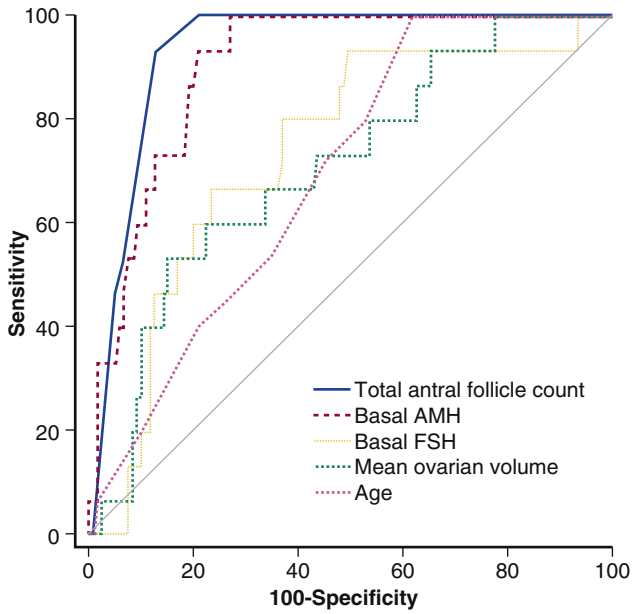


FIGURE 34.7 Prediction of poor response to COH: AFC and AMH are best; the combination is not better. *(Adapted from Jayaprakasan K, Deb S, Batcha M, et al. The cohort of antral follicles measuring 2-6 mm reflects the quantitative status of ovarian reserve as assessed by serum levels of anti-müllerian hormone and response to controlled stimulation.* Fertil Steril *94(5):1775–1781, 2010.)*

Measurement of Sex Steroid Hormones

Estrogens

Measurement of serum levels of E_2, the major active estrogen, plays an important role in clinical diagnostic and research laboratories. In women, diagnostic applications of E_2 assays include assessment of ovarian function in a wide variety of clinical situations such as menstrual disorders, precocious or delayed puberty, and assisted reproduction protocols. In men, E_2 measurements may be useful for evaluation of gynecomastia. In research laboratories, quantitation of E_2 has played an important role in many different studies (e.g., epidemiological studies of breast cancer).

Other estrogens such as estrone (E_1) and estrone sulfate (E_1S) are measured in clinical diagnostic laboratories much less frequently than E_2; this is especially true of E_1S. E_1 has substantial biological activity and is often measured together with E_2 when determining estrogen status in women or men. In contrast, E_1S has no biological activity but is quantitatively the most important estrogen in either premenopausal or postmenopausal women, or in men, and reflects the reservoir of estrogen in the circulation.[28]

Although direct RIA kits are available commercially to measure E_1 and E_1S, there are deficiencies associated with the assays obtained with these kits. Thus, conventional RIAs are recommended to measure E_1 and E_1S (measurement of E1 after sulfatase incubation of serum).

Estriol (E_3) is a very weak estrogen, which was measured frequently by RIA in the 1970s to assess fetal well-being in late pregnancy. However, diagnostic use of the E_3 assay

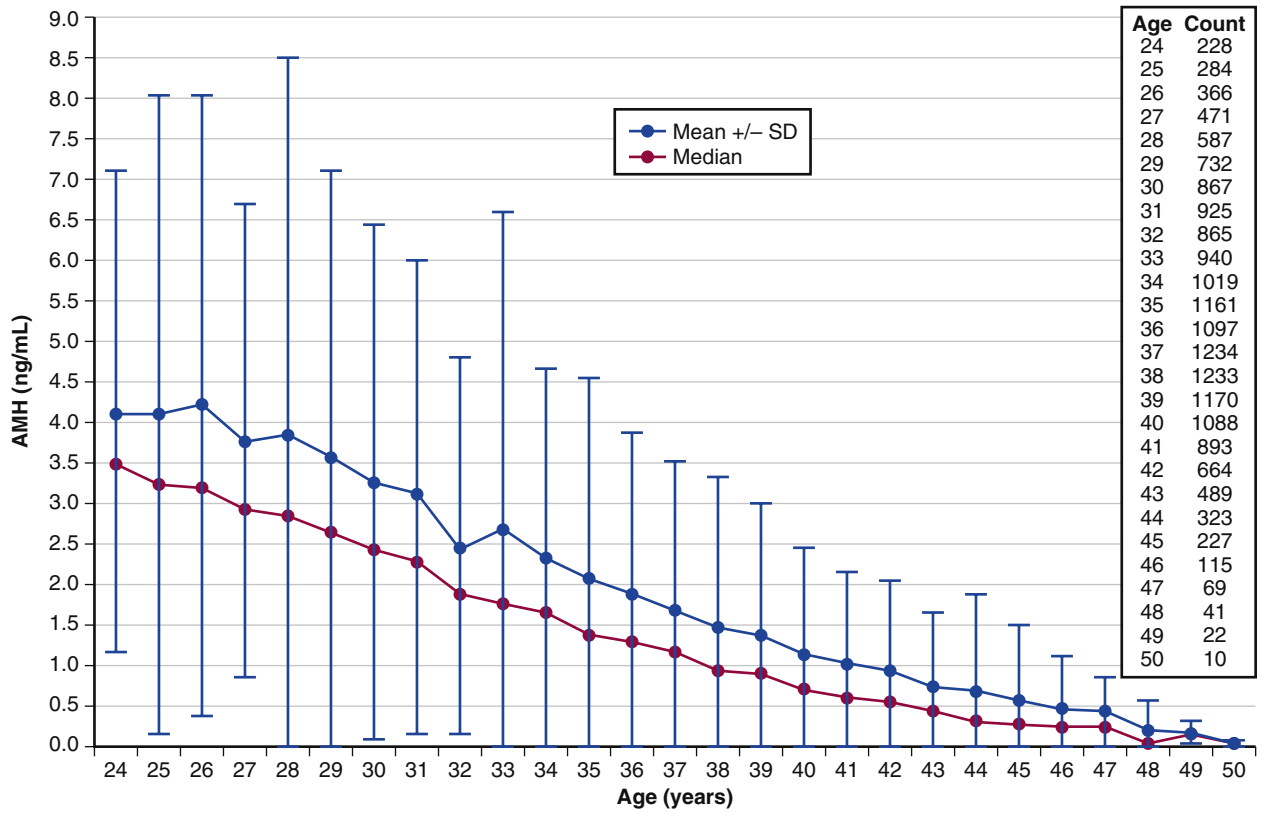


FIGURE 34.8 Data from 17,120 women—single assay site: BC-DSL. *(Adapted from Seifer DB, Baker VL, Leader B. Age-specific serum anti-müllerian hormone values for 17,120 women presenting to fertility centers within the United States.* Fertil Steril *95(2):747–750, 2011.)*

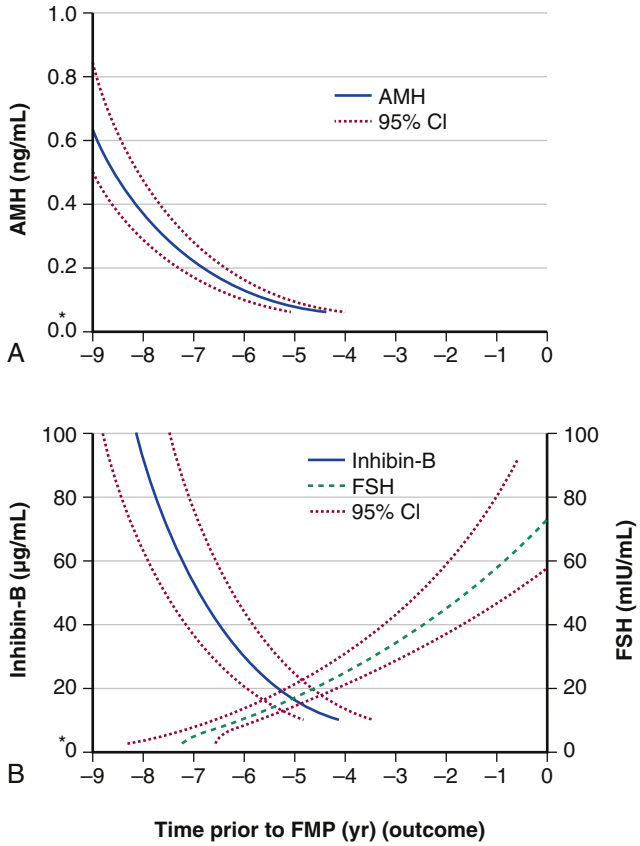


FIGURE 34.9 Anti-müllerian hormone (AMH) and inhibin-B concentrations in relation to the last menstrual period. *(Adapted from Sowers MR, et al.* J Clin Endocrinol Metab *93(9):L34768–L34783, 2008.)*

was replaced with ultrasound and fetal heart rate testing and now has re-surfaced as part of a genetic panel for aneuploidy testing.

As discussed earlier, E_2 was measured initially by conventional RIA in both clinical diagnostic and research laboratories. However, once immunoassays were developed for use on automated platforms, most clinical diagnostic laboratories began to use this methodology on a routine basis, and this methodology is still used by many of these laboratories. Although these assays can be used to measure higher E_2 levels (greater than 50 pg/mL), they lack the sensitivity to measure precisely low E_2 levels, such as those found in postmenopausal women and in men. In addition, direct E_2 immunoassays are not specific. This is evident when E_2 is measured in postmenopausal women using estrogen therapy (e.g., conjugated equine estrogens); the serum levels in these women are overestimated threefold to fivefold. Overestimation of serum E_2 levels may result in inappropriate treatment of a patient and potential adverse effects. This is especially important when E_2 is measured by direct immunoassay in patients treated with aromatase inhibitors. Some aromatase inhibitors cross-react with the antibody in those assays, resulting in falsely elevated E_2 levels. Based on these results, an inappropriate clinical decision may be made to increase unnecessarily the dosage of the drug.

To obtain accurate data, low E_2 levels should be measured by conventional RIA or by LC-MS and MS or GC-MS and MS.[29] These assays separate out potential interfering E_2 metabolites prior to the quantification step, as discussed earlier.

Circulating Estrogen Levels

It is important to realize that the reference ranges of the different estrogens will vary somewhat, particularly when different assay methodology is used. Serum levels of E_2 and E_1 during various stages of a woman's lifetime were established in the 1970s and 1980s using conventional RIAs. Laboratories using direct immunoassays have generally adjusted their reference ranges for E_2 and E_1 to match those obtained by conventional RIAs.

E_2 levels range from 20 to 80 pg/mL during the early to midfollicular phase of the menstrual cycle and peak at 200 to 500 pg/mL or higher during the preovulatory LH surge. During the midluteal phase, E_2 levels range from 60 to 200 pg/mL.

Before puberty, E_2 levels are less than 20 pg/mL, indistinguishable from cases of hypogonadism. After menopause, E_2 levels fall to prepubertal levels; the mean E_2 level is typically 10 to 20 pg/mL, and levels are lower than 10 pg/mL in women who have undergone oophorectomy.

In men, serum E_2 levels are less than 40 pg/mL, but may be increased in several testicular and non-testicular diseases. Male E_2 levels are relatively constant and, unlike testosterone, are unchanged with age.

As for serum E_1 levels, they generally follow the profile of E_2 levels but are a little lower, except in postmenopausal women where the levels are approximately threefold higher than E_2 levels. Also, when postmenopausal women are treated orally with any estrogen, E_1 levels are 3 to 5 times higher due to the high activity of 17β-hydroxysteroid dehydrogenase (type 2) present during hepatic first pass metabolism.

Progesterone

Serum progesterone levels may be measured by a variety of immunoassay methods. Although progesterone is metabolized to many different metabolites, use of direct immunoassays has generally not resulted in overestimated values due to the relatively high levels that are being measured and the development of highly specific antibodies against progesterone, which are used in these assays.

Serum progesterone is low during the follicular phase, (less than 1.5 ng/mL). Levels begin to increase just before the onset of the LH surge and then increase progressively to peak levels 6 to 8 days after ovulation. After menopause, serum progesterone of adrenal origin is less than 0.5 ng/mL.

The measurement of serum progesterone during the midluteal phase (days 21 and 22) is most frequently used to assess ovulatory status. During that time, serum progesterone levels are usually higher than 7 ng/mL. Some physicians have proposed using three luteal determinations with a total serum value of 15 ng/mL or more to indicate normal luteal function. For patients being monitored for fertility, other assessments may be used, including basal body temperature charts, urinary LH kits, and timed endometrial biopsy. In conception cycles, properly timed midluteal progesterone levels are more than 10 ng/mL.[30] Progesterone is also often used to assess ovulation after induction of ovulation. In clomiphene cycles, midluteal progesterone levels should be higher than 15 ng/mL.[31]

During pregnancy, serum progesterone may be useful to assess corpus luteum and placental function.[32] Maternal serum progesterone levels rise slowly to 40 ng/mL near the end of the first trimester and then increase progressively to reach 150 ng/mL, at term. Low levels of progesterone (less than 10 ng/mL) at 6 to 8 weeks signify an abnormal intrauterine pregnancy or an ectopic pregnancy.

Androgens

Androgens in women arise from three different sources: the ovaries, adrenal glands, and peripheral compartment. Most androgens are produced or metabolized in more than one compartment and, in evaluating women with androgen excess, several androgens are usually measured.[33] In the past, the measurement of urinary 17-ketosteroids was the most common method used to evaluate androgen production in women. However, 17-ketosteroid measurements reflect adrenal androgen production poorly reflect testosterone production, and are nonspecific; therefore, they have been abandoned for use in current practice.

Most commonly, serum testosterone (T) and dehydroepiandrosterone sulfate (DHEAS) levels are measured. These levels largely reflect ovarian and adrenal contributions, respectively.[34] Testosterone reflects mostly ovarian androgen production, with two thirds of circulating levels resulting from the peripheral conversion of androstenedione; therefore, increases in either ovarian or adrenal androstenedione production results in elevated serum testosterone levels. On the other hand, whereas DHEAS reflects adrenal androgen secretion well, its levels may be normal in certain cases of adrenal androgen hypersecretion (as in 21-hydroxylase deficiency).[34] The pathway of adrenal enzymatic blockade (affecting the Δ^4 pathway) explains this discrepancy. Conversely, serum DHEAS may be elevated in many patients with a prevalent ovarian source of hyperandrogenemia (as in PCOS).[34] Accordingly, increased levels of serum DHEAS do not predict androgen responses to dexamethasone suppression which has been used as a test to determine adrenal responsivity to suppression.

Total, Free, and Bioavailable Testosterone

When measuring total testosterone in female serum samples, it is essential to use either a conventional RIA or a MS assay. Direct immunoassays have no or poor validity for quantifying testosterone in female serum samples.[35] However, they can be used to measure testosterone in male serum samples due to the relatively higher circulating testosterone levels in men.

In premenopausal women, approximately 66% and 30% of total testosterone is bound to SHBG and albumin, respectively and the free fraction generally comprises less than 2% of the total.[36] Free testosterone is the fraction of circulating testosterone that is available for metabolism or binding to the androgen receptor in target cells.[37] The "gold standard" for quantifying free testosterone is the equilibrium dialysis method.

This method involves the addition of a small amount of ^{3}H-testosterone to a serum or plasma sample, which is then transformed to a vessel divided by a membrane and containing buffer. After a suitable incubation period, separation of the protein (SHBG and albumin)-bound fractions from the free fraction of testosterone by means of a membrane. The membrane retains the protein-bound fractions but allows free testosterone to pass through. The percentage of tritiated free testosterone is then calculated on the basis of the total ^{3}H-testosterone added.

Although the equilibrium dialysis method is commonly regarded as the "gold standard" for quantifying free testosterone, it is subject to technical limitations. The method is influenced by dilution of the serum sample and can be affected by impurities of tritiated testosterone not bound by SHBG or albumin; these impurities may increase the percentage of free testosterone. Also, the use of too large an amount of ^{3}H-testosterone in the assays may increase the concentration of total testosterone and possibly disturb the equilibrium of endogenous testosterone.

For many years it was accepted that only the free fraction of testosterone in the circulation can be taken up by tissues and that the protein-bound testosterone complex is inactive. However, some investigators observed that the fraction of testosterone bound to albumin dissociates rapidly and is taken up by tissues in a manner similar to that of the free steroid. Testosterone bound to the large pool of albumin, together with the small amount of the free steroid, most likely forms the circulating pool of bioavailable (non-SHBG-bound) testosterone. The bioavailable fraction of testosterone enters cells where it may undergo metabolism or binds to the androgen receptor and exerts biological activity.

A relatively simple method to determine bioavailable testosterone involves addition of a small amount of ^{3}H-testosterone to a serum sample and, after a suitable incubation period, precipitation of the globulins (including the SHBG-testosterone complex) with saturated ammonium sulfate, centrifugation, counting the tritium in the supernatant, and calculating the percentage of the tritium in the supernatant (which represents the "bioavailable" testosterone fraction) based on the total amount of ^{3}H-testosterone added initially. The most frequently encountered sources of error in the ammonium sulfate precipitation assay are due to use of impure tritiated testosterone, insufficient counting time of the small amount of radiolabeled testosterone, and incomplete precipitation of globulins.

Using the methods described above, the concentration of free or bioavailable testosterone is usually calculated from the percentage of free or bioavailable testosterone multiplied by the total testosterone concentration. Thus, it is essential to determine the total testosterone concentration accurately, either by a conventional RIA or MS assay.[38]

It is most practical, for clinical purposes, to calculate a free testosterone index (free androgen index [FAI]) using levels of testosterone and SHBG. The calculation is made using the ratio between testosterone and SHBG (in units of nanomole per liter): T [ng/mL] $\times$ 3.467/SHBG $\times$ 100).[39] At least in females, FAI values correlate well with free testosterone determined by equilibrium dialysis. However, FAI values have low validity in men[40] and are strongly dependent on the accuracy of testosterone and SHBG assays. In general, FAI should be used primarily for clinical rather than research purposes, when conventional RIAs or MS assays for total testosterone are not available.

Other Androgens

There are other androgens that may be useful to measure under different circumstances. These include 11β-hydroxyandrostenedione,[41] which reflects adrenal production of

androstenedione because 11β-hydroxylase activity is usually absent in the ovary. Also, assessment of peripheral androgen production requires the measurement of products of 5α-reductase activity. Although serum dihydrotestosterone (DHT) does not appropriately reflect increases in peripheral 5α-reductase activity, more distal metabolites may be helpful. Serum 3α-androstanediol glucuronide best reflects peripheral testosterone metabolism in hirsutism[42] and androsterone glucuronide best reflects the state in androgenic acne.[43]

Blood Levels of Androgens in Women

In women of reproductive age, an accurate range for serum testosterone is 20 to 50 ng/dL. With direct immunoassays, the upper end of the normal range is approximately 70 to 80 ng/dL, or even higher. Serum testosterone levels higher than 200 ng/dL are suggestive of ovarian neoplasms, but some tumors may present with lower levels.[44] Because upper normal ranges vary, a rule of thumb is to be concerned about a tumor in women whose values exceed *2.5* times the upper normal range of a particular assay. Patients with PCOS have mildly increased serum testosterone (usually less than 100 ng/dL), whereas markedly increased levels may be found in patients with hyperthecosis and in those who have adrenal enzymatic deficiencies.

The range for bioavailable testosterone is 0.6 to 5 ng/dL, whereas free testosterone levels are between 1 and *8.5* pg/mL. Normal ranges for SHBG in women are between 30 and 90 nmol/L.

Serum androgens are low before puberty, with adrenal androgen levels beginning to increase 1 or 2 years before the onset of puberty. Serum DHEAS values higher than 0.8 μg/mL, indicate the presence of adrenarche.[45] After menopause, serum androgens decline slightly. Aging is the principal determinant of this decline.[46] Serum DHEAS levels have been suggested to be useful in excluding adrenal deficiency in young women with autoimmune ovarian failure.[47]

The normal range of serum DHEAS is 0.8 to 2.8 μg/mL, Values greater than 8 μg/mL, are suggestive of an adrenal neoplasm; if the adrenal tumor produces testosterone, however, only modestly elevated values (3 to 4 μg/mL) may be found.[48]

Normal serum androstenedione levels range from 1 to *2.5* ng/mL. The pattern of serum androstenedione levels during the menstrual cycle is similar to that described for testosterone. However, in contrast to testosterone, androstenedione levels show some circadian rhythm.

Measurement of Circulating Androgen Precursors in Women

Measurements of intermediates in steroid metabolism are useful for the diagnosis of adrenal enzymatic deficiencies. Serum 17α-OHP is used in the diagnosis of 21-hydroxylase deficiency.[49] Because 17α-OHP is secreted by the corpus luteum, the measurement should be carried out in the morning during the follicular phase. In healthy women, serum 17α-OHP is generally lower than 1 ng/mL, but hyperandrogenic patients (mostly those with PCOS) generally have slightly higher levels. Therefore, values up to 2 to 3 ng/mL are not indicative of an adrenal enzymatic deficiency. Patients with classic neonatal forms of 21-hydroxylase deficiency have very high levels of 17α-OHP (50 to 200 ng/mL), while most patients with non-classic, late-onset 21-hydroxylase deficiency have serum 17α-OHP levels between 10 and 20 ng/mL. The adrenocorticotropin hormone (ACTH) stimulation test, which will be described later in this chapter, can be used to diagnose 21-hydroxylase deficiency. From a practical standpoint, most suspected patients can now have genotyping for the various mutations in the *CYP21β* gene, particularly when there is pertinent family history.[50]

The measurement of serum 11-deoxycortisol may be used to diagnose 11β-hydroxylase deficiency when 17α-OHP is elevated. This is a rarer condition that may be associated with hypertension. The measurement of serum 17α-hydroxypregnenolone (and the ratio 17α-OHP to 17α-hydroxypregnelone), as well as the ratio for DHEA to androstenedione is useful in the diagnosis of 3β-hydroxysteroid deficiency.[51] In adults, this latter diagnosis is controversial in that no genetic mutations in the *3βol* gene have been uncovered and most patients seem to have a variant of Polycystic Ovary Syndrome.[52]

Tests to Establish the Source of Hyperandrogenism

Because serum androgens may arise from different sources in women, several tests have been established to distinguish adrenal from ovarian hyperandrogenism. However, most tests are not specific and their use is generally limited. Currently clinical guidelines, for example for the evaluation of hirsutism, do not suggest the need to determine the source of androgen excess; however there may be an investigational interest in pursuing a more specific diagnosis.

The tests may be divided into two groups: tests to assess adrenal androgen secretion, and those that assess ovarian androgen secretion. The first group of tests includes the dexamethasone suppression test and the ACTH stimulation test.

Dexamethasone Suppression Test

For evaluation of hyperandrogenism, a prolonged test may be used in which dexamethasone (0.5 mg) is given orally every 6 hours for 3 to 7 days. Evaluation of the suppression of serum DHEAS requires 7 days because of the prolonged half-life of this steroid; for the evaluation of the suppression of testosterone and free testosterone, however, 3 days of dexamethasone administration is sufficient. For practical reasons, the measurement of testosterone and free testosterone are sufficient because DHEAS is always profoundly suppressed by dexamethasone unless an adrenal tumor is present. In most common forms of hyperandrogenism, a decrease of total and unbound testosterone of at least 60% of basal values suggests an adrenal cause of hyperandrogenism.[53] The test is not specific and is now rarely used.

Adrenocorticotropic Hormone Stimulation Test

The ACTH stimulation test is generally used for the diagnosis of cortisol deficiency or to uncover mild adrenal enzymatic deficiencies. Also, in the past this test has been used to distinguish between adrenal and ovarian sources of hyperandrogenism, but is not generally used for this purpose because of its lack of specificity. ACTH stimulation is not recommended for uncovering subtle defects in hyperandrogenism unless there is a strong suggestion that

21-hydroxylase deficiency (congenital adrenal hyperplasia) may be present. For example, the ACTH stimulation test would be indicated in adult women with hyperandrogenism who have baseline levels of 17α-OHP over 3 mg/mL but less than 10 mg/mL—the 17α-OHP level above which the diagnosis of 21-hydroxylase deficiency would be obvious.[54] Some authors have suggested a baseline 17α-OHP value cut-off of greater than 2 ng/mL as a threshold for ACTH stimulation testing. The index of suspicion should be higher in certain ethnic groups: Yupik Eskimos, Ashkenazi Jews, and certain Southern European groups. As stated earlier in the chapter, many patients now get genotyping done.[55]

The test is best performed between 8 and 9 AM, with 0.25 mg of cosyntropin injected intravenously. While the test may also be accomplished by intramuscular injection, more consistent results have been obtained with intravenous administration. Blood samples are generally obtained at 30 minutes and 60 minutes, but the 60-minute value suffices as a single time point for practical purposes. For the diagnosis of non-classic 21-hydroxylase deficiency, serum cortisol and 17α-OHP are measured. Serum 11-deoxycortisol may be evaluated if the rare 11-hydroxylase defect is suspected.

Non-classic 21-hydroxylase deficiency may be diagnosed using the New nomogram (Fig. 34.10),[50] in which the log of the baseline 17α-OHP level is plotted against the log of 17α-OHP at 60 minutes after ACTH administration. However, most laboratories diagnose 21-hydroxylase deficiency if peak 17α-OHP is higher than 10 ng/mL with an increase from baseline of at least 5 ng/mL.

Δ^5 and Δ^4 intermediate steroid measurements after ACTH stimulation have been used to diagnose 3-beta-hydroxysteroid deficiency,[52] however women so identified do not have a genetic form of enzyme deficiency, and it is likely that these women constitute a variant of PCOS.

Gonadotropin-Releasing Hormone-Agonist Test

GnRH-agonists (GnRH-A) are generally used to down-regulate the secretion of pituitary gonadotropins and to inhibit ovarian steroid secretion. However, prior to down-regulation, the early agonistic action of GnRH-A causes prolonged stimulation of gonadotropin secretion, which in turn, stimulates the ovary. The test has been used in the setting of PCOS.[56]

The original test was performed using nafarelin,[56] but other GnRH agonists may be used with no substantial differences. Generally, leuprolide (500 or 1000 μg) is given subcutaneously in the morning between the fifth and the eighth day of the cycle. Blood samples are taken at 0 minutes and after 8 and 24 hours. A delta (increase from baseline) in serum 17α-OHP greater than 2.6 ng/mL suggests an increased or exaggerated response that is characteristic of women with PCOS.

Hormonal Evaluation of Hirsutism

Hirsutism is excessive hair growth in women where it is not normally found, usually with a central body distribution. When this biological signal is manifest, laboratory tests may be indicated to pinpoint the abnormality.

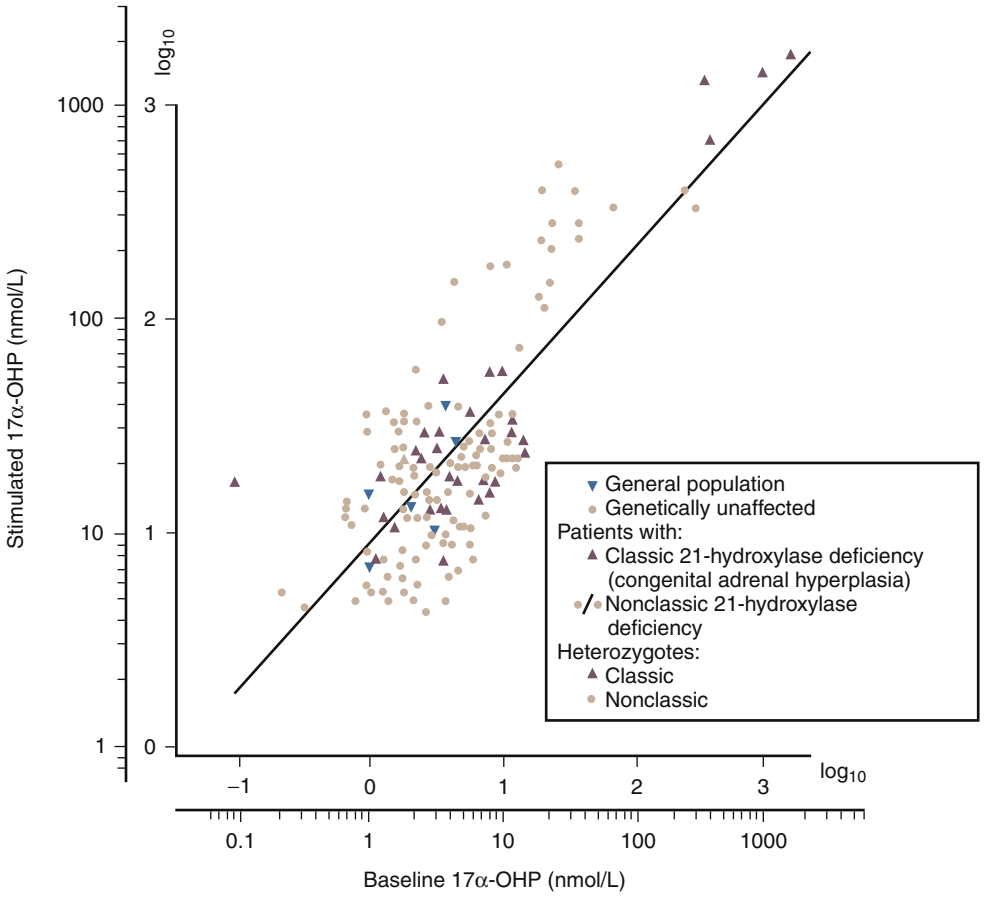


FIGURE 34.10 Nomogram of 17α-hydroxyprogesterone (17α-OHP) in different types of patients with congenital adrenal hyperplasia. *(Adapted from New MI, Lorenzen F, Lamer AJ, et al. Genotyping steroid 21-hydroxylase deficiency: hormonal reference data.* J Clin Endocrinol Metab *57:320–326, 1983.)*

Most commonly, total testosterone, DHEAS, and SHBG are measured (as well as 17α-OHP to exclude non-classic congenital adrenal hyperplasia). In about 10% to 15% of hirsute women, all of these hormone levels are in the normal range and a diagnosis of "idiopathic hirsutism" is made.[57] While it is not our intent to discuss the differential diagnosis of hirsutism, a simple algorithm is provided in Figure 34.11.

Blood Levels of Androgens in Men

In adult men, total testosterone and unbound testosterone are the only androgens measured in the evaluation of testicular function. Serum testosterone levels range between 300 and 1000 ng/dL. Serum testosterone decreases during the day and in the evening the levels are approximately 15% lower.

The normal range for bioavailable testosterone is between 66 to 417 ng/dL; for free testosterone, normal values are between 50 and 210 pg/mL. These assays may be useful in patients with low testosterone values. Serum DHT values range between 30 and 86 ng/dL, but this assay is not recommended on a routine basis. In evaluating children and neonates for 5α-reductase deficiency, serum DHT assays have been useful.

Assessment of Glucose Metabolism and Insulin Activity

Normal fasting blood glucose levels are lower than 100 mg/dL and values of 125 mg/dL and higher are diagnostic of diabetes mellitus. Prediabetes represents an elevation of plasma glucose above the normal range but below that of clinical diabetes.[58] Prediabetes is diagnosed as either impaired fasting glucose (IFG) (fasting blood glucose levels between 100 and 125 mg/dL) or impaired glucose tolerance (IGT). The latter is detected by oral glucose tolerance testing (see later in the chapter) with blood glucose levels greater than 140 mg/dL 2 hours after glucose administration indicating IGT. Both IFG and IGT are risk factors for type 2 diabetes, and the risk is even greater when IFG and IGT occur together.

Glycated haemoglobin A1c (HbA1c) represents a useful tool for predicting and diagnosing diabetes mellitus.[59] HbA(1c) has slightly lower sensitivity than fasting plasma glucose (FPG) in detecting diabetes, but slightly higher specificity with a cut-off value of greater than 6.1% for the diagnosis of diabetes mellitus. However, because optimum cut-off values vary by ethnic group, age, gender, and population prevalence of diabetes, a slightly higher value of 6.5% may be used.[60]

Measurement of Serum Insulin

Serum insulin may be measured by different immunoassays. Use of heparin causes falsely elevated values, while hemolysis of the blood may result in falsely low values. Insulin antibodies in blood result in low levels in some assays and high levels in others.

Using commercial assays, normal fasting insulin levels range between 5 and 15 μU/mL but with more sensitive assays normal fasting insulin should be lower than 12 μU/mL. Obese subjects have increased values, while very high circulating levels are found in patients with severe insulin resistance. 40% to 50% of women with a normal body mass index who have classic PCOS have slightly increased insulin levels,[61] higher levels are observed in obese women with PCOS.[62]

Evaluation of Insulin Resistance

Insulin resistance is considered to be extremely important in health and disease and has an important role in the pathophysiology of PCOS. There are several ways to assess peripheral insulin sensitivity.[62,63] The most precise methods involve the use of glycemic clamps.[64] However, these methods are very cumbersome and should be reserved for research purposes. Use of the minimal model of Bergman applied to a frequently sampled intravenous glucose tolerance (FSIGT) test is considered to be the "gold standard" for assessing insulin resistance.[65] This method is as sensitive as the euglycemic insulin clamp.[66,67] An insulin tolerance test (ITT) is a good alternative for use in clinical practice.

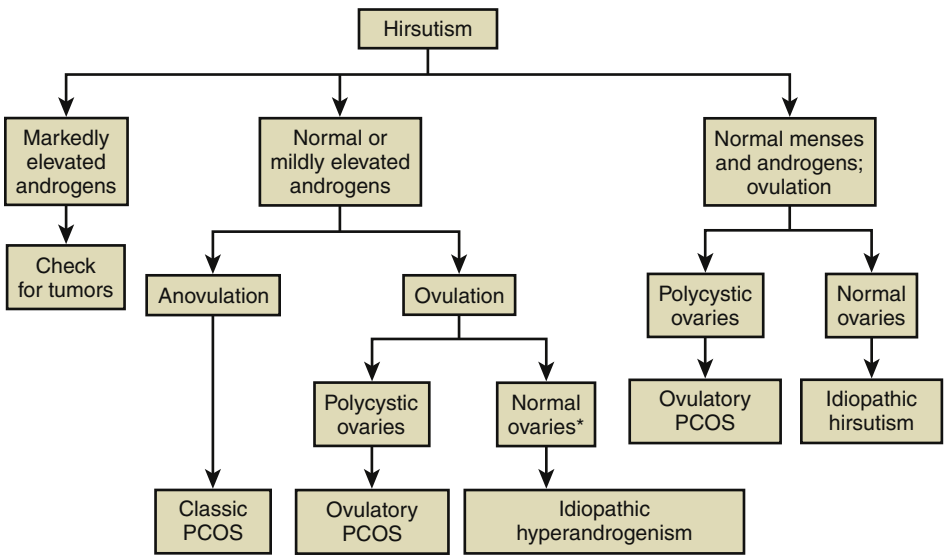


FIGURE 34.11 Diagnosis of hirsutism. *Note that the ultrasound diagnosis of polycystic ovaries is somewhat subjective. Therefore, the diagnostic distinction between ovulatory polycystic ovary syndrome (PCOS) and idiopathic hyperandrogenism is fluid.

Methods based on fasting blood concentrations for glucose and insulin with the use of various calculations, are highly dependent on the quality of the insulin assay and should not be used when β cell function is impaired as occurs in diabetes mellitus, or in the elderly. In addition, these methods measure mostly hepatic, and not peripheral, insulin sensitivity. With all these limitations, these methods may be useful in a clinical setting, for example in patients with PCOS without diabetes. Methods based on OGTT have similar limitations and clinical usefulness but presents the advantage of giving other important clinical information such as the presence of glucose tolerance.

Frequently Sampled Intravenous Glucose Test (FSIGT)

Two different catheters (one for glucose and insulin administration and one for blood samples) are placed in two antecubital veins.[68] Glucose (0.3 g/kg) is injected over 1 minute intravenously, followed 20 minutes later by an intravenous insulin bolus (0.03 U/kg). In the original method, blood samples were taken at 15, 10, 5, and 1 minute before the glucose load; additional samples were taken every minute for 10 minutes after loading, and again at 12, 14, 16, 20, 22, 23, 24, 25, 27, 30, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 minutes. Because of the great number of blood samples required ($n = 33$), the test has been modified to require only 12 samples: 5 minutes before the glucose load, then 2, 4, 8, 20, 22, 30, 40, 50, 70, 100, and 180 minutes afterwards. In all samples, glucose and insulin are measured and insulin sensitivity (SI), which is reciprocally inversely related to insulin resistance, is calculated with the minimal model method by using a computerized algorithm. According to ADA, this test and the euglycemic insulin clamp are the only two methods that accurately estimate peripheral resistance to insulin.

Insulin Tolerance Test

The ITT also has been used to assess insulin resistance[69] and has shown a good correlation with both the FSIGT and the clamps.[70] After an overnight fast, regular insulin (0.1 U/kg) is infused intravenously over 1 minute. Blood glucose is measured at 0, 2, 5, 8, 10, and 15 minutes after insulin administration. Hypoglycemia generally occurs after 15 minutes, and therefore a solution of dextrose 50% in water ($D_{50}W$) is injected at 15 minutes to block this reaction. Because the test is so rapid (15 minutes), the usual counter regulatory factors (growth hormone, cortisol, catecholamine release) that can affect insulin sensitivity are not yet in play. The linear slope of the declining blood glucose concentrations (between 2 and 15 minutes after insulin administration) is calculated and expressed as KITT % per minute. Young healthy women have KITT values between 5 and 6, while patients with PCOS generally have values lower than 4 (Fig. 34.12). Although the ADA considers the ITT to be less sensitive than the FSIGT test, several researchers have reported that it gives comparable results. We have used this test widely because it requires only six blood samples for blood glucose determinations—thus reducing time and cost, particularly for repeated testing in clinical research (Fig. 34.13).

Oral Glucose Tolerance Test

The OGTT is generally used to evaluate glucose tolerance, but may also be used to obtain an estimate of insulin resistance. The test is generally performed giving orally 75 g of glucose, after an overnight fast. Blood samples are taken before the glucose administration and each 30 minutes for 2 to 3 hours. For evaluating glucose tolerance, a blood glucose measurement 120' after glucose administration is sufficient with values >140 mg/dL indicating IGT and values >200 mg/dL indicating type 2 diabetes. For assessing insulin resistance, serum insulin should also be measured and blood samples at 30', 60' and 120' are needed. At least nine indices have been proposed to measure insulin sensitivity by OGTT but only few show a sufficient correlation with the clamps. The Matsuda index (fasting glucose × fasting insulin) × (mean glucose × mean insulin during OGTT) and the log sum insulin give the best estimate of insulin sensitivity.[71]

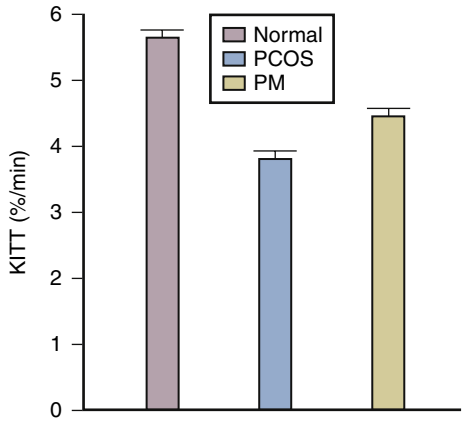


FIGURE 34.12 Insulin tolerance test to evaluate insulin sensitivity. PCOS, polycystic ovary syndrome; PM, postmenopausal women. *(Adapted from Carmina E, Lobo RA. Dynamic tests for hormone evaluation. In Lobo RA, Mishell DR, Paulson Rj, Shoupe D [eds].* Mishell's Textbook of Infertility, Contraception, and Reproductive Endocrinology, *4th ed. Oxford, Blackwell Science, 1997.)*

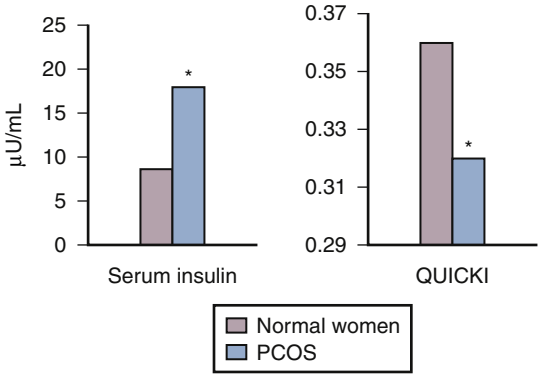


FIGURE 34.13 Serum insulin levels and quantitative insulin sensitivity check index (QUICKI) values in normal women and women with polycystic ovaries. QUICKI values less than 0.33 are suggestive of insulin resistance. PCOS, polycystic ovary syndrome. *(Data from Carmina E, Longo RA, Rini GB, Lobo RA. Phenotypic variation in hyperandrogenic women influences the finding of abnormal metabolic and cardiovascular risk parameters.* J Clin Endocrinol Metab *90:2545–2549, 2005.)*

Fasting Glucose and Insulin Calculations

These methods are based on mathematical calculations applied on simultaneously obtained fasting glucose and insulin values. The most frequently used methods are:

- Glucose-to-insulin ratio[72]
- Homeostasis model assessment of insulin resistance (HOMA-IR)[73] = (glucose × insulin) ÷ 22.5
- Quantitative insulin sensitivity check index (QUICKI)[74] = 1 ÷ (log glucose + log insulin)

As already stated, these methods are easy to perform and useful in a clinical setting but present several limitations. In general, methods using logarithmic transformation should be preferred because the relationships between the insulin and the glucose level are hyperbolic rather than linear. We generally prefer to use the QUICKI method because it is easy to perform and correlates reasonably well with the euglycemic clamp. Values lower than 0.335 suggest insulin resistance and may be found in most women with PCOS (Fig. 34.13).[62]

Measurement of Adipose Tissue Hormones

Adipose tissue is a complex endocrine organ that secretes hormones, cytokines, free fatty acids and various proteins that have profound effects on metabolism and the cardiovascular system.[75] In recent years, many of these factors have been characterized and measured in various endocrine disorders, including PCOS.[76] While most assays are useful only for research purposes, the measurement of two adipose hormones, leptin and adiponectin, also may be useful in clinical practice.

Leptin is a 146 amino acid protein which acts mostly as a signaling factor from adipose tissue to the central nervous system to serve as a metabolical indicator of energy sufficiency.[77] It may be measured by RIA or ELISA and in our laboratory, in adult women with normal body weight, leptin values are between 10 and 30 pg/mL. Serum levels of leptin increase in obese subjects, probably because in obesity there is a condition of leptin resistance. However, some massively obese subjects have a genetically determined leptin deficiency and the finding of low leptin in severe obesity suggested the possibility of using leptin in the treatment of these subjects. In PCOS, the levels of leptin are strictly related to body weight, and are not different when compared to weight matched normal women. Finally leptin levels are lower in children than in adults and increase progressively during puberty.

Adiponectin is a large protein comprised of 244 amino acids which has a major role in preventing or counteracting the development of diet-induced insulin resistance.[78] Moreover, adiponectin has been reported to have an important function in protecting endothelial cells from injury.[79] Adiponectin may be measured by RIA or ELISA and in our laboratory, in adult women with normal body weight, adiponectin values are between 8 and 18 μg/mL. Adiponectin is decreased in metabolically active obesity, probably because of the increased cytokine production in visceral and ectopic adipose tissue,[80] and this reduction has an important role in determining the effects of obesity on insulin resistance, endothelial disease, and cardiovascular risks.[81]

In PCOS, adiponectin is lower than that in weight matched controls, and serum levels less than 8 μg/mL may suggest the particular risk for developing early endothelial disease.[82]

Evaluation of Fat Quantity and Distribution

Because excess fat and/or altered fat distribution represent very important factors in determining insulin resistance and metabolical and cardiovascular risk,[83] many methods have been developed to assess these parameters.

The most simple way to assess fat quantity is to calculate the body mass index (BMI) by the formula: body weight (kg)/height^2 (cm). Values of 30 or higher indicate obesity with values over 40 indicating severe obesity. Values between ≥25 and less than 30 suggest an overweight status while values between 19 and less than 25 indicate normal body mass. However, BMI does not take in account differences in lean and bone mass and in particular patients it may be useful to measure fat quantity directly by total body dual x-ray absorptiometry (DEXA).[84,85] This method permits the measurement of fat quantity in the total body and in several different areas. Particularly important is the measurement of fat quantity in the trunk because this represents a way to assess abdominal fat mass. An increase in abdominal fat, mainly visceral fat, has been suggested to be the main cause of cardiovascular and metabolical risks of obesity.

Because of the role of abdominal fat, it has been suggested that the measurement of waist circumference is more important than the evaluation of body weight in assessing the health problems linked to obesity. In women, values ≥88 cm indicate excessive abdominal fat accumulation and increased metabolical and cardiovascular risks. However, in young women, a normal waist circumference is less than 80 cm and values greater than 80 and less than 88 cm may indicate some abdominal fat increase.

To assess abdominal fat more accurately, other methods may be useful. Abdominal CT or MRI are very sensitive tests but cannot be used routinely in clinical practice for this purpose, and, therefore, abdominal ultrasound or DEXA are generally the preferred methods. Ultrasound is a simple method to assess abdominal fat but is very skills dependent. With this method, omental thickness in centimeters is assessed by using the distance between the posterior surface of the aponeurosis of the rectus abdominal muscle and the anterior wall of the aorta, 5 cm above the umbilicus.

DEXA scans are simple and sensitive but the software used generally measures fat in the trunk that includes the thorax. The sensitivity of this method may therefore be enhanced by measuring abdominal fat in an area of 50 cm^2 around the central point of the midline between the lateral iliac crests and the lowest rib margins at the end of a normal expiration.[125] This point generally corresponds to the umbilicus (but may not) and is located in the midline. Other authors have preferred to measure fat quantity between L1 and L4. Because DEXA measures total body fat and trunk (or central abdominal) fat, it is possible to calculate with the same examination, the percentage of abdominal fat.

We have recently determined normal values and the upper limits (Table 34.1) of fat parameters (by DEXA) in young normal weight women. In general, vales ≥40% of trunk fat indicate the presence of abdominal obesity.[85]

Depending on the population studied, 30% to 60% of women with PCOS may be considered obese and most of them will have abdominal obesity. However, 60% of overweight women with PCOS and 30% of normal weight women with PCOS will exhibit an increase in abdominal fat.

Table 34.1 Evaluation of Abdominal Fat by Dual X-Ray Absorptiometry (DEXA)*

	Mean	Upper Normal Limits	
Trunk fat (g)	5200	8000	High values indicate abdominal fat accumulation
Trunk fat (%)	31.4	38	High values indicate abnormal fat distribution (abdominal obesity)
Central abdominal fat (g)	300	520	High values indicate abdominal fat accumulation. More sensitive index than trunk fat

*Modified from Reference 125.

Metabolical Syndrome

In recent years, the term metabolical syndrome has become popular to denote a condition of increased risk for atherosclerosis and cardiovascular risk. This is based primarily on the findings of insulin resistance and/or abdominal obesity.[86] According to National Cholesterol Education Program Adult treatment guidelines,[87] the syndrome is present if an individual has altered values of at least three of five parameters (blood glucose, blood triglycerides, blood HDL-cholesterol, waist circumference, and blood pressure). The concept of metabolical syndrome has been debated because the health risk may merely be the sum of the single risks. However, the diagnosis of metabolical syndrome may be useful in that it helps to identify subjects who have a particularly high metabolical and CV risk. In PCOS, metabolical syndrome is very common and its prevalence ranges between 10% and 50% depending on body weight and the lifestyle variables of the different populations.[88,89]

Measurement of Growth Hormone and Growth Factors

Circulating growth hormone (GH) may be measured by conventional RIA or immunoradiometric assays. With any method, because of very low basal values and pulsatile secretion, measurement of an unstimulated random GH is usually not very meaningful.[90] Overnight urinary GH may be useful,[91] but the urinary concentrations of GH are also very low and may not be detectable in some assays. There is also a high degree of variability in GH renal excretion.

Insulin-like growth factor 1 (IGF-1) is measured by specific immunoassays and, because these values reflect the integrated 24-hour concentrations of serum GH, IGF-1 may be used for screening of GH-deficiency.[92] However, circulating IGF-1 levels are low in prepubertal children, making differentiation between healthy and GH-deficient children difficult and also in adults IGF-1 determination has a low accuracy for diagnosis of GH deficiency.[93] Moreover, several conditions result in low IGF-1 concentrations—including malnutrition, hypothyroidism, renal failure, and diabetes mellitus—and therefore the confirmation of the diagnosis requires at least two GH provocative tests.

Serum IGF-1 is more useful in the screening evaluation of GH hypersecretion.[94] Persistently elevated serum IGF-1 can identify acromegaly better than several random GH serum measurements and overnight urinary GH tests; however, some acromegalic patients may have normal IGF-1 levels, requiring other investigations to be done if acromegaly is suspected. IGF-1 assessment is particularly useful in evaluating the effects of treatment in acromegalic subjects: finding increased levels of IGF-1 indicates that the disease is not well controlled. Finally, because IGF-1 is low in malnutrition, this assay also may be useful in assessing the general nutritional status of patients.

Insulin-like growth factor 2 (IGF-II) may be evaluated by specific IRMAs, but this level is less dependent on growth hormone secretion. The measurement of IGF-II is useful only in islet-cell tumor hypoglycemia (NICTH), an uncommon syndrome caused by some tumors (generally mesenchimal tumors) and associated with low levels of insulin, IGF-I, and IGF-BP3.[95]

There are six IGF-binding proteins. Among these, circulating IGFBP-1, IGF-BP2, and IGFBP-3 have been the subject of evaluation for clinical purposes. IGFBP-3 is GH-dependent and reflects changes in GH levels. Its long half-life and nonpulsatile secretion make IGFBP-3 measurements an alternative to IGF-1 measurement for the screening of perturbations in GH.[95a]

Levels of serum IGFBP-1 are significantly influenced by hyperinsulinemia, with low levels being found in insulin resistance; in PCOS, where IGF-1 levels are found to be normal, "free" IGF-1 is increased because of a reduced level of IGFBP-1.[96] Serum IGFBP-1 may be measured in diabetes to evaluate whether hyperglycemia is due to inadequate insulin dosage or to other factors.

Serum IGFBP-2 correlates inversely with growth hormone secretion and the ratio IGFBP-2 to IGFBP-3 is a marker of growth hormone action. Serum IGFBP-2 also increases in patients with some tumors (mostly gliomas and prostate cancers) and may be used to assess the growth of these tumours and in conjunction with PSA in the management of certain patients with prostatic carcinoma.[97]

Dynamic Tests for Evaluation of Growth Hormone Secretion

Many provocative or inhibitory tests to diagnose GH-deficiency or excess have been described.[98] Reduced responses to at least two provocative tests are diagnostic of GH deficiency. Although differences exist in expert recommendations and guidelines, using new GH assays, the cut-off for a normal peak GH level in response to provocative tests is generally 7 ng/mL.[99]

Insulin Tolerance Test

This test is the preferred first choice for the diagnosis of GH deficiency. Blood samples are taken at 0, 15, 30, 60, and 90 minutes and glucose and GH are measured. The measurement of glucose is required for validation of hypoglycemia (less than 40 mg/dL), which is necessary to evoke a GH response.

Clonidine Stimulation Test

Clonidine, a well-known antihypertensive drug, may be used for the diagnosis of GH deficiency. Oral clonidine (0.15 mg/m^2) is administered after an overnight fast. Blood

pressure must be monitored during the test because a modest decrease in blood pressure is often observed.

Growth Hormone Releasing Hormone *(GHRH)* and Arginine Test

This test is considered to be an alternative to the ITT for the diagnosis of adult growth hormone deficiency (AGHD) in patients for whom insulin hypoglycemia is contraindicated.[100] GHRH (1 μg/kg) and arginine (0.5 g/kg to a maximum of 30 g in 30 minutes) are combined in the same stimulation test.

Oral Glucose Tolerance Test

The OGTT is the primary test used for the diagnosis of GH hypersecretion. After an overnight fast, 75 to 100 g of oral glucose will cause a suppression of serum GH. Blood samples are taken every 30 minutes for 2 hours. With usual polyclonal RIAs, a GH decrease to below 2 ng/mL is found in normal subjects while 80% of acromegalic subjects will not suppress to this level. The monoclonal assays are much more sensitive for testing, and failure of GH to suppress to below 1 ng/mL is diagnostic of acromegaly.[101]

Measurement of Calcium-Regulating Hormones and Bone Markers

In humans, the main calcium-regulating hormones are parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D. The measurement of PTH is complicated by the complex metabolism of this hormone, which results in a multiplicity of circulating molecular forms with different bioactivities as well as the presence in blood of PTH fragments. The bioactive forms (intact PTH molecule and the amino-terminal fragment) circulate in small quantities because of their shorter half-lives. Therefore, immunoassays using antibodies that recognize the mid or carboxylterminal regions of PTH may be useful in subjects with primary hyperparathyroidism because they provide an index of integrated secretory function of the parathyroid gland. On the contrary, assays that use antibodies against the intact molecule or amino-terminal fragment should be preferred in studies of patients with renal failure, or when studying physiological changes of PTH function.

An important advance has been the development of two site IRMAs for intact PTH. This assay uses antibodies directed against the amino-terminal and mid-region (or carboxyl-terminal) fragments.[102] Because intact PTH is labile at room temperature, specimens should be frozen within 2 to 4 hours of collection. Normal PTH values range from 10 to 65 pg/mL but have to be correlated with circulating calcium levels. The finding of increased serum PTH and calcium is diagnostic of hyperparathyroidism, although some patients may have normocalcemic hyperparathyroidism that results in a form of osteoporosis that is often clinically indistinguishable from postmenopausal osteoporosis. This condition has to be differentiated from the more common mild deficiency of vitamin D, where PTH levels are moderately increased but calcium blood values are normal. In these patients, the administration of vitamin D normalizes serum PTH values.

The clinical assay techniques for circulating monohydroxylated (25-OH) and dihydroxylated (1,25-OH) D-vitamin have progressed from competitive protein binding assay to radioimmunoassay (RIAs) that utilize both ^{125}I and chemiluminescent reporters. These methods are used in the screening of osteoporotic women for underlying vitamin D deficiency.[103] It has been suggested that a large percentage of the female population, particularly in the northern hemisphere, is vitamin D deficient but disagreement exists on 25-OH-D values indicating vitamin D deficiency. The Institute of Medicine has suggested that serum 25-hydroxyvitamin D values lower than 20 ng/mL suggest vitamin D deficiency while guidelines of the Endocrine Society have indicated that all 25-OH-D values lower than 30 ng/mL are to be considered low.[104] This disagreement may depend on different perspectives on vitamin D function with IOM experts focusing on the role of vitamin D in bone metabolism[105] and the Endocrine Society experts also considering extra bone activities of vitamin D.

Determination of Vitamin D Status

The principal circulating form of vitamin D is 25-hydroxyvitamin D [25(OH)D], and is overall the best indicator of vitamin D status. There are two forms of 25(OH)D, namely $25(OH)D_2$ and $25(OH)D_3$. The predominant form is 25(OH)D, which is derived mainly from actions of ultraviolet light on the skin, whereas $25(OH)D_2$ is derived solely from dietary and pharmaceutical sources. Since the two parent compounds provide various contributions to the overall vitamin D status of an individual, it is essential that both the D_2 and D_3 forms of 25(OH)D be recognized equally by the testing system used in treatment monitoring. Failure of the testing system to detect the D_2 form may lead the clinician to the erroneous conclusion that additional supplementation is required, which could possibly result in hypervitaminosis D and hypercalcemia.

Two assays that have been widely used to measure 25(OH)D include a manual RIA and an automated LIAISON® chemiluminescent immunoassay (DiaSorin, Stillwater, Minnesota). Both assays utilize an antibody-based detection system with 100% recognition of both the D_2 and D_3 forms. However, in the last few years the state of the art assay method utilizes LC-MS and MS. This assay measures both forms of 25(OH)D with high accuracy and precision, as well as high throughput. In comparisons with LC-MS and MS as the gold standard, several automated assays in clinical use were variable in terms of performance. However, the LIASON system performed well.[106]

Several biochemical markers of bone turnover are measurable and may be useful in assessing bone formation and resorption (Box 34.1).[107] Improved immunoassays have increased their role in studying bone metabolism and clinical consequences of osteoporosis. The most sensitive and specific markers of bone formation include serum bone alkaline phosphatase, total osteocalcin, and the procollagen type I N-terminal propeptide assay. Among the various markers of bone resorption, serum C-terminal cross-linked telopeptides are the most sensitive and specific. Markers of bone turnover can be used to predict the rate of bone loss in post-menopausal women and can also be used to assess the risk of fractures. In osteoporosis-treatment studies, markers of bone turnover appear even more strongly associated with fracture risk reduction than bone mineral density (BMD).[108]

Box 34.1 Markers of Bone Turnover

FORMATION (SERUM)
- Bone alkaline phosphatase
- Osteocalcin
- Propeptides of type I collagen:
  - Carboxyterminal (PICP)
  - Aminoterminal (PINP)

RESORPTION (SERUM)
- Telopeptides of collagen cross-links:
  - Carboxytelopeptides (CTX)
  - Aminotelopeptides (NTX)
  - Tartrate resistant acid phosphatase (TRAP)

RESORPTION (URINE)
- Pyridinolines cross-links (PyD)
- Deoxypiridinolines cross-links (DpD)
- CTX
- NTX
- Hydroxyproline

Methods for Measurement of Bone Density

Measurement of bone density is essential for the diagnosis of osteopenia and osteoporosis. While many methods have been widely used, including ultrasound evaluation of the calcaneus, a precise determination of bone density requires DEXA. The lumbar spine, the hip, and the femoral/neck are the most important regions to be evaluated. The data are expressed as a T-score, which provides the number of standard deviations below the mean peak bone density of a normal adult. Values between −1.0 and −2.5 are indicative of osteopenia, and values less than −2.5 indicate osteoporosis. DEXA of peripheral bones (e.g., forearm) is possible and is sometimes used as an additional assessment, particularly in metabolical disease. Osteoarthritis and other degenerative bone diseases may result in artificially higher T scores, therefore this condition can be suspected when there is a discrepancy in T scores between different lumbar vertebrate in the spine.

Measurement and Blood Levels of Thyroid Hormones

Circulating TSH is measured by two-site non-competitive immunometric assays (IMAs) utilizing enzymes, luminescent compounds, or ^{125}I as labels.[109] These assays have completely replaced the original RIAs because of the need for greater sensitivity to distinguish the low levels in hyperthyroid individuals from suppressed levels carrying some other significance.

Normal serum TSH ranges between 0.5 and 4.0 μU/mL. Values between 0.1 and 0.45 and 4.5 and 10 μU/mL, although abnormal are usually associated with normal levels of thyroid hormones.[110] On the basis of population based large surveys, a much narrower range of TSH levels, between 0.3 and 2.5 μU/mL has been suggested.[111] These observations have been confirmed by longitudinal studies that have shown a low risk of developing hypothyroidism (defined as TSH greater than 4 μU/mL) in subjects with a baseline TSH of 2.5 or less followed for 13 years but a significant risk in subjects presenting with TSH levels between 2.5 and 4 μU/mL (mostly if antithyroid antibodies are also present).[111] However, because increased morbidity is not present in patients with these mild TSH elevations and TSH determinations are subject to modulations due to endogenous and exogenous factors including substantial variations due to the laboratory methods, there is a general agreement that therapeutic intervention should not be considered when TSH levels are below 4 μU/mL. In such patients, only careful follow up is needed. Nevertheless, there remains some controversy about treating women with infertility who have values above 2.5 μU/mL. While this has not been definitively settled, there are some data suggesting that treatment outcomes in infertility are not different with values less than 2.5 and greater than 2.5 μU/mL.

During pregnancy, it is particularly important to maintain serum levels of TSH in the strict normal range (less than 2.5 μU/mL) because subclinical maternal hypothyroidism is associated with complications of pregnancy and adverse effects on the fetus.[112-113] The upper range in pregnancy is 2.5 μU/mL, in part because of some TSH activity afforded by hCG, particularly in the first trimester. In these women, l-thyroxine at low doses is started immediately at the beginning of pregnancy and serum TSH is monitored each month, increasing the treatment if TSH levels increase. For a more complete evaluation of thyroid function the measurement of thyroid hormones is necessary. Circulating total thyroxine (T_4) and triiodothyronine (T_3) and their free fractions may be measured. Many laboratories prefer to measure only total T_4, but in some conditions (such as pregnancy), free thyroid hormones have to be evaluated. Any condition that increases circulating thyroxine-binding globulin (TBG)—as occurs in pregnancy and with estrogen treatment—also increases total thyroid hormone. During pregnancy, TBG increases approximately 2.5 times, with peak levels at 15 to 20 weeks' gestation and normal T_4 levels are higher than 7.8 μg/dL. Free thyroid hormones remain in the normal range; the only exception being late in the first trimester, when a transient increase may occur.

The most precise determination of free T_4 is obtained by equilibrium dialysis, while some commercial RIA kits—which use a T_4 analogue—may give inaccurate results.[114]

Because the most common causes of thyroid disorders are autoimmune disorders, assay of thyroid autoantibodies is often useful.[115] Several autoantibodies may be measured but the most used in clinical practice are the measurements of thyroid peroxidase antibody (TPO) and thyroid stimulating immunoglobulins (TRab or TSI). TPO are useful for diagnosing autoimmune chronic thyroiditis but may be increased also in Graves disease. With improved immunoassays and high sensitivity, the TRab assay has become a hallmark in the diagnosis of Graves disease.[116] During pregnancy, the TRab assay also may be useful to predict neonatal Graves disease.

Thyrotropin-Releasing Hormone (TRH) Stimulation Test

With the improvement and sensitivity of TSH assays, the TRH stimulation test is not required in the evaluation of primary hypothyroidism or hyperthyroidism with low TSH levels. The test is still useful in the evaluation of central hypothyroidism, in rare patients with TSH-dependent hyperthyroidism, and in patients with pituitary tumors.

After an overnight fast, a 200-μg dose of TRH is administered intravenously. Blood samples for TSH are then taken

at 0, 30, and 60 minutes. The mean peak TSH response is a value 8 to 9.5 times the basal values; however, the range in euthyroid subjects is large (3 to 23 times baseline).

Evaluation of Glucocorticoids and Adrenal Function

Cortisol, the main glucocorticoid produced by the adrenal, is conveniently measured by immunoassay. Several corticosteroids, including prednisone, prednisolone, and cortisone, cross-react in the assay.[117] Because cortisol secretion is highly pulsatile and exhibits circadian variations, with the highest values occurring in the morning and the lowest levels in the late evening, a single random serum cortisol determination is not diagnostic for adrenal disorders.

Plasma ACTH is measured by immunoradiometric assays. Plasma ACTH levels over 100 pg/mL in the presence of low serum cortisol are suggestive of primary adrenal insufficiency (Addison syndrome).[118] In patients with Cushing syndrome, a plasma ACTH level above 20 pg/mL at 9 AM is highly indicative of ACTH-dependent Cushing syndrome. A level below 10 pg/mL suggests ACTH-independence and primary adrenal pathology.[119] However, in many individuals, the evaluation of blood ACTH and cortisol are not sufficient to diagnose adrenal disorders; therefore specific diagnostic procedures have to be performed.

Diagnosis of Pathological Hypercortisolism (Cushing Syndrome)

Cushing syndrome results from prolonged and inappropriately high exposure of tissues to glucocorticoids. The clinical features are variable and include abnormal fat distribution, proximal muscle weakness, wide purple striae, and decreased linear growth with continued weight gain in a child. Clinical presentation of the disorder may be florid and in this case the diagnosis is usually straightforward. However, the diagnosis is often difficult particularly in states of mild or cyclical or periodical hypercortisolism (Fig. 34.14).

Several tests have been used for diagnosing Cushing syndrome, but none has proven fully capable of distinguishing all affected patients from normal and/or pseudo-Cushing individuals. Three first-line diagnostic tests are currently used to screen for Cushing syndrome:[120-121] measurement of free cortisol in 24-hour urine (UFC), cortisol suppression by low doses of dexamethasone, and assessment of cortisol circadian rhythm using late-night serum and/or salivary cortisol. While in the past, urinary free cortisol assessment has been the screening test of choice, late-night salivary cortisol is actually preferred.

If the diagnosis is confirmed, the search for the responsible tumor then relies on the assessment of corticotroph function, and imaging studies[122]: suppressed ACTH plasma levels indicate an 'adrenal' source of the hypercortisolism while the distinction between unilateral adrenal tumour, primary pigmented nodular adrenal dysplasia associated with Carney complex, and ACTH-independent macronodular adrenocortical hyperplasia will require a careful imaging study by CT.

Measurable or increased ACTH plasma levels indicate either Cushing's disease (pituitary adenoma) or the ectopic ACTH syndrome and requires further dynamic tests (high-dose dexamethasone suppression test and/or the CRH test). When the results of these tests are equivocal, and/or the imaging is nondiagnostic, it may be difficult to distinguish between the two. This is the situation where sampling ACTH plasma levels in the inferior petrosal sinus may be necessary.

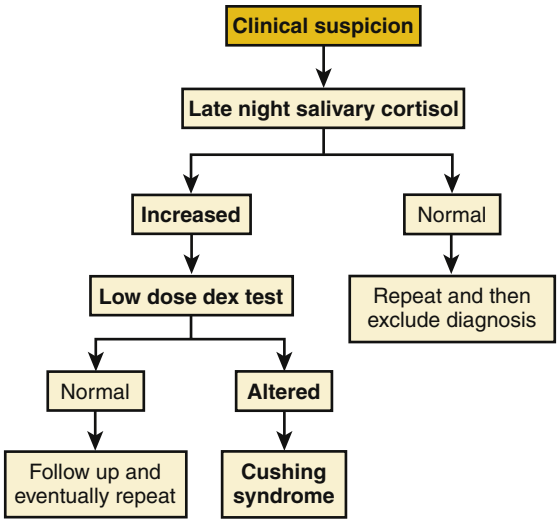


FIGURE 34.14 Diagnostic algorithm for Cushing syndrome

In general for the clinician the main problem is to distinguish patients with pathological hypercortisolism from normal subjects. Therefore, we will describe shortly only the screening tests. Because the complexity of the complete diagnosis, if pathological hypercortisolism has been demonstrated, the patient should be evaluated by a specialized endocrinological center.

Late Night Salivary Cortisol Measurement

Loss of circadian rhythmicity is the most sensitive indicator of pathological hypercortisolism. Ideally, midnight sleeping cortisol should be a very sensitive indicator of Cushing syndrome but it is impractical for screening purposes. Assessment of late-night salivary cortisol is being increasingly considered as the simplest and more convenient outpatient procedure. This method is also useful for the diagnosis of early or mild Cushing syndrome.[123]

Saliva samples are collected at LATE-NIGHT (11 PM) in a commercially available device and assayed for CORTISOL by radioimmunoassay. The cut-off level of 1.5 μg/mL shows high sensitivity and specificity in both subclinical and overt Cushing syndrome.

Urinary Free Cortisol

Twenty-four-hour urinary free cortisol provides an index of cortisol secretion during the day. While the upper normal range of urinary cortisol should be 50 to 60 μg/day, some commercial assays report normal ranges as high as 100 to 130 μg/day. Urinary free cortisol may be measured by high-performance liquid chromatography combined with RIA or a competitive binding assay. In general, 3 to 4 completely normal 24 hours collections exclude the diagnosis, while a value more than 4 times the upper limit of the normal suggests the finding of Cushing syndrome. Usually urinary creatinine is also measured to account for the completeness of the urinary collection.

The methodological difficulties in 24-hour urine collection and in assay precision have rendered this screening technique increasingly unpopular.[124,125] Furthermore, the increased prevalence of mild, preclinical, or cyclic Cushing syndrome along with the fact that cortisol is not uniformly secreted during the day do not support its use as a screening test, although strikingly high levels can be useful.

Low Dose Dexamethasone Suppression Test

As a simple screening test for hypercortisolism, overnight dexamethasone suppression has been largely used. Dexamethasone (1 mg) is given at 11 PM and serum cortisol is measured the morning after. Serum cortisol below 5 μg/dL generally indicates a normal response, but one out of five of patients with Cushing syndrome have been shown to suppress serum cortisol to these levels after overnight dexamethasone. Although lower cut-offs have been suggested, the specificity of the test remains low and is therefore not helpful for screening purposes.

The classic low-dose dexamethasone suppression test has greater diagnostic accuracy and may be used in screening of Cushing syndrome, often together with late night salivary cortisol measurement. The test is performed reliably even in outpatients and involves the administration of dexamethasone, 0.5 mg every 6 hours for 2 days, then collection of blood for cortisol measurement on the morning of the third day. Serum cortisol levels greater than 1.8 μg/dL is a sensitive indicator of Cushing syndrome. Some drugs that increase hepatic clearance of dexamethasone, such as carbamazepine, phenytoin, nifedipine, rifampicin, and phenobarbital may interfere with the dexamethasone tests and should be stopped prior to investigation. Estrogens may give falsely elevated cortisol levels (increasing CBG) and should be stopped at least 6 weeks before testing.

Tests for Adrenal Insufficiency

ACTH Test

Stimulation with ACTH (250 μg), administered intravenously with measurements of cortisol at 0, 30, and 60 minutes, is an excellent test for primary adrenocortical insufficiency. Serum cortisol levels lower than 13 μg/dL after ACTH ensure the diagnosis of adrenal glucocorticoid insufficiency. Levels between 13 and 18 μg/dL are intermediate and require additional diagnostic evaluation. Serum cortisol levels higher than 18 μg/dL after ACTH stimulation excludes the diagnosis. Low-dose ACTH (1 μg intravenously) may also be used, and researchers have suggested that this protocol uncovers milder forms of adrenal insufficiency that are not diagnosed with the classic ACTH stimulation test.

Tests for Secondary Adrenal Insufficiency

Insulin Tolerance Test

After an overnight fast, regular insulin (0.1 U/kg) is infused intravenously over 1 minute. Blood glucose and cortisol are measured at 0, 15, 30, 45, 60, 75, and 90 minutes. Once hypoglycemia (blood glucose of less than 40 mg/dL) has to be documented, serum cortisol levels over 18 g/dL excludes adrenal insufficiency. The test is contraindicated in elderly patients and in patients with cardiovascular, psychiatric, or seizure disorders.

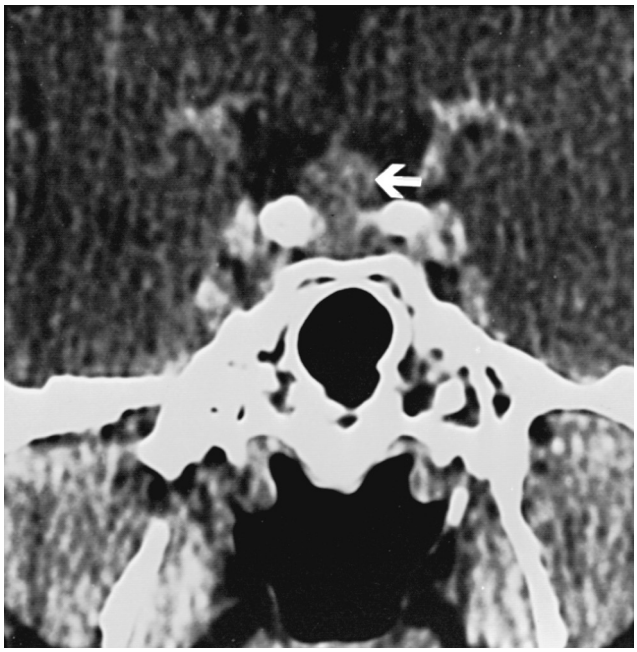


FIGURE 34.15 Typical close-up coronal view of a pituitary microadenoma by computed tomography (CT). The neoplasm cannot be distinguished from normal pituitary. Thus, the arrow indicates an enlarged abnormal pituitary bulging superiorly. The stalk is seen superior to the gland in the midline. The bone appears white on CT scan. Magnetic resonance imaging may allow better resolution of neoplasms from normal pituitary. *(Adapted from Rebar RW. Practical evaluation of hormonal status. In Yen SC, Jaffe RB, Barbieri RL [eds].* Reproductive Endocrinology, Physiology, Pathophysiology, and Clinical Management, *4th ed. Philadelphia, WB Saunders, 1999, p 709.)*

Metyrapone Test

Metyrapone, which blocks 11β-hydroxylation, is used to test the adrenal response to endogenous ACTH. This agent further reduces serum cortisol, however, and may induce adrenal insufficiency. For this reason, many endocrinologists prefer to perform an ITT test. Metyrapone is generally used when an ITT test is contraindicated. Metyrapone, 30 mg/kg, is given orally at midnight with a snack; a blood sample for cortisol and 11-deoxycortisol are obtained at 8 AM the following morning. After the blood sample, a prophylactic dose of prednisone or hydrocortisone is usually given. To be valid, cortisol has to decrease to less than 5 μg/dL. Peak values of 11-deoxycortisol below 7 μg/dL suggest reduced activity of the pituitary adrenal axis.

Diagnostic Procedure in Suspected Pituitary Tumors

Patients with pituitary tumors seek evaluation because of the symptoms (e.g., headache, diabetes insipidus, visual field alterations) or because of the finding of increased hormone secretion (e.g., acromegaly Cushing syndrome, amenorrhea with or without galactorrhea). Once a pituitary lesion is suspected, radiographical investigation is required.

Magnetic resonance imaging (MRI) with gadolinium enhancement is the preferred technique to evaluate hypothalamic—pituitary lesions; however, CT scanning with intravenous contrast and thin coronal sections may also be used. Examples of MRI and CT techniques may be found in Figures 34.15 and 34.16. Table 34.2 lists the major MRI

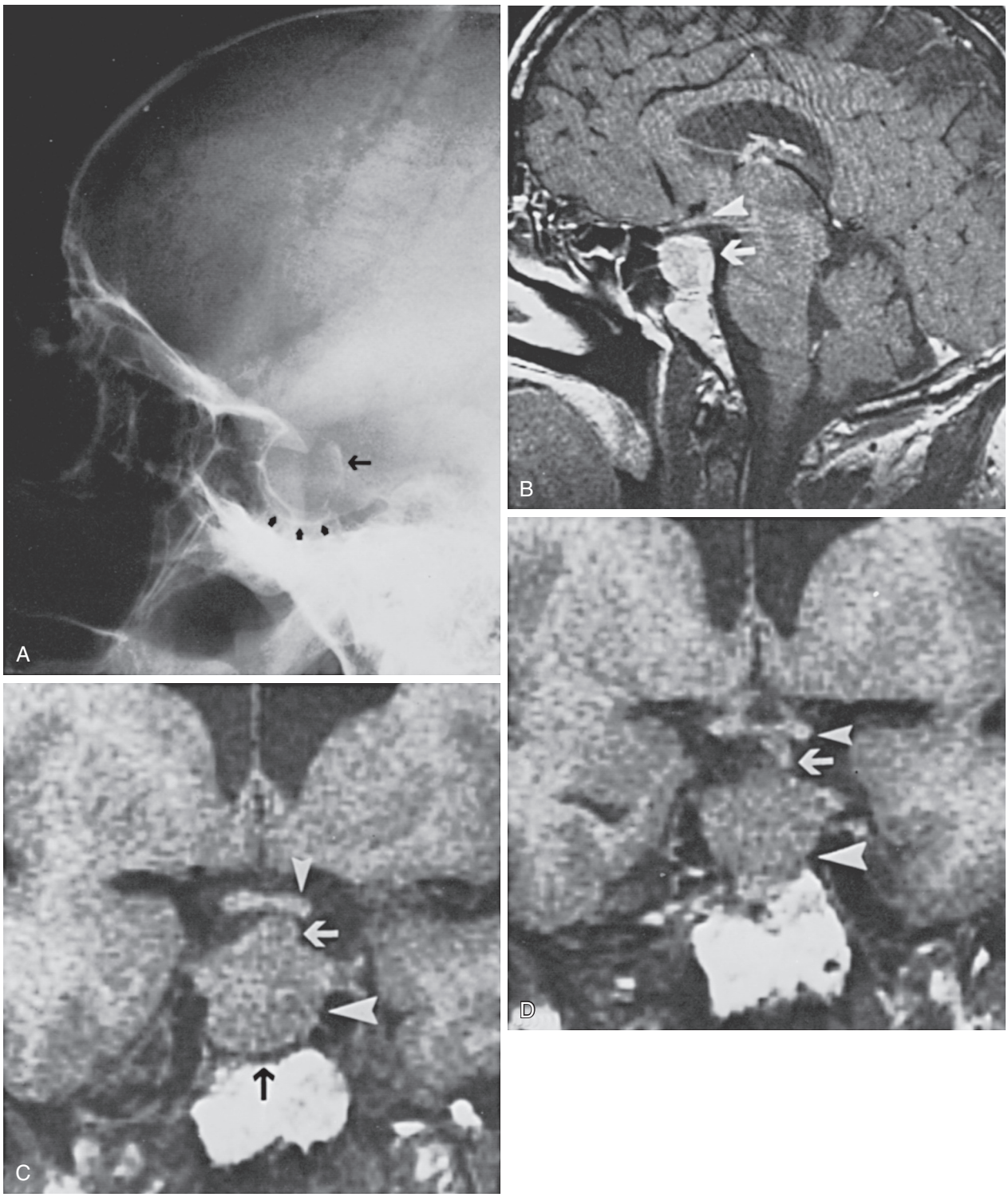


FIGURE 34.16 Selected views in a 39-year-old woman with a probable nonsecreting neoplasm who has amenorrhea, galactorrhea, and prolactin levels of approximately 50 ng/mL. The radiographs suggest that the mild hyperprolactinemia is due to stalk compression. **A,** Lateral skull film showing ballooning of the sella turcica with a thin double floor *(small arrows)* and erosion of the clinoid processes posteriorly *(large arrow)*. **B,** Sagittal view of magnetic resonance imaging with contrast material showing the large pituitary tumor *(arrow)* and normally positioned optic chiasm *(arrowhead)*. **C,** Coronal view showing the large neoplasm *(large arrowhead)* bulging superiorly *(white arrow)* toward the optic chiasm *(small arrowhead)*. The *black arrow* shows the diaphragm sella. **D,** Another view of the neoplasm shows displacement of the optic chiasm *(arrow)* below the optic nerves and chiasm *(small arrowhead)*. The *large arrowhead* indicates the lesion. The diaphragm is indistinct and there is the suggestion of bony erosion. *(Adapted from Rebar RW. Practical evaluation of hormonal status. In Yen SC, Jaffe RB, Barbieri RL [eds].* Reproductive Endocrinology, Physiology, Pathophysiology, and Clinical Management, *4th ed. Philadelphia: WB Saunders, 1999, p 709.)*

Table 34.2 Magnetic Resonance Imaging Characteristics of Some Hypothalamic-Pituitary Lesions

Type of Mass	*MRI Images*	*MRI with Contrast Images*	*Particular Features*
Pituitary adenoma	Hypointense	Hypointense	Hyperintense with hemorrhage
Pituitary cyst	Hyperintense	No change	Cystic; may be hypointense if CSF is in the cyst
Craniopharyngioma	Hyperintense	No change	Cystic with iso-hypointense solid portions
Meningioma	Isointense	Hyperintense	Hyperostosis of adjacent bone

MRI, magnetic resonance imaging; CSF, cerebrospinal fluid.

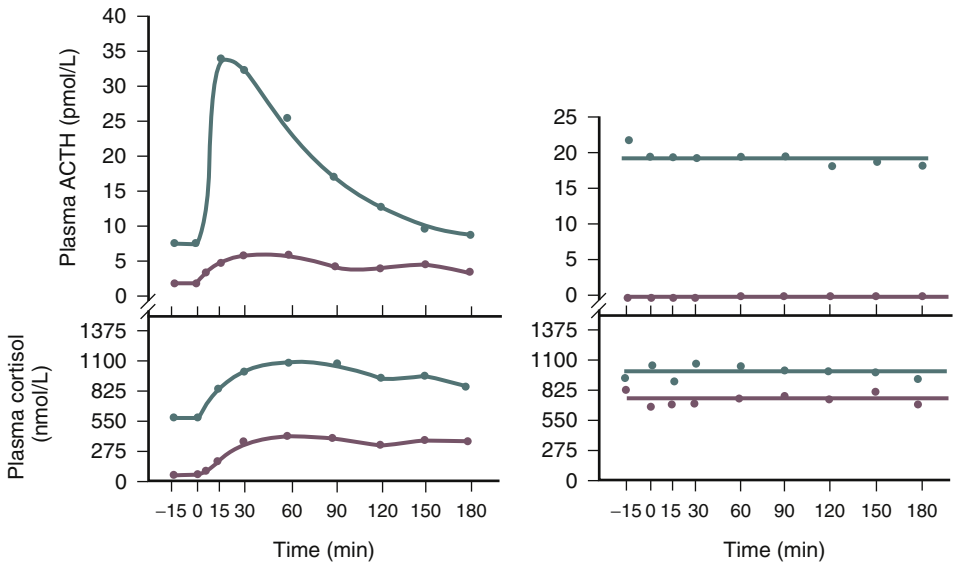


FIGURE 34.17 Adrenocorticotropin hormone (ACTH) response to corticotropin-releasing hormone. Colored lines represent different patients. *(Adapted from Kaye TB, Crapo L. Cushing's syndrome: an update on diagnostic tests.* Ann Intern Med *112:435-444, 1990.)*

characteristics of the more common hypothalamic-pituitary lesions.[126-128] Dynamic pituitary MRI may increase the number of microadenomas that may be visualized.

If hypothalamic or pituitary lesions are found, detailed evaluation of all pituitary hormones should be performed. Regardless of the size of the lesion, patients should initially be evaluated for hormone hypersecretion. Serum PRL, GH with IGF-1, and 24-hour urinary free cortisol could be used for the initial screening. In selected patients, basal and TRH-stimulated gonadotropins and α-subunits should be measured for the evaluation of possible gonadotroph adenomas. Hyperthyroid patients should be screened for rare TSH adenomas. In a large series of 1043 pituitary tumors, the most common pituitary hormone secreting adenomas were prolactinomas (27.2%), followed by GH-secreting adenomas (14%), GH- and PRL-secreting adenomas (8.4%), and ACTH-secreting adenomas (8%).

Some pituitary adenomas produce only α-subunit, a component of glycoprotein pituitary hormones (LH, FSH, TSH). Therefore, in patients with pituitary tumors and no increase of pituitary hormones, the assay of α-subunit may be used. The subunit is measured by an RIA method and in young adults the circulating values are less than 1 ng/mL. Some gonadotropin secreting pituitary tumours produce high quantities of α-subunit and this assay may be useful in postmenopausal women with pituitary adenomas. However, it has to be remembered that normal values of α-subunit are higher in postmenopausal women (less than 3.6 ng/mL) than in young women.

Pituitary microadenomas do not generally cause disruption of pituitary function; therefore, pituitary hypofunction should be assessed only in those patients with large sellar masses that do not have increased secretion. GnRH, TRH, growth hormone-releasing hormone, and CRH tests (all four releasing factors) may be performed together for contemporaneous evaluation of gonadotropins TSH, PRL, GH, and ACTH[129] (Fig. 34.17).

Pelvic Imaging Evaluation

Pelvic ultrasound has become an extremely important tool for the reproductive endocrinologist. Assessment of ovarian function and monitoring for endometrial development are useful for clinical management and have been discussed elsewhere (see Chapters 9, 20, and 21). The finding of polycystic ovaries on ultrasound now have been included as a criterion for the diagnosis of PCOS and therefore a pelvic ultrasound evaluation should be performed in all patients with androgen excess, as well as those with persistently irregular cycles.

The ultrasound diagnosis of polycystic ovaries has shifted somewhat from the classical criteria to the newer Rotterdam criteria where ovarian size, alone, is sufficient for the diagnosis;[130-132] this, too, has been scaled back recently from requiring a size of 10 cc to that of 7.5 cc.[131-134] Figure 34.18 depicts the transvaginal ultrasound appearance of a polycystic ovary with assessment of ovarian blood flow, which is increased in PCOS. If androgen excess is the central issue in the disorder of PCOS, then a more meaningful criterion in the ovarian stromal and total surface area (S/F) ratio.[133] As shown in the Figure 34.19, some authors have suggested that a cut-off value to 0.32 correlate well with the presence of androgen excess (values greater than this being significant).[134]

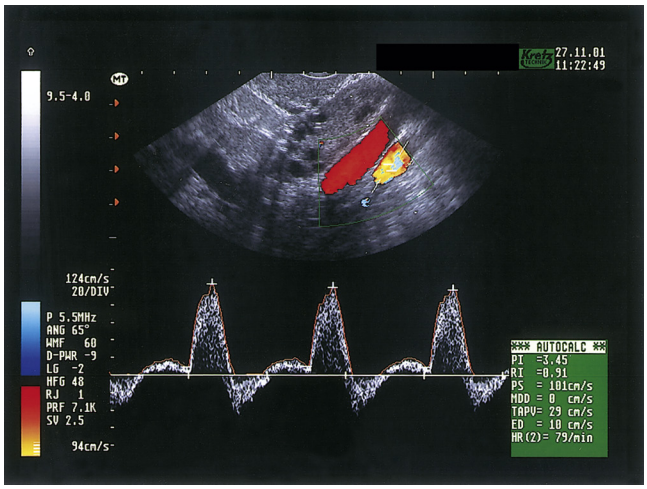


FIGURE 34.18 Typical color Doppler ultrasound of a polycystic ovary showing increased blood flow.



FIGURE 34.19 Example of median ovarian section with outlined ovarian and stroma areas. A1 is the total area and A2 is the stroma area. *(From Fulghesu AM, Angioni S, Frau E, et al. Ultrasound in polycystic ovary syndrome—the measuring of ovarian stroma and relationship with circulating androgens: results of a multicentric study.* Hum Reprod *22:2501–2508, 2007.)*

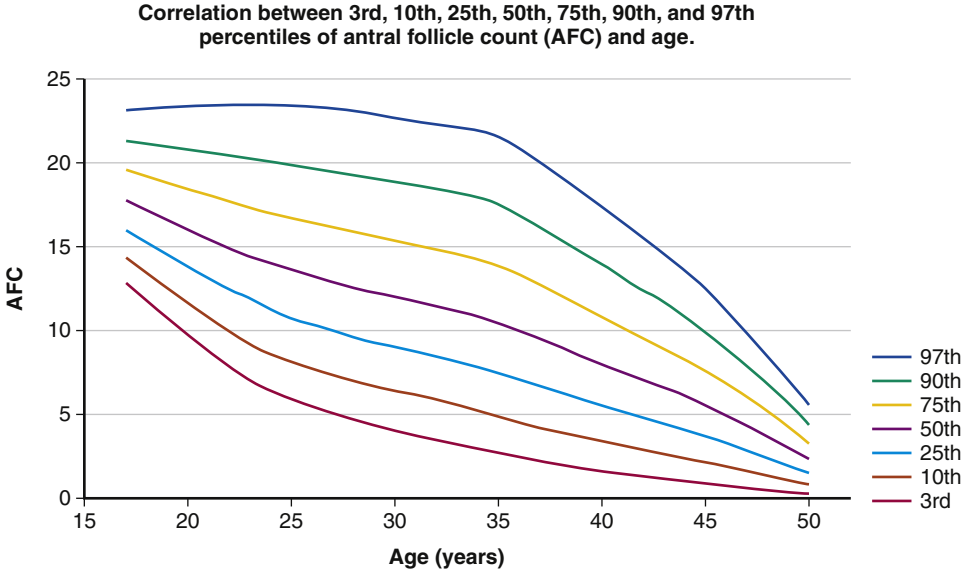


FIGURE 34.20 McGill nomogram for antral follicle count (AFC). *(From Almog B, Shehata F, Shalom-Paz E, et al:* Fertil Steril *95[2] 663-666, 2011).*

Using three-dimensional ultrasound (3-D US), which has also been found to be useful for the diagnosis of PCOS, müllerian anomalies can be detected. While routine two-dimensional ultrasound is not able to distinguish between various müllerian anomalies, 3D US is as sensitive as MRI in this regard. Nevertheless, MRI may still have an advantage over US when differentiating between fibroids and adenomyosis.

More recently, ultrasound evaluation of antral follicle counts has been used as an assessment of ovarian reserve. In this regard, current data suggest that it has equal efficacy to serum AMH, and yet the combination of both does not necessarily improve predictability of ovarian response to stimulation. An effort has been made to standardize the technique because of its more subjective nature. By consensus it has been suggested that it be carried out on days 2 to 4 of the cycle, have well trained personnel using vaginal ultrasound and a 7 MHz transducer.[135] Systematic counting of all follicular structures should be carried out, and then those over 10 mm should be subtracted.[136] Each ovary should be measured in turn. A normal AFC is usually greater than 8, and age specific nomograms have been established (Fig. 34.20).[136]

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 35

Pelvic Imaging in Reproductive Endocrinology

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Vaginal Ultrasound and In Vitro *Fertilization*

The advent of vaginal ultrasound a quarter century ago revolutionized imaging of the pelvic cavity and in particular visualization of the ovaries and uterus. A limiting principal in ultrasound is that lower frequency ultrasound has greater penetration (less attenuation) but lower spatial resolution, while higher frequency ultrasound has lower penetration (greater attenuation) but greater spatial resolution. Vaginal ultrasound takes advantage of the ability to place a probe close to the structures of interest. This permits the use of higher frequency ultrasound and improves the resolution over that possible with lower frequency abdominal ultrasound probes. The original purpose of vaginal ultrasound was not diagnostic imaging, but to simplify oocyte aspiration for *in vitro* fertilization (IVF), which was originally performed by a transvesical route.[1] To facilitate this process, probes were designed for vaginal use and equipped with needle holders to permit transvaginal oocyte aspiration.[2-4] The practical advantages of vaginal ultrasound for guiding oocyte retrieval were obvious and it rapidly became the primary mode of oocyte retrieval.[5]

Improvements in image resolution that resulted from the use of high-frequency probes sparked an instant interest for use of vaginal ultrasound in general gynecology.[6-8] Subsequently, all the technological improvements incorporated into general ultrasound equipment, such as pulsed, color and power Doppler functions,[9,10] and later, three-dimensional (3D) capabilities[11,12] became available on vaginal probes. Ultimately, the increase in image resolution provided by vaginal ultrasound and its multiple refinements allowed for meticulous assessment of parameters that reflect the anatomical characteristics of pelvic organs, but also their functional status.[13] For example, the antral follicular count (AFC) stands at par with hormonal parameters (anti-müllerian hormone [AMH] and/or day-3 follicle stimulating hormone [FSH] and estradiol [E_2] levels) for assessing the functional state of the ovaries or *ovarian reserve*, and vaginal ultrasound is the main tool for monitoring ovarian response and follicular growth in controlled ovarian stimulation (COS).[14]

Vaginal Ultrasound and Pelvic Examination

Beyond improving image resolution and quality, it rapidly became evident that vaginal ultrasound could provide information about functional interactions that exist between pelvic organs and nearby structures. This possibility stems from the fact that a vaginal ultrasound examination, as opposed to abdominal ultrasound, does not require a full bladder preparation. Consequently, it is possible to freely slide pelvic organs against one another, and move them about the pelvic cavity under direct ultrasound vision by gentle pressure applied to the vaginal probe. This approach offers a glimpse of the functional status of the pelvic cavity not available with static images.

- It can precisely determine the limits of pathological structures, such as an ovarian cyst. This is done by determining how the identified structure—the ovarian cyst in our example—moves with respect to other nearby organs when pelvic structures are mobilized by the vaginal probe. At times, the help of pressure exerted by an "abdominal hand" may be necessary in order to mobilize the organ(s) being studied.
- The ability to slide organs also provides clues about the possible presence of pelvic adhesions. For example, the ovary and nearby loops of bowel may move together when mobilized by the probe.
- While pelvic organs are mobilized by pressure exerted either by the vaginal probe and/or with the help of an abdominal hand, the examiner can assess pain that this maneuver may generate. The dynamic vision provided by vaginal ultrasound allows the examiner to pinpoint the cause of the pain and its anatomical location.

This functional dimension provides a true pelvic examination with a view, fusing various forms of information to provide a global assessment of pelvic status.

Technical Refinements of Vaginal Ultrasound: Doppler and 3D Reconstruction

As vaginal ultrasound progressively became a primary diagnostic tool in gynecology, two technical refinements have

come to enrich its capabilities and scope: Doppler-based analyses of organ perfusion and the *off-line* and later *built-in* 3D image reconstruction systems. In the case of 3D image reconstruction from saved 3D volumes, the technical novelty also offers new possibilities for improving the quality control of diagnostic ultrasound through secondary exploration of saved 3D volumes.

Contrast-Enhanced Vision of the Uterus and Tubes

It struck many that the ultrasound images of the uterine cavity obtained in early pregnancy largely surpass in quality those of the nonpregnant uterus. The improved image quality of the pregnant as opposed to nonpregnant uterus was attributed to the presence of amniotic fluid, playing the role of a contrast enhancer that facilitates embryo visualization. Being sono-transparent like water, amniotic fluid creates a *black* interphase between the walls of the gestational sac and the developing embryo, which enhances image resolution.

To reproduce the early pregnancy conditions, infusion of isotonic saline or other solutions into the uterine cavity of the nonpregnant uterus was introduced to create greater contrast. This procedure has several designations including saline infusion sonography (SIS), hysterosonography (HySo), or hysterosalpingocontrastsonography (HyCoSy).[15,16] Numerous variants of the original procedure have been proposed that employ either sono-transparent[17,18] or sono-refractory contrast media, such as Echovist®, Levovist® or Albumex®,[19-21] which provide *black* and *white* contrast enhancement, respectively. Black contrast exploration of the uterine cavity has been found to be particularly helpful for exploring uterine malformations,[22] favorably comparing with magnetic resonance imaging (MRI).[23] While *black* contrast is superior for exploring the uterine cavity, *white* contrast offers the advantage of imaging the fallopian tubes.[21] Another approach to contrast-enhanced ultrasound utilizes microbubbles manufactured with a hydrophilic shell of albumin, galactose, lipids or polymers and a hydrophobic gas core. When the gas bubbles interact with ultrasound energy, they alternately compress and expand, generating a strong, unique signal. Ultrasound devices can be specially tuned to enhance the detection of these unique signals.

Endometriosis and Adenomyosis: Ultrasound and Magnetic Resonance Imaging (MRI)

Endometriosis can affect all pelvic organs, associated with various degrees of extension and infiltration. Exploring endometriosis often requires MRI. Likewise, a variant of endometriosis, adenomyosis—the diffuse and focal forms—can be reliably diagnosed on MRI with good correlation with anatomical findings.

Functional Pelvic Imaging in Reproductive Endocrinology and Infertility

Anatomy and Morphological Measurements

The two primary constituents of the uterus—the outer muscle or myometrium and its mucosa, the endometrium—present an interphase that is easily identifiable on ultrasound. The lower end of the endometrium delineates the internal os of the cervix, which provides a landmark for individual measurements of the cervix and uterine corpus. The total length of the cervical canal is measured after identifying the external os of the cervix. The endometrium, which is hormonally sensitive, changes in thickness, volume, and aspect (echogenicity) during the menstrual cycle, and in response to hormonal treatments.

The myometrium is composed of three layers.[24,25] The inner, or sub endometrial layer of the myometrium, is of müellerian origin. It is endowed with estrogen receptors (ER) and progesterone receptors (PR) showing cyclical changes that mimic those encountered in the endometrium.[26] The two outer nonmüllerian layers of the myometrium have constant concentrations of ER and PR throughout the menstrual cycle.[24] In the nonpregnant uterus, the outer layer of the myometrium participates in uterine contractions encountered at the time of menses in response to progesterone withdrawal, but not in the retrograde contractions seen in the late follicular phase of the menstrual cycle. Disturbances of the contractile processes at the time of menses may cause dysmenorrhea or endometriosis, whereas alterations in the follicular phase may interfere with sperm transport.[27]

Current imaging is capable of identifying the subendometrial layer of the myometrium where the peristaltic contractions that culminate in the late follicular phase are initiated. High-resolution vaginal ultrasound defines this myometrial segment, the intermediate layer of lighter echogenic appearance, or *subendometrial halo*. It lies between the echoic basal layer of the endometrium and the underlying myometrium. Similarly, but using a different terminology, MRI identifies the subendometrial layer of the myometrium as a hypointense layer that separates the endometrium from the myometrium on T_2-weighed images or the *junctional zone* (JZ) (see section on endometriosis and adenomyosis).[28]

Video recordings have identified that uterine contractions during the late follicular phase under the stimulus of rising E_2 levels originate from the *subendometrial halo* or JZ area.[29,30] In an ex-vivo trial, Lesny and colleagues[29] studied six uteri obtained from fertile women 32.9 to 48.5 years old. Following hysterectomy, all uteri were needle-biopsied in the subendometrial halo under ultrasound guidance. The results indicate that the subendometrial halo, or JZ area, is a distinct section of the myometrium with increased vascularitiy and more tightly packed myometrial cells that is now also identifiable on ultrasound.[31] As discussed later, endometriosis and adenomyosis are thought to be associated with dysfunction of the endometrial-subendometrial unit.[24]

The boundaries between the cervix and the uterine corpus, and between the endometrium and myometrium, are easily identifiable, so that the uterus can be measured on ultrasound scans (Fig. 35.1). In adult women, uterine length ranges from 5 to 8 cm[32] following growth at the time of puberty. In a prospective trial examining 139 girls between the ages of 1 and 13 years of age with radiological bone age measurements, uterine volume was calculated from transabdominal scan images taking the measured values of length, width, and depth and using the ellipse formula.[33] Uterine measurements and volume were markedly smaller in pretelarchial girls at 1.8 ± 1.2 cm^3 as compared to 8.1 ± 6.6 cm^3 after telarche.[34] In a large prospective trial conducted on 380 school girls 6 to18 years of age, Holm

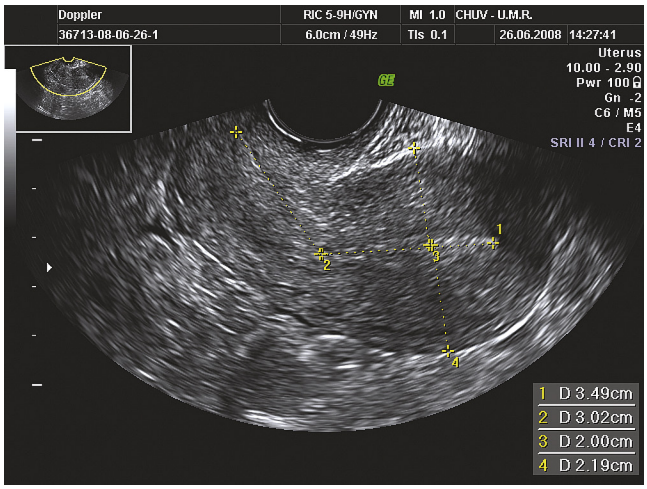


FIGURE 35.1 Uterine size. The interphase between the endometrium and myometrium is identifiable on ultrasound images. The lower end of endometrial development identifies the internal os of the cervix, which delineates the limits of the uterine corpus and cervix. The yellow marks define the cavity interphase in the inner cervix and uterine corpus. The two outer marks define the outer uterine limits at the level of the corpus.



FIGURE 35.2 Effects of *in utero* exposure to diethylstilbestrol (DES): T-shaped uterus with characteristic mid-uterine ridges.

and colleagues observed no age-related differences in uterine and ovarian size in 44 prepubertal girls, whereas a direct correlation with Tanner stages was observed in 163 prepubertal girls.[35] A further enlargement of the uterus was observed between Tanner stage 3 and adult girls (mean age of 19).

Looking at 114 premenarchial girls, Orsini and colleagues observed that until the age of 7, the uterus and cervix were of similar size with the corpus gradually becoming larger than the cervix thereafter.[36] These authors observed that the uterus continued to grow after menarche for several years, with uterine size correlating with the number of postmenarchial years, but not with height, a finding that has been challenged by others.[35]

There are clinical conditions in which the uterus was believed to be smaller than normal, a finding not always verified by measurements. Doerr and colleagues report on a series of 75 women affected by Turner syndrome. Of 50 women with a karyotype 45,X0 who had received estrogen replacement, 42 (84%) had a normal size uterus, whereas it was smaller (length less than 5 cm) in the remaining 8 women (16%).[37] In this study, uterine size might have been affected by the age at which estrogen therapy was initiated, but showed no correlation with other factors, notably the final height.[37] The impact of growth hormone (GH) treatment on uterine size remains a matter for debate, with Sampaolo and colleagues[38] showing a positive effect whereas, Snajderova and colleagues found none.[39]

We know from Herbst and colleagues[40,41] that *in utero* exposure to diethylstilbestrol (DES) may affect the genitalia. These authors linked seven cases of adenocarcinoma of the vagina, an otherwise excessively rare tumor, with *in utero* DES exposure. All these cases occurred at a single hospital in DES-exposed women between the ages of 7 and 22 years,[41] a finding later confirmed by others.[42] Further studies of DES-exposed women revealed many benign morphological abnormalities of the genital tract. These abnormalities, stemming from epigenic alterations[43] of the *HOX* gene family[44] include transverse ridges in the vagina, vaginal adenosis, and morphological anomalies of the uterus, such as the characteristic T-shaped uterus (Fig. 35.2).[45] Viscomi and colleagues analyzed the echographic appearance of the uterus in cases of DES exposure.[46] An abnormal T-shape uterus was found in 3 of the 18 (16%) women exposed to DES, a percentage in agreement with an incidence of 12% observed on hysterosalpingogram (HSG).[47] Furthermore, Viscomi and colleagues reported a reduced uterine size with a mean uterine length of 6.8 ± 0.4 cm in 18 DES-exposed women as compared to 8.1 ± 0.8 cm in 20 age-matched controls, even in the absence of gross morphological anomalies.[47] Calculated uterine volume was 49.4 and 90 cm^3 in DES-exposed women and controls, respectively. Salle and colleagues have reported altered uterine vascularity in women exposed to DES, with higher uterine artery pulsatility (PI) and resistance index (RI), and an absence of the cycle related changes.[48] However, the true impact of these uterine flow differences are difficult to interpret.

Several authors have proposed surgical correction (metroplasty) of uterine anomalies related to DES exposure, including T-shaped distortion[49] or simple overall reduction of uterine size.[50] Although authors reporting on small series recount positive impressions about the impact of surgery on subsequent reproductive outcome,[51] the efficacy of these procedures remains to be rigorously evaluated.

The Uterine Cervix

Vaginal ultrasound studies can precisely delineate the anatomical limits of the cervix. The cervical canal can easily be identified. When aqueous mucus is produced—under the influence of E_2 in the follicular phase—the cervical canal appears sono-transparent and hypoechoic. Early in the luteal phase, the mucus changes its ionic constitution under the influence of progesterone and the canal appears hyperechogenic (white) on ultrasounds.

We use a semiquantitative grading system in which hyperechogenic mucus is either not seen (–) or seen and graded as +, ++, and +++ when a sonotransparent dilatation of the cervical canal can be measured at less than 1 mm, 1 to 2 mm,

and greater than 2 mm, respectively, an approach close, if not similar, to that of others.[52]

The presence of cervical mucus visible on ultrasound serves as a biomarker for estrogen exposure, as well as an indicator of the absence of progesterone effects.[21] This latter parameter is particularly helpful when following patients treated with assisted reproductive technologies (ART). Identifying sonotransprent (black) cervical mucus on ultrasound makes it possible to disregard increases in plasma progesterone levels. This biomarker of progesterone's effects is particularly handy when monitoring ovarian responses.[21] An abrupt disappearance of cervical mucus observed concomitantly with plasma progesterone elevation in late stages of controlled ovarian hyperstimulation (COH), as sometimes encountered in poor ovarian responders, is an ominous sign.[53] The sudden disappearance of cervical mucus on ultrasound associated with pre-human chorionic gnonadotropin (hCG) progesterone elevation constitutes grounds for canceling an ART cycle, or freezing embryos and deferring transfer.[54]

Changes in echographic characteristics of the cervix have received little attention.[55] As expected from visual examination of the outer cervix, 3D assessment of the whole cervix reveals size differences between nulliparus and multiparous women.[56]

Links between cervical length and the risk of preterm labor (PTL) and delivery have been evaluated with new image-based screening for PTL.[57-63] More pertinent to infertility is the early identification of cervical insufficiency and its corollary, clinical indications for preemptive cervical cerclage. This clinical topic remains controversial, in spite of the quality cervical length measurements and the number of well designed studies. A well-established indication for cervical length measurement in the decision for cervical cerclage is during the midtrimester in women with a past history of pregnancy losses due to cervical insufficiency and prior short cervix measurements on ultrasound.[64] Various technical refinements may help in assessing cervical length using sonoelastography[65] or harmonic imaging.[66] A recent study indicated that assessing cervical length for reinforcing an existing cerclage may actually be counterproductive.[67]

The Endometrium: Biomarker of the Hormonal Environment

Endometrial Thickness: a Biomarker for Estrogen Action

By convention, the endometrial thickness is measured on ultrasound from one myometrial-endometrial interphase to the next, on the thickest point of the endometrium (Fig. 35.3). This widely accepted practice amounts to measuring a double endometrial thickness, something that has become a universally acepted convention.

From being relatively thin at the time of menses, the endometrium progressively thickens during the proliferative phase of the menstrual cycle, commonly peaking at 7 to 9 mm on the day of the luteinizing hormone (LH) surge.[68-70] Prior to ovulation, the endometrium takes on a multilayered or three line appearance formed by the echogenic basal layers and the two hypoechoic functional layers, separated by the hyperechoic interphase of the virtual uterine cavity (Fig. 35.4). The increase in endometrial thickness seen

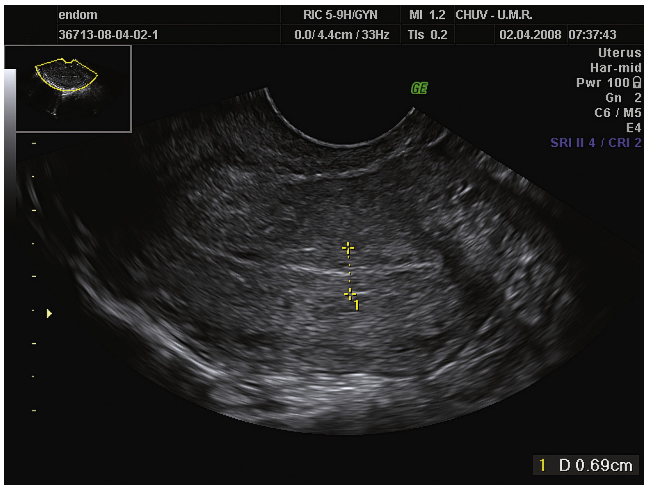


FIGURE 35.3 Endometrial thickness. Measurements are made from one endometrial to myometrial interphase to the one on the opposite side, therefore amounting to a double endometrial thickness measurement.

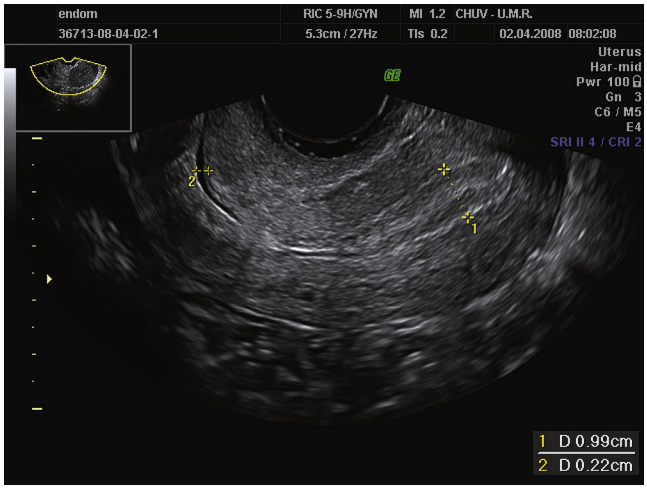


FIGURE 35.4 Hypoechogenic or type I endometrium: Echographic appearance of the endometrium during the follicular phase. Between the hyperechogenic layer of the endometrial basalis and the echogenic line of the virtual endometrial cavity lies a characteristically hypogenic endometrium. Taken together, this constitutes the characteristic three-layer pattern of the proliferative endometrium. On this figure one can also identify mucus present in the cervical canal. With our semi-quantitative grading system, this corresponds to a +++ score for the quantity of mucus because the mean diameter of the dilated cervical canal is dilated at 2.2 mm.

throughout the follicular phase represents the endometrial proliferation induced by E_2. This effect of estrogen, leading to the deployment of ER and PR, primes the endometrium for its response to progesterone. Under the influence of E_2, endometrial glands develop vertically with strait lumens. The donor-egg ART model revealed the respective roles of E_2 and progesterone in the priming of endometrial receptivity. Donor-egg ART taught us, in particular, the exceptional leeway that exists for the duration of the E_2 priming phase, which can range from 10[71] to 100 days without consequences on ART outcome.[72] Provided that E_2 priming is sufficient,[54] the duration of E_2 priming duration has

little impact on endometrial thickness or other endometrial parameters of estrogen effects on the uterus such as contractility.[73] As discussed later in this chapter, an endometrial thickness of ≥7 mm, or a volume of 2.9 mL,[74,75] is seen as reflecting sufficient endometrial priming by E_2. Extending treatment duration rather than increasing E_2 administration is seen as the better option for managing the excessively thin endometrium.[76]

McWilliams and Frattarelli looked at the dynamic changes in endometrial thickness in ART rather than static data (endometrial thickness at a single given point).[77] Fresh IVF cycles were studied in which the changes in endometrial thickness were analyzed from baseline to day 6 and from there to the day of hCG administration. Increments in endometrial thickness from baseline to day 6 and from day 6 to day of hCG were of 3.6 ± 2.4 and 2.0 ± 2.2 and of 2.3 ± 2.6 and 2.5 ± 2.1 in women who became pregnant (n = 70) and those who did not (n = 62), respectively. This comparison suggested that increments in endometrial thickness from baseline to day 6 were more predictive of pregnancy since there were no differences in the increment taking place from day 6 to the day of hCG. These dynamic changes in endometrial thickness point out the positive bias that exists with the quality of the ovarian response to COS, which can be wrongly interpretated as reflecting endometrial receptivity. This, therefore, constitutes a bias that must be remembered when interpreting an apparent link between endometrial thickness and endometrial receptivity[78] drawn from analyses of ART data. Ng and colleagues studied standardized endometrial preparations used for frozen embryo transfers (FET).[78a] These authors found no differences in endometrial thickness or Doppler-assessed blood flow between pregnant and nonpregnant women after receiving similar E_2 priming,[78a] a finding confirmed by others.[79]

Comparisons of endometrial thickness in the late follicular phase of the menstrual cycle, following physiological E_2 and progesterone replacement or mild ovarian stimulation for FET revealed similar findings.[80,81] This suggests that similar forms of hormonal priming have similar effects on endometrial thickness. Furthermore, exposure of the endometrium to E_2 levels that are more than 10 times higher than menstrual cycle values, as encountered in ART[82] and following the vaginal administration of 2 mg of E2/day[83] barely resulted in a 20% thicker endometrium. This suggests that E_2 priming that results from menstrual cycle levels of E_2 is nearly maximum. As noted earlier, the same leeway that exists for the amount of E_2 used for endometrial priming is also seen for the duration during which the priming is applied. In a retrospective analysis of their donor egg data, Pellicer's group showed that extending the E_2 priming phase for up to 100 days had no significant impact on ART outcome[84] and endometrial thickness[72] a finding confirmed by others.[85,86] The minimal effects of increased and/or prolonged E_2 priming on endometrial thickness conflicts with reports indicating that excessive ovarian responses to COS negatively impacts on pregnancy and embryo implantation rates.[84,87] To account for these divergent findings, we proposed that in strong responses to COS, it is the excessive production of ovarian factors other than E_2 that are responsible for any adverse endometrial effect, not a direct action of high E_2 levels per se.[54,88]

A consensus exists today that women whose endometrium is less than 7 mm in ART have markedly decreased chances of becoming pregnant,[89,90] particularly if no triple line pattern was seen.[91] In rare cases, albeit disturbing ones, this condition may persist, and be found in menstrual cycles and stimulated and E_2-supplemented cycles.[92] This condition is typically resistant to markedly elevated estrogenic exposure.[83] It may be encountered in the aftermath of total body irradiation or other cancer treatments,[93] or in the absence of readily available explanations. The treatments that have been proposed for these women include low dose aspirin,[94] an estriol challenge test,[95] locally active vasodilators,[96] and the combination of pentoxifylline and tocopherol (vitamin E).[92,97] The latter products, pentoxifylline and tocopherol, were tested on the grounds that they had been reported to be effective at reducing fibrosis induced by radiation therapy.[98] While some reports speak for possible improvements in results in donor-egg ART recipients,[99] no rigorous evidence for efficacy exists.

The possible poor predictive value of a thickened endometrium in ART reported by Casper's team[100] remains a matter for debate. While confirmed by some,[89] a larger group of publications failed to observe that a thicker endometrium has an overt negative impact on ART outcome.[101-104]

Endometrial Echogenicity: a Biomarker for Progesterone Action

Echogenicity reflects the different tissue interactions with ultrasound. Tissue echogenicity ranges from near sonotransparent as encountered in certain constituents of the body, notably water, to the highest echogenicity, produced by air (present in the bowel). On gray scale imaging, low echogenicity, as that of water, is commonly depicted in black, while high echogenicity appears in white. Typically, solid tissues with intermediate echogenic characteristics are depicted by degrees of gray with final grade depending on the water and/or air content and the number of such interphases. In the uterus, the echogenicity of the myometrium remains constant throughout the menstrual cycle and following various hormonal treatments. In contrast, the echogenicity of the endometrium varies between the follicular and luteal phases of the menstrual cycle and in response to exogenous hormones.

In a prospective trial on 80 infertile patients, Forrest and colleagues[105] showed that endometrial echogenicity increases after ovulation under the influence of progesterone. These authors were first to recognize the hypoechoic characteristics of the follicular phase endometrium (Fig. 35.4). The endometrium is characterized by a hypoechoic functional layer bordered by the hyperechoic basal layers with the center hyperechoic line produced by the interphase of the virtual endometrial cavity. Together, the hypoechoic functional layer of the endometrium and its outer limits produce the typical *three-line pattern*.

Changes in endometrial echogenicity are seen soon after ovulation. The endometrium progressively acquires its hyperechoic features characteristic of the luteal phase, starting from the endometrium basalis layer and expanding upward. Forrest and colleagues[105] observed a hyperechoic endometrium in 78% of women scanned during the luteal phase. Likewise, Templeton's group identified a similar

sequence of changes from hypoechogenic endometrium in the follicular phase to a primarily hyperechogenic pattern in the luteal phase of stimulated cycles.[68] The sonographic changes from the follicular phase to those characteristic of the luteal phase start to be visible within 48 hours of ovulation. The hyperechoic transformation of the functional layer of the endometrium takes 4 to 7 days to complete.

Grunfeld and colleagues studied the changes in endometrial echogenicity that took place in 18 women receiving sequential E_2 (0.2 to 04 mg/day, transdermally) and progesterone (50 mg/day IM) treatment in preparation for donor egg IVF.[106] Vaginal ultrasounds were performed starting before the onset of progesterone administration, and every 3 days thereafter until the 8th day of progesterone, when an endometrial biopsy was performed. Endometrial echogenicity was scored as pattern I, showing a triple-line pattern before the start of progesterone treatment, to pattern III, showing full hyperechoic transformation of the layer fucntionalis from the lamina basalis all the way to the endometrial lumen (Fig. 35.5). These authors concluded that endometrial thickness was a poor discriminator between advanced and retarded stromal changes on endometrial biopsies. Conversely, the degree of hyperechogenic changes on ultrasound (complete or partial) correlated with the degree of advancement of luteal changes (condensation, predecidualization) in the endometrial stroma. All women whose endometrial biopsy revealed delayed secretory changes in endometrial glands and stroma had ultrasounds showing partial hypoechogenic images (pattern II) (Fig. 35.6).

Analyzing endometrial echogenicity on the day after hCG administration in ART, Gonen and Casper observed a typical hypoechogenic triple-line pattern in 49% (60/123) of subjects.[107] In this subgroup of ART participants, the pregnancy rate was 30% (18/60), which was significantly higher than the figure obtained in the whole cohort (19.5%). Conversely, a full and partially hyperechogenic endometrium was found in 33% (41/123) and 18% (22/123) of women in whom pregnancy rates at 9% and 9.1%, respectively, were markedly lower than in their hypoechogenic counter parts. The original data of Casper and Gonen on the poor prognosis value of a hyperechoic endometrium in the late follicular phase of ART were largely confirmed in other publications,[108-113] while others failed to find this relationship.[114-115]

In an effort to analyze how ultrasound data, namely, echogenicity, could reflect endometrial receptivity, we studied the endometrial appearance on ultrasound on the day of hCG administration in 228 consecutive COH cycles.[116] We only included women younger than 38 years of age whose uteri were morphologically normal, and in a position that offered optimal visualization on ultrasound. Ultrasound images were digitized and analyzed using a computer-assisted system designed for measuring the degree of endometrial echogenicity (Fig. 35.7). Specifically, we studied the degree of the hyperechogenic changes that develop from the basal endometrium upward during the follicular-luteal transition, as described in E_2 and progesterone cycles,[106] but at times reported in ART before exposure to progesterone, at the time of hCG administration.[107] Our results indicated that in the selected population of IVF candidates 34/228 (14%) had a fully hypoechoic *triple-line* endometrium (less than 30% hyperechoic at the base of the endometrium) while 28/228 (12%) had a fully hyperechoic endometrium (greater than 70% of hyperechoic invasion of the functionalis layer).[116] The remaining 166 women had 4 degrees of progressive hyperechoic changes that extended from 31% to 40% to 61% to 70% of the full functionalis layer. Demographic, hormonal and biological characteristic of each of these groups were similar, including, endometrial thickness and plasma progesterone levels,

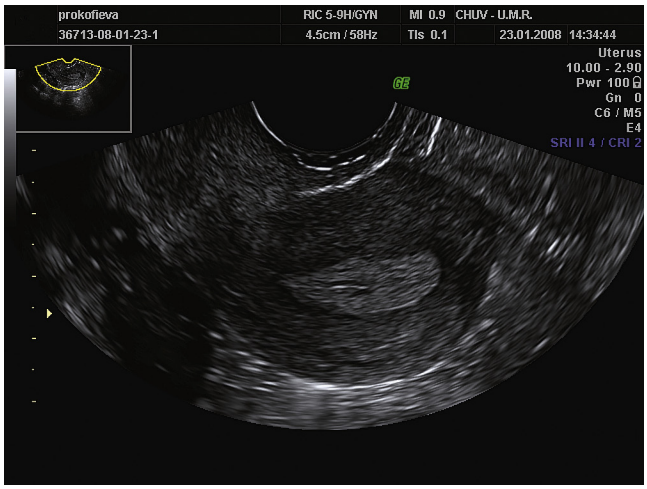


FIGURE 35.5 Hyperchogenic or type III endometrium: Under the influence of progesterone, the endometrium becomes hyperechogenic in relation to the surrounding myometrium during the luteal phase. From studies conducted in estradiol (E_2) and progesterone substitution cycles, it has been determined that the endometrium becomes fully hyperechogenic, usually after 4 days of exposure to luteal phase levels of progesterone. The hyperechogenic characteristics of the secretory endometrium are believed to result from the abundance of mucus-filled tortuous glands, which generate many ultrasound interphases and, therefore, increase the overall echogenicity of the endometrium.

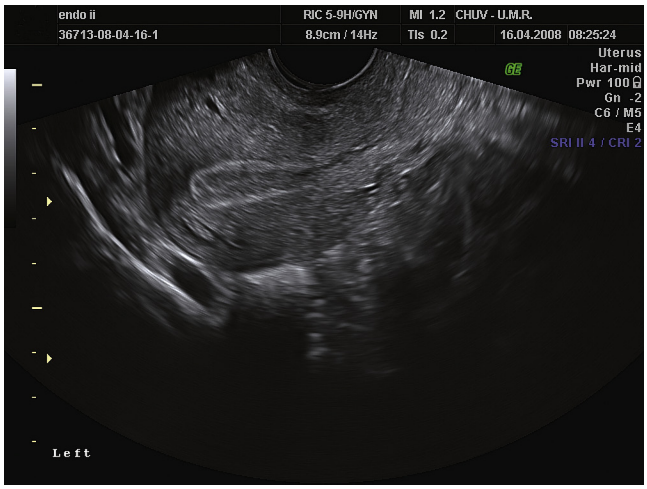


FIGURE 35.6 Intermediate or type II endometrium: During the first days of the luteal phase there is a characteristic thickening of the endometrial basalis, which progressively spreads upward to ultimately reach the hyperechogenic interphase of the virtual endometrial cavity. The condition encountered after 2 days of exposure to progesterone when the hyperechogenic endometrial basalis extends over approximately 50% of the whole endometrial layer is described as a type II endometrium.

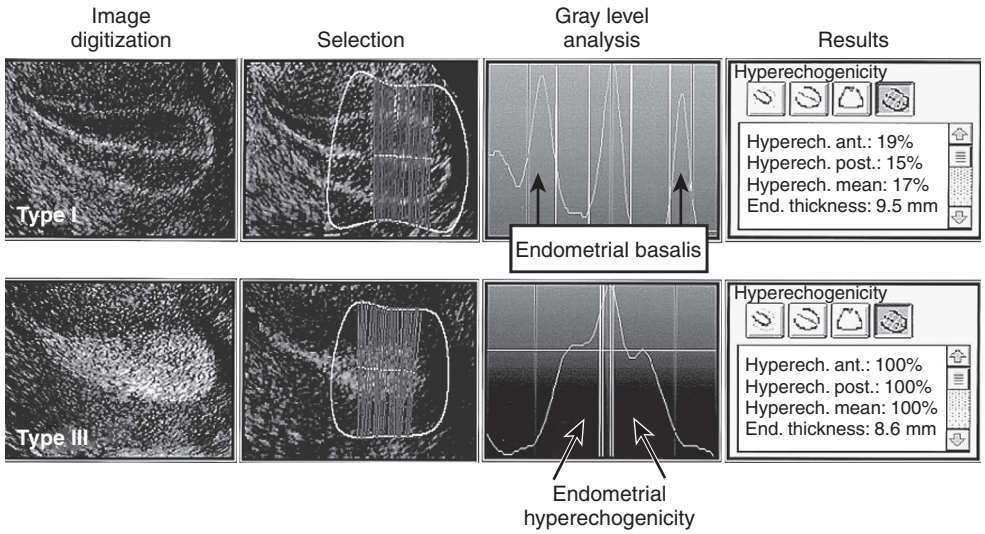


FIGURE 35.7 Computer assisted measurement of endometrial echogenicity. Endometrial echogenicity was studied on transverse slices. In the follicular phase (type I endometrium), one recognizes the characteristic hyperechogenicity of the endometrial basalis and that generated by the virtual endometrial cavity, outlining the hypoechogenic functionalis. In the luteal phase (type III endometrium), the hyperechogenicity pattern spreads to ultimately reach the virtual endometrial cavity, producing the characteristic hyperechogenic appearance of the endometrium. *(From Fanchin R, Righini C, Ayoubi JM, et al. New look at endometrial echogenicity: objective computer-assisted measurements predict endometrial receptivity in in vitro fertilization-embryo transfer.* Fertil Steril *74:274, 2000.)*

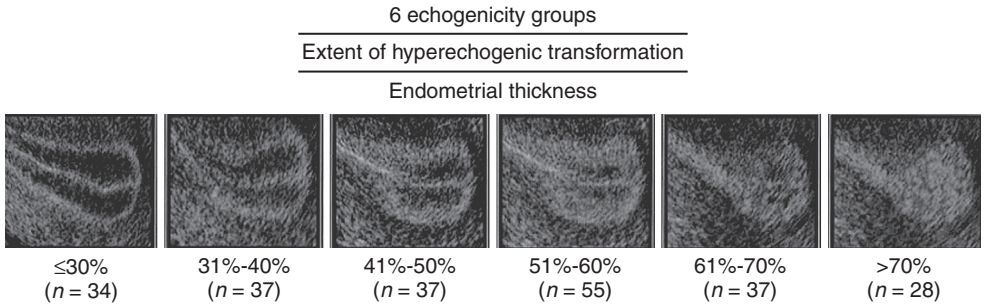


FIGURE 35.8 Computer assisted endometrial echogenicity showing the extent of hyperechogenic transformation on the day of human chorionic gonadotropin (hCG) and pregnancy and implantation rates. The figure shows six echogenic groups of increasing endometrial echogenicity. Pregnancy and implantation rates (59%, 57%, 35%, 20%, 16%, and 11%, respectively) and implantation rates declined from 59% to 11% and 35% to 3%, respectively when hyperechogenicity ranged from <30% to >70% of endometrial thickness. *(From Fanchin R, Righini C, Ayoubi JM, et al. New look at endometrial echogenicity: objective computer-assisted measurements predict endometrial receptivity in in vitro fertilization-embryo transfer.* Fertil Steril *74:274, 2000.)*

which remained less than 1 ng/mL in each group. This latter observation indicates that the *premature* increase in echogenicity seen in certain IVF patients is not the result of premature luteinization with increased progesterone production. Finally, the number of embryos transferred was also similar in all six groups; in each case two to four embryos were transferred (as commonly done in the 1990s). Pregnancy and embryo implantation rates varied greatly, however, between the different echogenicity-based groups. From a highest value of 59% and 16%, respectively, for the hypoechogenic group, it decreased to 23% and 3%, respectively, for the hyperechoic group, with progressively diminishing results for increasing echogenicity in the intermediate groups (Fig. 35.8).

In a different study, we assessed endometrial echogenicity on the day of hCG administration, oocyte retrieval, and embryo transfer (day 2) in patients whose plasma progesterone was below or above the cut off value of 0.9 ng/mL.[117] On the day of hCG, the mean hyperechoic transformation of the functionalis layer of the endometrium was similar in the two groups at 40% and 41%, respectively. During the 4 days that followed hCG administration, the hyperechoic transformation was much faster, however, in the high-progesterone group, with values of 70% versus 63% at oocyte retrieval and 90% versus 79% at the time of embryo transfer (ET), respectively.

The histological basis for the premature hyperechogenic transformation seen in certain IVF women remains unclear. During the follicular-luteal transition, it has been hypothesized that the development of spiral arteries and the coiling of the glands which takes place under the influence of progesterone causes the increase in echogenicity. The fact that progesterone was low (less than 1 ng/mL) in women showing premature echogenicity excluded this simple explanation. Work from Devroey's team performing endometrial biopsies on the day of oocyte retrieval in the cycle of embryo transfer confirmed that premature lutenization did not explain the early hyperechogenic transformation.[118,119] This leaves us with no explanation for the increase in echogenicity found in certain women on the day of hCG

administration in ART, an observation bearing ominous predictive value for ART outcome.

There are considerable inter-study differences in the predictive value of various echogenic patterns on the day of hCG, oocyte retrieval and embryo transfer for which no explanations are readily available.[120] We believe that some of the variability in the ultrasound patterns reported stems from methodological issues. For example, the endometrium of women whose uteri are in an intermediate position is likely to erroneously appear hyperechoic due to the fact that the ultrasound beam tends to hit endometrial glands at an angle rather than being parallel with the glands when the uterus is in a marked anteverted or retroverted position. In an unpublished trial, we performed manual rotations of freshly removed uteri before an ultrasound probing. This confirmed that the hypoechoic pattern obtained when the uterus is held in the anteverted or retroverted position before the probe becomes hyperechoic when the intermediate position is mimicked.[73] Recently, Dietterich and colleagues indicated that transabdominal screening likely limits the possibility of false interpetation.[101]

Three-Dimensional Volume Reconstruction of the Endometrium

Interest in measuring endometrial volume rather than its thickness is based on obtaining a more global parameter for assessing endometrial priming by estrogens.[11] Progress made in 3D technology allows the subendometrial junctional zone to be assessed.[121] Early assessment of endometrial volume estimated from 2D ultrasound images by simple formulae such as the prolate ellipsoid[122,123] were of little help for studying the uterus, probably because of the irregular shapes of this organ which made volume calculation based on theoretical formulas inaccurate. Off-line and later, built-in 3D volume reconstruction systems incorporated in the ultrasound machine offer supperior approaches for ultrasound-based volume measurements.

3D-based volume calculation of territories such as the endometrium use either of the two following methods: (1) The simplest or *multiplanar approach* consists of scrolling through multiple serial slices, which allows more measurements of irregularly shaped objects.[124] (2) A more elaborate approach is based on using a virtual organ computer-aided analysis imaging program (VOCAL) developed by a 3D ultrasound manufacturer, GE-Kretz, Zipf, Austria. A detailed description is provided by Raine-Fenning and colleagues.[12] Briefly, 3D reconstruction encompasses two primary steps. First, the relevant boundaries of the volume to be studied are identified on 2D gray-scale images in order to delineate the limits of the 3D reconstruction. For the endometrium volume, the endometrial to myometrial interface is either manually, or semi-automatically identified by the examiner on 2D images, using the B (transverse) or C (coronal) plane. Second, the 3D volume is rotated about a central axis through sets of user-defined steps ranging from 6 degrees to 30 degrees at a time. It takes from 30 to 6 planes, depending on the rotating pattern chosen, to complete a full 180-degree rotation. Finally, volume acquisition is generated automatically upon completion of a full 180-degree rotation (Fig. 35.9A-C). Experimental calculations indicated that the volume is measured with increasing precision with the increased number of rotation steps.[125] Practically, 9-degree rotations leading to a total of 15-degree rotation steps[73] are preferred.

From a first volume of reference (i.e., the endometrial volume), it is possible to generate secondary volumes that extend to set distances of the primary reference. For example, 1 to 5 mm outer extensions of the primary endometrium volume, using the *shelling* properties of the VOCAL system, can define subendometrium volumes.

In a prospective trial, Raine-Fenning and colleagues scanned a population of 30 presumably fertile volunteers on alternate days until evidence of ovulation was obtained and collapse of the follicle was witnessed, and every 4 days thereafter.[126] 3D volumes of the uterus were acquired using a Voluson 530D ultrasound machine from an appropriate longitudinal scan of the uterus. 3D volume measurements were performed off-line using the VOCAL system with endometrium boundary identification conducted on the coronal C plane in successive manual 9-degree rotation steps. As illustrated in Figure 35.9B, the changes in endometrial thickness and volume throughout the menstrual cycle followed similar patterns with progressive increments during the follicular phase and stable values throughout the luteal phase. The changes in endometrial thickness and volume were highly correlated ($R^2 = 0.767$; $P < 0.001$). Studies looking at correlations between endometrial volume and ART outcome revealed the same kind of relationship already described for endometrial thickness. Like their endometrial thickness counterparts, 3D endometrial volume studies revealed poor pregnancy rates in cases of marginal endometrial volume. Specifically, pregnancy rates were directly related to endometrial volume for values from 2 to 4 mL, significantly reduced when the endometrium was less than 2 mL, with no pregnancy if less than 1 mL. Conversely, further increases in endometrial volume greater than 4 mL were not associated with better outcomes, as compared to 2 to 4 mL.[11] In a study of 47 women, Schild and colleagues showed no correlation between endometrial volume and pregnancy rates,[124] as did Yaman and colleagues.[127]

Uterine Doppler

Principles and Applications

The Doppler effect (named after the German physicist, Christian Doppler) refers to the phenomenon whereby an apparent change in frequency is perceived when relative motion exists between the wave source (in our case, the reflected ultrasounds) and the receiver. Yet, the emitted frequency (here, the ultrasounds refected to the receiver) does not change. The frequency change is only perceived by the receiver, as the source-receiver distance changes due to movement. If ultrasounds are emitted (or reflected) from a source that moves toward the receiver, an increase in frequency will be perceived. The reverse will be perceived if the ultrasound source moves away from the receiver. In both cases, the perceived frequency change (i.e., the intensity of Doppler effect) is proportional to the speed of displacement of the target on which ultrasounds are reflected.

The primary application of the Doppler effect has been for analyzing the movement of blood particles on which ultrasounds are reflected for studying blood flow. Depending on the methodological approach used, Doppler-based

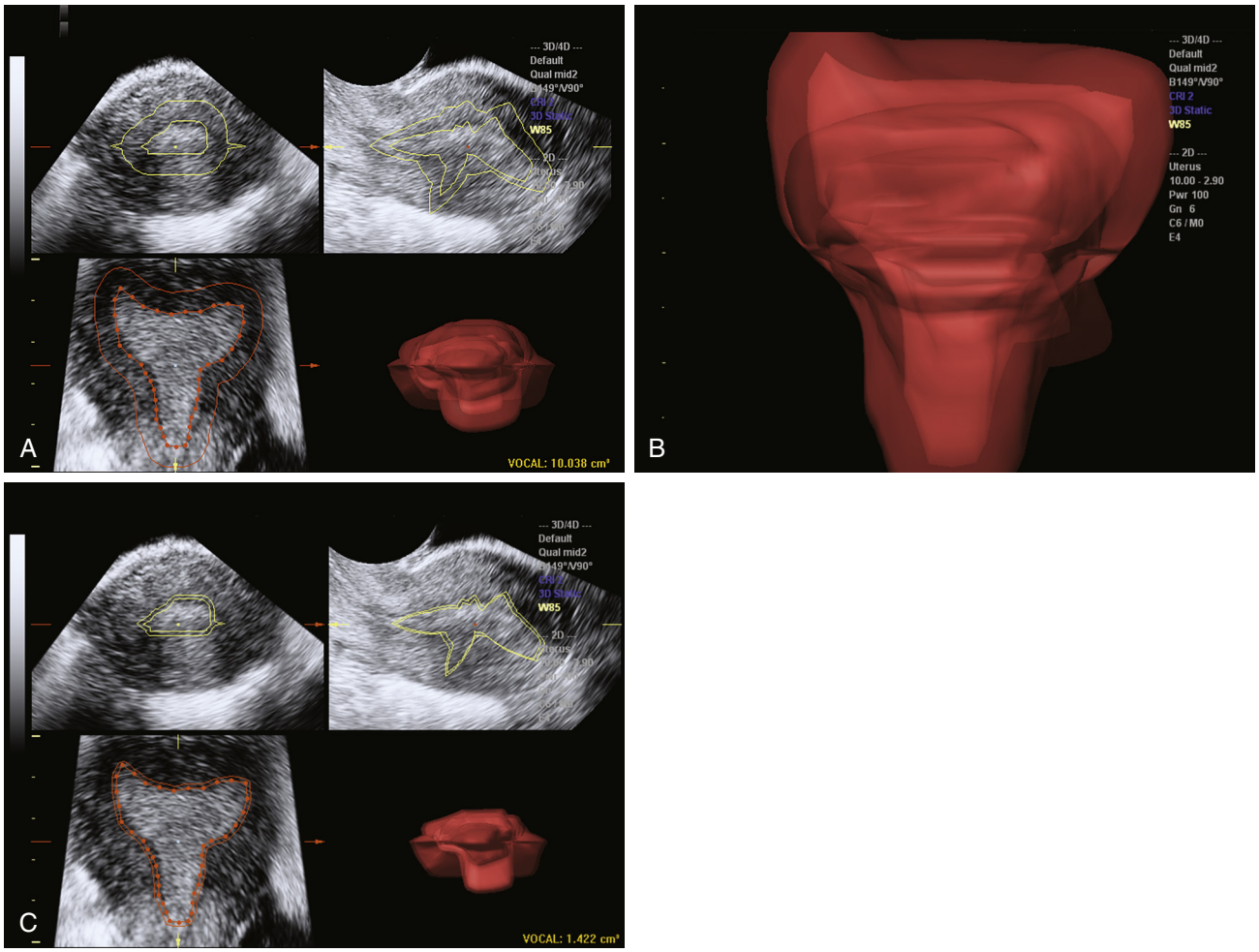


FIGURE 35.9 Endometrial volume calculation using a virtual organ computer-aided analysis (VOCAL) imaging program also allows the calculation of a secondary subendometrial volume extending at 5 mm **(A** and **B)** or 1 mm **(C)** outside from the endometrial myometrial interphase.

analyses may (1) look at individual vessels or; (2) measure perfusion in a whole tissue area. The former approach assessing flow in specific vessels (i.e., the uterine artery) rely on the pulsed-Doppler technique. This consists of sending sound signals at timed intervals in a defined volume or *sampling gate*, and studying the perceived frequency changes of the return signal or Doppler *waveform*.

Doppler-based blood flow analyses stem from advances in cardiology, which established the methodological foundation for all Doppler analyses performed today.[128] Pulse Doppler (Fig. 35.10) involves first physically locating the vessel to be studied in order to properly position the Doppler sampling gate. This can be done using a duplex system, which provides concomitant gray scale or *color Doppler* imaging for vessel identification (see later in the chapter). Once the emitting signal is targeted at the designated vessel, blood flow and resistance are calculated by analyzing the frequency changes perceived or Doppler waveform. The intensity of the Doppler effect reflects the speed of displacement of the blood particles toward or away from the probe, which emits and receives the return signal. An exact computation of blood flow and resistance implies, however, that the angle between the ultrasound wave and the great axis of the vessel is known. This is needed for determining the true displacement speed of the blood-borne particles.

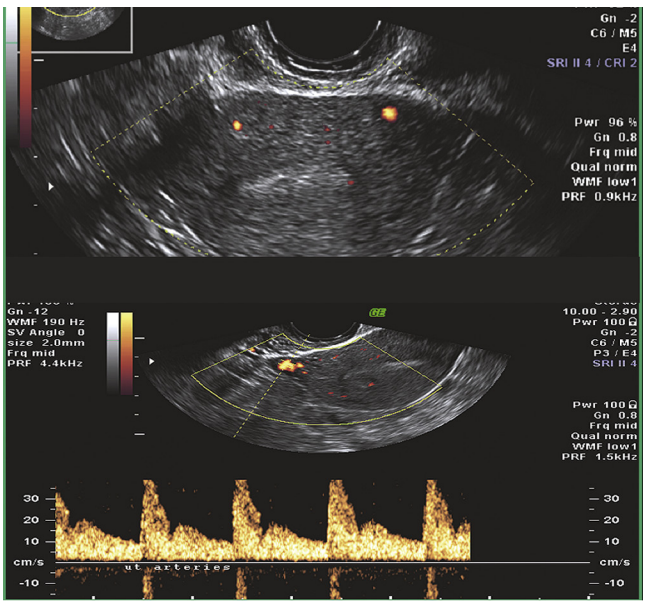


FIGURE 35.10 Pulsed Doppler waveform analyzed by computation of the semiquantitative pulsatility index (PI), which reflects the impedance to flow in the territory downstream from the area of measurement.

This calculation is, unfortunately, impossible in gynecological organs because uterine and ovarian vessels are far too tortuous.

The pulsed-Doppler technology in gynecology must, therefore, rely on approximations for assessing the Doppler signal, as an accurate determination of blood flow and resistance are impossible.[9,10,129,130] Practically, the impedance to flow, a reflection of vascular resistance, is assessed by descriptive analysis of how the *Doppler flow wave* is modulated through the systolic and diastolic phases of the cardiac cycle. This permits semiquantitative measurements of systolic and diastolic signal ratios, or Doppler indices. The most common of these indices are the resistance indices (RI) and pulsatility indices (PI). RI (values from 0 to 1) and PI (values from 0 upward) are directly related to vascular resistance, with higher values corresponding to lower flow.

The alternate approach for Doppler analysis uses more complex real-time assessment of the Doppler effect directly on the return ultrasound signal serving gray scale imaging. This approach applies color-coding to voxels that are subjected to Doppler effect, while the rest of the voxels serve for gray scale imaging. The end result or *color Doppler* function and its variant angle-independent *power Doppler,* provide direct imaging (or mapping) of vascular flow overlying gray-scale ultrasound images. Color and power Doppler, therefore, offer a novel way for assessing vascularity in a given organ or territory. Color and power Doppler also permit global assessment of vascularization in a given area by direct computer-based visual analysis of colored voxels present over the total number of voxels, or *power* Doppler *angiography* (PDA).[87] Unlike PI and RI, PDA values are directly related to local flow, with higher values indicating higher flow. Various systems have been developed for filtering nonspecific color Doppler signals or "flash" artifacts that are triggered by displacements of the targeted tissue as a result of bowel or respiration-linked movements.

More recently, computer-assisted power Doppler assessments have been coupled with 3D volume reconstruction (3D-PDA) for automated operator-independent measurement of vascularity in a precisely defined volume.[131] (Fig. 35.11A and B). This approach offers improved reproducibility, because measurements are conducted in an anatomically and electronically defined territory, such as the endometrium or ovary.[12] 3D-PDA is measured in the volume of interest previously defined using VOCAL.[12] The results of 3D-PDA are displayed on a histogram from which three indices, the vascular (VI), flow (FI), and vascular/flow indices (VFI), are derived. VI (%) represents the proportion of lighted voxels over all voxels, thus reflecting the degree of vascularity of the studied volume. FI (0-100) represents the mean power Doppler intensity of the lighted voxels, a parameter meant to reflect the vascularization flow rate. VFI (0-100) is a product of the two indices VI and FI (Fig. 35.12).

The Doppler function of ultrasound makes it possible to extend the diagnostic usefulness of gray-scale imaging. Doppler data have been used for two main purposes: (1) refining cancer detection by identifying or excluding suspicious vascularitiy in relation with gray-scale findings (i.e., an ovarian cyst); (2) assessing the effects of hormones (endogenous or exogenous) on blood flow in an attempt to identify markers of endometrial receptivity.

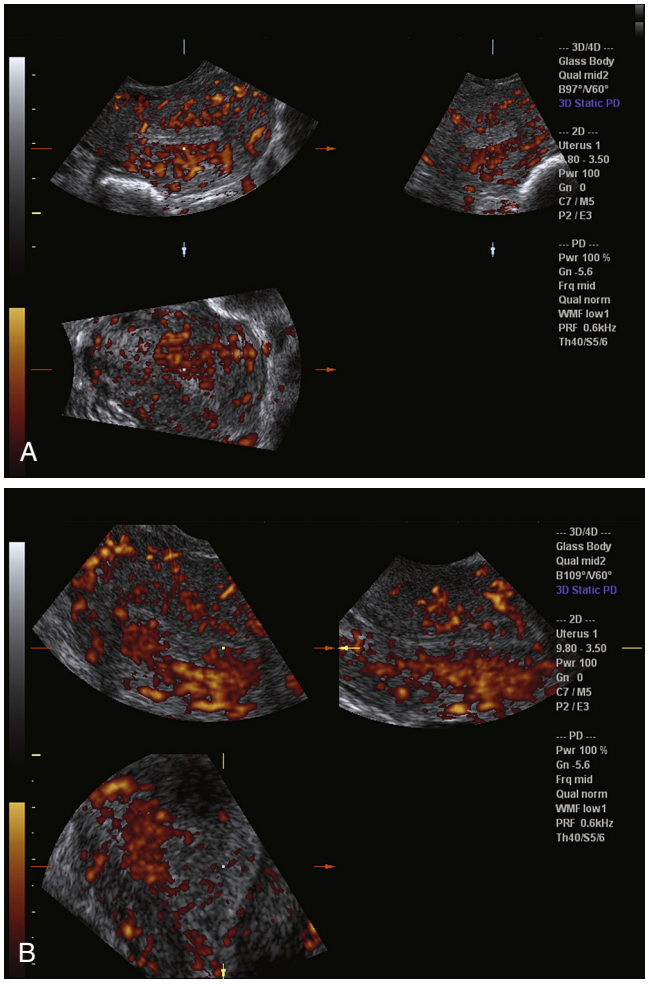


FIGURE 35.11 Computation of color Doppler finding in a given area by computation power Doppler angiography (PDA) in a 3D defined territory, as for example, the endometrium and subendometrial volume using the virtual organ computer-aided analysis (VOCAL) and shelling functions. PDA score is directly related to flow. Three primary indices are computed, the vascularization (VI), flow (FI), and vascularization-flow index (VFI). VI reflects the number of vessels in a given area whereas FI is proportional to blood flow in these vessels and VFI constitutes a product of VI and FI. **A**, In normal conditions, color Doppler shows no to minimal vascular entries in the endometrium. **B**, Endometrial polyp vessels are shown entering inside a given territory of the endometrium (right cornua).

Vascular Changes Related to the Menstrual Cycle and Hormonal Effects

Investigators have tried to follow the changes in uterine vascularization that are tied to hormonal changes in the menstrual cycle. Early studies using pulsed Doppler looked at changes in Doppler waveforms recorded from the uterine artery[9] and its major branches.[129,132,133] Later, Raine-Fenning and colleagues used 3D-PDA to quantify changes taking place in endometrial and subendometrial blood flow during the menstrual cycle.[126] These authors observed that both endometrial and subendometrial blood flow increased during the follicular phase, peaking 3 days prior to ovulation, and decreased to a nadir 5 days post-ovulation (Fig. 35.13). This pattern differs, however, from prior findings made with pulsed Doppler showing a steady increase of blood flow throughout

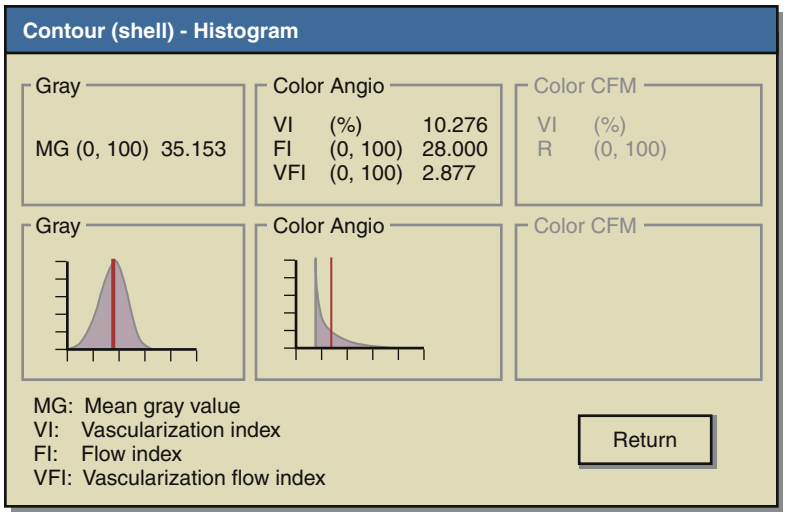


FIGURE 35.12 Computer assisted analysis of "lighted" voxels in a given territory leads to the computation of two distinct indices, the vascular (VI) and flow indices (FI), with VFI being of a product of both. VI represents the number of vessels in a given territory whereas FI is proportional to the intensity of blood flow in these vessels.

the menstrual cycle.[126,129,134-137] In Raine-Fenning's trial, smoking was associated with significantly lower VI and VFI throughout the menstrual cycle, whereas parous women had higher values than their nulliparus counterparts.[126]

Exogenous hormones administered to women suffering from premature[10,133] or normally occurring menopause[132] induced a prompt and profound decrease in uterine PI and RI indices. This was interpreted as reflecting the vasodilatory effects of E_2. Menopausal[138] and donor-egg IVF regimens[10] were associated with similar vasodilation of uterine arteries, suggesting that maximal vasodilation is achieved at levels of E_2 found within menopause treatments. Conversely, exposure to higher E_2 doses as used in donor-egg regimens caused no further increase in flow. In these studies, the vasodilation of uterine arteries induced by E_2 administration was partially antagonized by synthetic progestins,[138] but not by progesterone administration.[10]

Vascular changes observed in the uterus during COH do not parallel those that would be anticipated from the mere increased exposure to E_2. These findings indicate that the stimulated ovaries produce vasoactive factors other than E_2. In a prospective trial, Ng and colleagues[138a] compared endometrial and subendometrial blood flow measured using the 3D-PDA approach in menstrual and COH cycles of the same women. Their findings showed a significant decrease of endometrial and subendometrial blood flow during the stimulated cycle,[138a] with excessive responders having further reductions in endometrial blood flow as compared to moderate responders.[138a] These findings suggest that vasoconstrictive substances are released by the stimulated ovaries that antagonize the vasodilatory properties of E_2.[88,139] Among the putative factors possibly responsible for the decrease in uterine flow during COH are ovarian androgens, which are produced in increased amounts under the influence of exogenous follicle-stimulating hormone (FSH).[53,140] Supporting the concept that ovarian androgens interfere with the vasodilatory properties of E_2 is the observation of increased uterine artery resistance in case of polycystic ovary syndrome (PCOS) made by some,[141] but not all investigators.[142] Further supporting the vasoconstrictive role of androgens, or of a substance released in conjunction with androgens, is the report of an improvement of uterine flow in PCOS following administration of the antiandrogen, flutamide.[141] In a different study, Ng and colleagues[142a] observed a negative correlation between E_2 levels and blood flow suggesting that the putative vasoactive factor produced during COH is triggered by FSH administration in a proportional manner to that of E_2. This is further supported by the observation that the decrease in endometrial flow is more pronounced in women whose response to COH is excessive as compared to moderate responders.[142b]

The promising findings of early Doppler studies indicating that Doppler values predicted endometrial receptivity[143] have not been confirmed by recent 3D-PDA data from either fresh[5] or frozen embryo transfer cycles.[5] Yet, increased vascularitiy may be predictive of lesser risk of miscarriage.[82]

Dysfunctional Uterine Bleeding: Benign and Malignant Endometrial Pathology

Endometrial pathology, such as polyps or submucosal fibroids, are sought in cases of: (1) dysfunctional uterine bleeding (DUB) or; (2) as part of an infertility workup.

Odeh and colleagues prospectively evaluated DUB in 89 perimenopausal and 56 menopausal women.[144] In their hands, endometrial volume measurement more accurately predicted endometrial hyperplasia and/or carcinoma than simple endometrial thickness measurement. Receiver Operating Characteristics (ROC) curve analysis indicated that a sensitivity of 100% with a cut off value of 1.45 mL, which resulted in low sensitivity of 0.08%. A cut off value of 3.56 mL for the endometrium volume offered a sensitivity of 93% and a specificity of 36%. With this value, two carcinomas would have been missed in the patient cohort; one with alarming 3D-PDA data, the other with negative 3D-PDA results. Consequently, new tools such as 3D-based volume measurements should not cause us to ignore careful analysis of the endometrium. Irrespective of endometrial thickness or volume values, a heterogeneous echogenicity with close juxtaposition of hyperechoic and hypoechoic aspects warrants histological exploration. When endometrial polyps are suspected based on an irregular endometrium on plain ultrasound, direct power Doppler may identify the feeding vessel entering the polyp. Conversely, endometrial vessels are normally not viewed on power Doppler.[145] Suspicious findings on plain ultrasound justify a contrast-enhanced study.

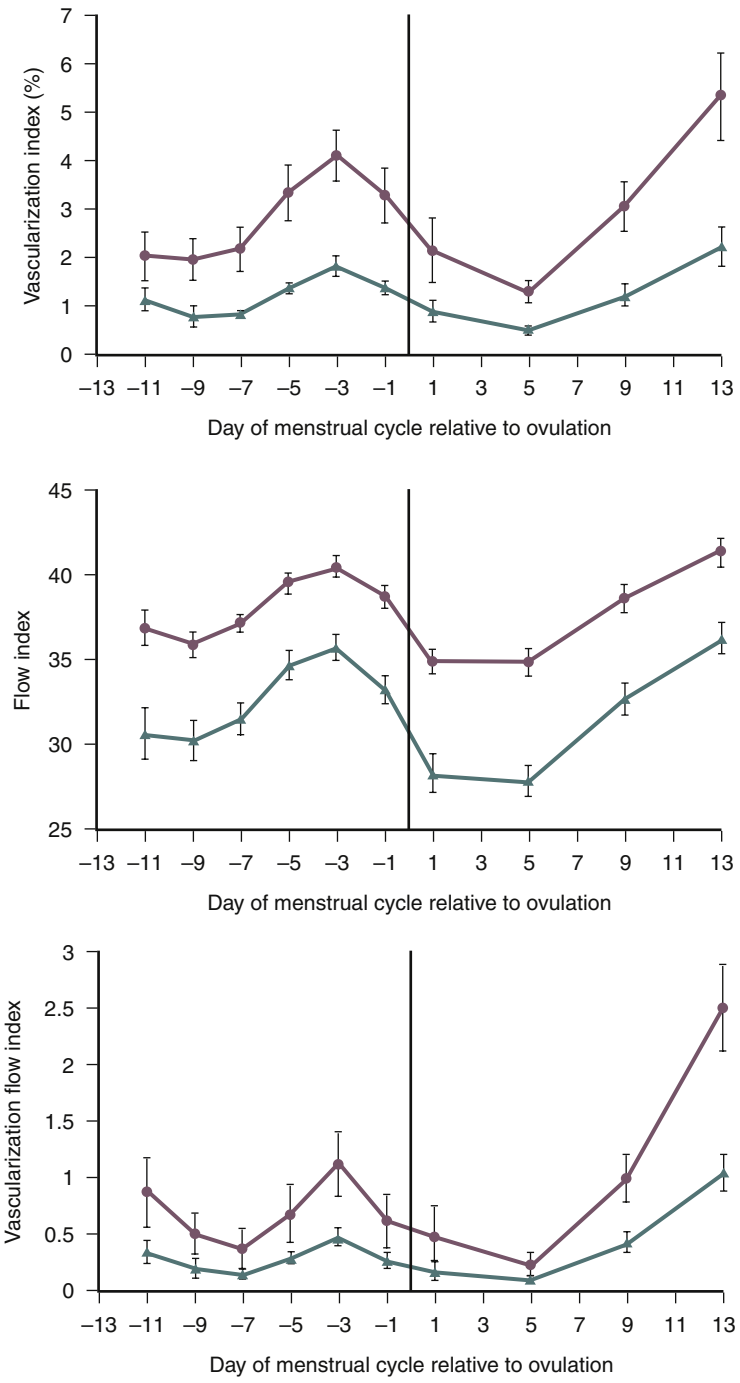


FIGURE 35.13 Temporal variation of three-dimensional-power Doppler angiography (3D-PDA) vascularization index (VI), flow index (FI), and vascularization flow index (VFI) throughout the menstrual cycle in endometrial and subendometrial territories. A characteristic postovulatory decrease in blood flow is observed in both endometrial and subendometrial territories. *(Raine-Fenning NJ, Campbell BK, Kendall NR, et al. Quantifying the changes in endometrial vascularity throughout the normal menstrual cycle with three-dimensional power Doppler angiography.* Hum Reprod *19:330–338, 2004.)*

The cardinal sign of endometrial carcinoma is an abnormally thick endometrium,[146,147] often with irregular and erratic echogenicity.[148] In 339 menopausal women presenting with DUB, there was no endometrial cancer developing over a 10-year follow-up with an endometrial thickness of ≤4 mm.[149] A more recent meta-analysis concluded that earlier studies probably overestimated the diagnostic accuracy of endometrial thickness.[150] A more conservative cutoff level of 3 mm was recommended for exclusion of endometrial carcinoma in women with postmenopausal bleeding.[150] Doppler has been claimed to differentiate between cancer and noncancerous lesions. While noncancerous lesions tend to have higher Doppler indices, there is an overlap between the PI and RI values of benign and malignant lesions.[151] Based on these findings, Tabor and colleagues concluded that postmenopausal bleeding requires an endometrial sampling irrespective of the thickness measured on ultrasounds.[152] Finally, in cases of endometrial cancer, vaginal ultrasound and MRI were equivalent for determining the depth of myometrial invasion.[153]

The Myometrium: Physiological and Pathological Findings

Uterine leiomyomas or fibroids are the most common solid tumor, occurring in 20% to 40% of women in their

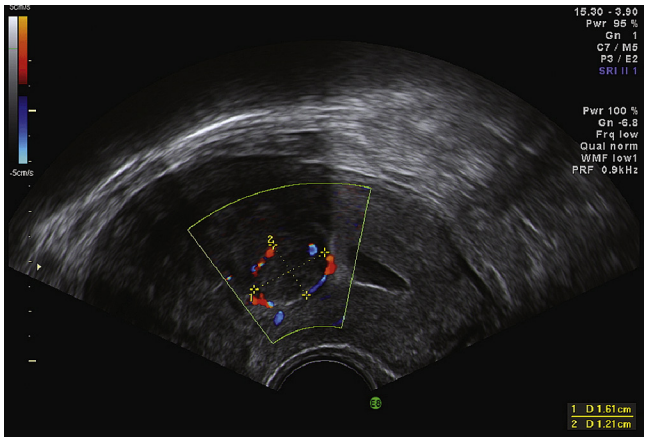


FIGURE 35.14 Uterine fibroid. Characteristically, vessels run around the surface of the fibroid from which radiating branches penetrate vertically into the tumor.

reproductive life.[154] Fibroids are well-circumscribed lesions, often with a pseudo capsule containing feeding vessels running at the surface of the fibroid, which penetrate into the fibroid in a radiating manner (Fig. 35.14). This pattern differs from that of adenomyosis, in which vessels are sparse and scattered, and the borders of the lesion typically indistinct. Fibroids originate from the middle layer of the myometrium from which they tend to expand either inward toward the cavity to become submucosal or outward toward the serosa, possibly to the point of becoming pedunculated.

The possibility that fibroids may cause infertility and constitute a predisposing factor for miscarriages depends primarily on their location and degree of extension. First, fibroids may alter fertility by interfering with sperm transport and embryo implantation.[155] This detrimental effect of fibroids on sperm transport has recently been supported by MRI-based studies of uterine contractility.[156,157] Kido and colleagues observed that submucosal fibroids were more likely to affect uterine peristalsis than their intramural counterparts.[157] Second, the possibility that fibroids alter endometrial receptivity has been entertained for many years. Fibroids may hamper embryo implantation, in part by physical effects when present in a subendometrial location, in part by focal endometrial disturbances[158] and local inflammation through the release of vasoactive and other substances.[159]

The possibility that fibroids cause miscarriages remains a subject of debate. In a recent study, miscarriages were more frequent in a population that experienced fibroids than in their unaffected counterparts.[160] There are many other studies, however, that failed to find such an association.[161] There are new therapeutic avenues for treating fibroids medically using selective progesterone receptor modulators (SPRMs).[162] However, the role of SPRMs in infertility treatment remains to be explored.

Contrast-Enhanced Imaging of the Uterine Cavity

History, Principle and Practical Issues

Early in the use of vaginal ultrasound it became apparent that uterine exploration in early pregnancy offered images of the developing embryo of outstanding quality.[163,164] The remarkable definition of fetal images obtained in early pregnancy using vaginal ultrasound is crucial for early detection of fetal malformations.[165-167] However, ultrasound images of the nonpregnant uterine cavity yield lower quality because of the lack of intrauterine fluid playing the role of contrast enhancer.[15] This led to the design of a new diagnostic procedure in which 0.9% NaCl or similar products are instilled in the uterine cavity for improving image quality. This procedure has been called hystero salpingo-contrast-sonography (HyCoSy), saline infusion sonography (SIS), or hysterosonography (HySo) (Fig. 35.15).[15,17,18,168,169]

Two types of contrast products provide either negative (black) or positive (white) contrast enhancement of the uterine cavity on grey scale imaging.[170] Negative contrast solutions have the acoustic properties of water (sonotransparent) with normal saline being the most commonly used agent. Positive contrast solutions consist of preparations that gain sonorefracting properties from air entrapped in various microparticle systems from which it is progressively released over time.[171-173] This is achieved through albumin (Albumex®) or saccharide microparticles (Echovist® later replaced by Levovist®, BR1, EchoGen, etc.). These positive ultrasound contrast products are injectable solutions available for various medical indications, but are used intrauterinely for gynecological imaging.[174]

Positive contrast solutions instilled in the uterine cavity provide *white* contrast enhancement of the uterine cavity. Unfortunately, this often obscures vision of the far-sided uterine wall because of shadowing effects created by the contrast product in the uterine cavity.[175] Negative contrast solutions, such as normal saline, provide *black* contrast enhancement of the uterine cavity. This leaves the vision of the far side of the uterus totally unobstructed which is better for identifying intracavitary lesions.[173,176,177] From these findings, it was rapidly recognized that negative contrast using saline is the preferred option for exploring the uterine cavity, whereas, positive contrast solutions are best for visualizing the fallopian tubes, organs that evade scrutiny on ultrasound examinatiton.[178]

Positive echographic contrast solutions have also been employed for enhancing the Doppler signal and improving the assessment of tissue perfusion when administered systemically.[179] Yet, Doppler enhancement that requires IV administration of these contrast products has not yet provided clinically relevant advantages that would justify their use for diagnostic purposes. Lastly, a variety of harmonic imaging techniques with and without pulse sequencing methods have been developed for improving the signal-to-noise relationship.[180,181] The approaches currently in development in various domains of ultrasonography may generate practical applications in gynecological assessment in the future.[182]

The relative complexity of the contrast-enhanced procedures, which requires the help of an assistant, limits the use of these methods, despite the quality of the images obtained and the reliability of the diagnoses.[169,183] Two options exist for assuring the proper distension of the uterine cavity during the procedure: (1) A simple catheter of the type used for intra-uterine insemination (IUI) or one equipped with a specially designed cone-shaped stopper for better fit against

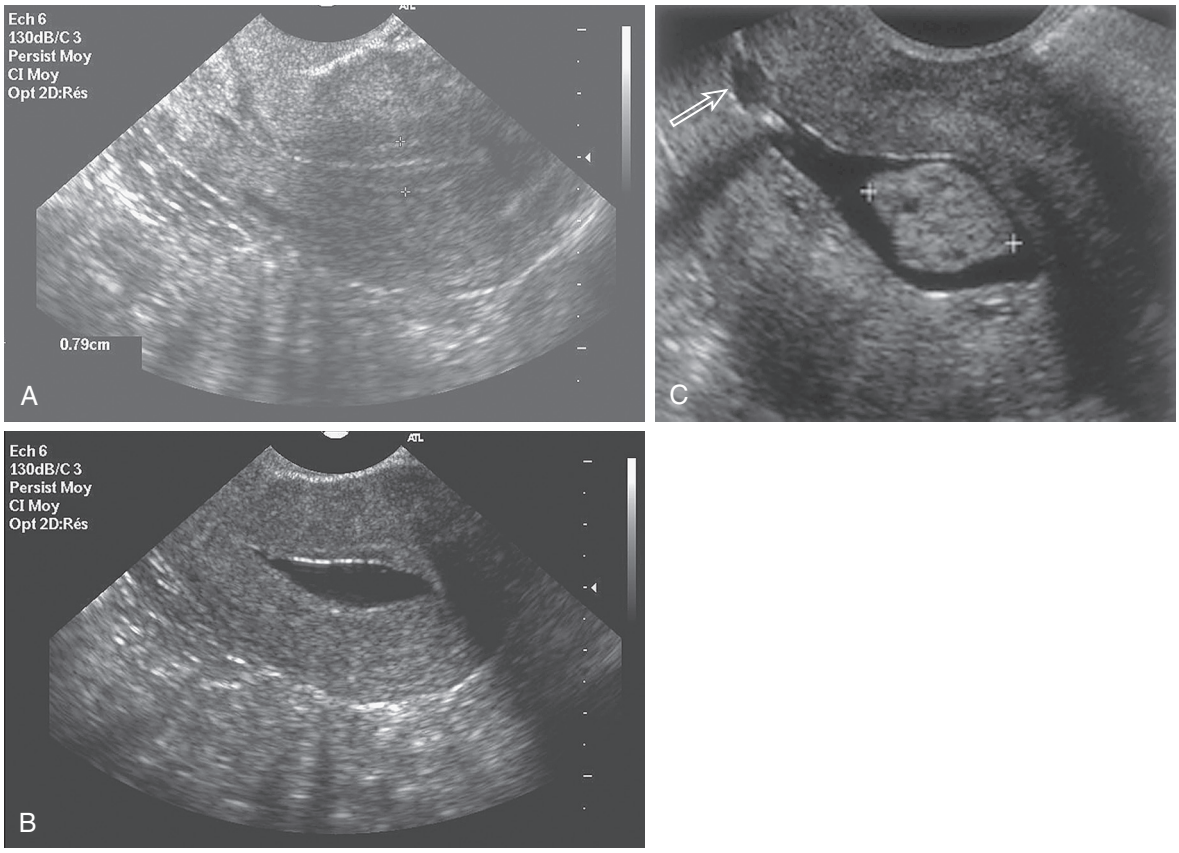


FIGURE 35.15 Hysterosonography (HySo): Intrauterine infusion of a contrast solution improves vision of the uterine cavity provided by plain ultrasound **(A).** Provides an interphase that enhances contrast **(B).** Facilitates the detection of intrauterine pathologies such as polyps or fibroids **(C).**

the external os of the cervix.[8] (2) A balloon-equipped catheter, which is inflated after positioning in the uterine cavity in order to prevent displacement. With the former, the speculum needs to be removed very carefully after placement of the catheter in order to not displace it from its proper intrauterine position. If the catheter is not firmly held in place, it most often justifies using a speculum that is articulated on one side only, which facilitates extraction, but is notoriously uncomfortable for the patient. The balloon catheter is itself capable of creating some degree of discomfort when it expands in the lower uterine segment. Moreover, it may hinder visualization of pathology lying in the lower uterine segment. In short, albeit providing images of remarkable resolution, HySo is a three-hand procedure (one for holding the probe, one for instilling medium, and the third for making image adjustments and measurements). HySo, therefore, most often requires the help of an assistant. Recently, an innovative option has been proposed in which a phase shifting medium is used.[168] This medium has a gel consistency at room temperature and the acoustic characteristics of water. Intrauterine instillation of 1 to 3 mL creates a slight distension of the cavity, thus offering negative or *black* contrast vision during the time necessary for ultrasound examination, which is conducted in the absence of any instrument present in the uterus. The medium later liquefies and is expelled naturally. In a pilot trial, this novel technique provided classical HySo-like images.[184]

Hysterosonography for Visualizing Endometrial Polyps and Fibroids

Using HySo for visualizing intrauterine pathologies, as in cases of DUB, has advantages and pitfalls that have been recognized.[185,186] In these women, HySo is in direct competition with other noninvasive diagnostic procedures such as diagnostic hysteroscopy.[187]

In a prospective trial, de Kroon and colleagues[188] reported that HySo provided conclusive information in 84% of 180 consecutive women presenting with DUB. In all these cases, the positive and negative findings of HySo were confirmed in women undergoing surgery, for a sensitivity and specificity of 100%. In 5.6% of cases, however, HySo could not be performed because of cervical stenosis and its results were judged inconclusive in a further 10.3%. A meta-analysis of trials looking at the value of HySo in women with DUB reported pooled sensitivity and specificity of HySo in the 24 retained trials of 0.95 (95% CI: 0.93, 0.97) and 0.88 (95% CI: 0.85, 0.92), respectively.[188] These results were confirmed by further trials and meta-analyses.[189,190] Furthermore, HySo-targeted endometrial biopsies have been described that allow direct endometrial sampling in the area of interest.[191] A survey found, however, that patients generally preferred office hysteroscopy over HySo.[192]

Concomitent use of 3D-reconstruction of uterine imaging further enhances the clinical value of HySo.[193] 3D volume acquisition allows for limited duration of uterine

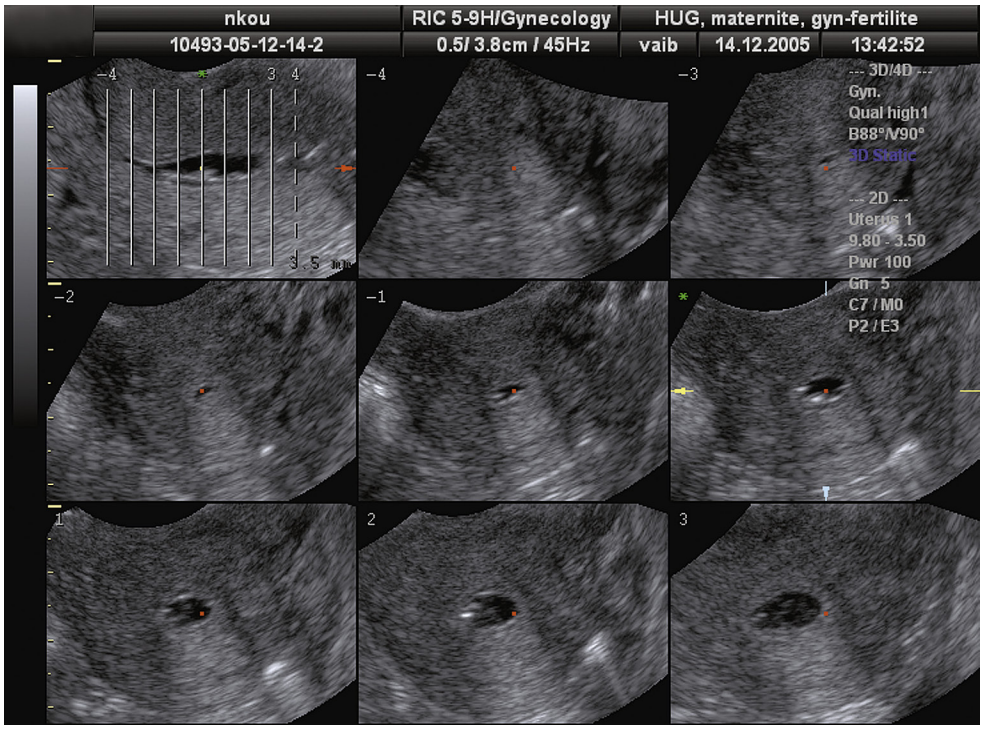


FIGURE 35.16 Three-dimensional-hysterosonography (3D-HySo) allows secondary review of images for detailed analysis of intrauterine contents.

distension to the time necessary for carrying out the electronic acquisition of a 3D volume. Furthermore, 3D reconstruction permits review of HySo images after uterine distension has subsided (Fig. 35.16).

HySo can also benefit from the added information provided by Doppler, such as the detection of a vessel entering into a polyp or encircling the surface of a submucosal fibroid.[193] Unusual uses of Doppler in conjunction with HySo includes the identification of an uteroperitoneal fistula[194] and intrauterine vascular malformation.[195]

Besides DUB, other indications for HySo include evaluation of women receiving tamoxifen treatment,[196,197] and according to some authors, evaluation of asymptomatic women entering menopause and considering hormonal therapy.[198]

Discomfort generated by HySo examination is usually mild to minimal. In a prospective trial, Dessole and colleagues[199] looked at the side effects and complications of 1153 HySo procedures. In 93% of cases, HySo was successful. Side effects of moderate to severe pain, vaginal reactions, and nausea and vomiting occurred in 102 (8.8%) of cases. Severe complications such as fever and clinical signs of peritonitis were encountered in 0.95% of cases, with tubo-ovarian absesses having been reported.[200] We do not routinely perform cervical cultures or use routine antibiotics; a recommendation shared by the Amercan College of Obstetricians and Gynecologists (ACOG).[201]

The risk of propagating cancer in case of endometrial cancer has been examined.[202] Fluid spilling from the fallopian tubes was collected while HySo was conducted preoperatively in 32 women diagnosed with endometrial cancer. Malignant and suspicious cells were recovered from tubal spills in 2 (6.25%) and 6 (18.8%), respectively, for a total of 8 (25%) findings of suspicious or malignant cells recovered from the 32 subjects. These authors strongly recommended that HySo not be performed in case of proven or suspected endometrial cancer.[202]

The value of routine pre-ART screening of the uterine cavity using hysteroscopy or HySO is being discussed.[203-205] In our program, women are synchronized with a short 7- to 14-day course of oral contraceptive (OC) pills during which time routine HySo is performed. We commonly remove incidental polyps while the patient is still on the OC pill. Performing HySo while the patient is on the pill offers the advantage of having an atrophic mucosa which precludes confusing a mucosal fold for a polyp. HySo or hysteroscopy should be part of the regular workup in case of repeated miscarriages.[206]

It is important to underscore that a comparison between HySo and office hysteroscopy goes beyond the ability to detect polyps, as each technique offers respective advantages. On the one hand, office hysteroscopy provides information about possible chronic inflammation or infection of the endometrium not otherwise identifiable,[88] including by cervical cultures.[207] On the other, HySo combined with pelvic ultrasound offers information about organs other than the uterus, namely the ovaries and possible cysts, that may be pertinent for sorting out the cause of DUB. Hence, the choice between HySo and office hysteroscopy should result from a process that takes into account all the issues pertinent to each clinical situation.

Hysterosonography in ART

In light of ART costs, many recommend routine assessment of the uterine cavity for the presence of polyps prior to ART using either HySo or office hysteroscopy, which are equally effective.[208,209] A recent meta-analysis reported data that collectively supports the value of removing even small polyps.[210] Hysteroscopic removal of endometrial polyps of 16 mm (mean) detected by ultrasound doubles the pregnancy

rate in patients undergoing intrauterine insemination.[210] In patients with two failed ART cycles, diagnostic or operative hysteroscopy before a subsequent attempt appears to improve outcomes.[210,211] It should be noted, however, that such benefit may result from nonspecific effects of any type of endometrial injury.[212] Fearing that systematic pre-ART HySo may lead to false positive findings generated by mucosal folds, it is recommended that HySo be performed either in the first 10 days of the follicular phase or better while on OC pill.

Müllerian Anomalies and Recurrent Pregnancy Loss

The true prevalence of uterine anomalies is uncertain because of the disparate reported numbers, with incidence ranging from 0.16% to 10%,[213] mainly based on interpretation of hysterosalpingograms. A selection of better structured studies reported a similar incidence of approximately 1% in infertile or presumably fertile women.[214-216] A higher incidence, approximating 3%, was found, however, in women having a history of recurrent pregnancy loss.[217-219]

Early in development (approximately 9 to 10 weeks), the common understanding has been that paramesonephric ducts fuse caudally while the uterine septum starts regressing in an unidirectional manner from the lower part of the uterocervical canal upward.[220] An alternate bidirectional regression theory exists, however.[221]

In a recent study, Mazouni and colleagues undertook a full evaluation of 110 women suspected of having a müllerian anomaly, using vaginal ultrasound, hysterosalpingography (HSG), computarized tomography (CT) scans, and in cases diagnosed in the later years, MRI.[222] Müllerian duct anomalies were reported according to the American Fertility Society (AFS) classification (Fig. 35.17).[223] Of the 110 patients referred for müllerian anomalies, 73 women had a septate uterus, 20 a bicornuate uterus, 10 uterine hypoplasia, 4 a unicornuate uterus, and 3 Mayer-Rokitansky-Kuster-Hauser syndrome. In 33% of the cases, the diagnosis was made as part of the infertility work-up, in 18.2% because of repeat early spontaneous abortion, and in 12.7% on the occasion of ultrasound performed during pregnancy. In cases of septate vagina, dyspareunia was a presenting symptom, accounting for 8.2% of all müllerian anomalies in this series.

The issue of how müllerian anomalies are best diagnosed has not yet been resolved. HSG was the traditional approach, and remains a useful if not indispensable examination in many cases. MRI, although helpful, should not be looked at as the primary means for diagnosing müllerian malformations, but rather used secondarily for defining cases too complex or atypical for definitive diagnosis on ultrasound.[222] 3D ultrasound with contrast enhancing solution stands as the diagnostic tool of choice for first-line screening for uterine malformations. Yet, admittedly, this novel approach has not been sufficiently challenged by use in low-prevalence populations. However, Woelfer and colleagues prospectively studied 1089 women with no history of infertility or recurrent early fetal losses using a 3D ultrasound.[224] The scanned uteri were analyzed in reconstructed coronal planes, looking at a series of parallel transverse sections and analyzed according to the AFS classification (Fig. 35.17). Briefly, normal and arcuate uteri were recognized by their convex external contour with indentation less than 10 mm. The fundal contour, which was straight or convex in the normal uterus, was concave in the case of arcuate uterus (indentation at obtuse angle) and showed the presence of a septum with the central point of the septum at an acute angle. In the bicornuate uterus, the external contour showed an indentation greater than 10 mm dividing the two cornua with a convex fundal contour in each. Of the original 1289 women recruited into the study, 200 were not suitable for analysis because of poorly visualized uterus primarily due to fibroids distorting the cavity. Of the remaining 1089 women, 983 had a normally shaped uterine cavity. Of the 106 (9.7%) uterine anomalies, there were 72 (68%) arcuate, 29 (27%) septate, and 5 (4.7%) bicornuate uteri.[211]

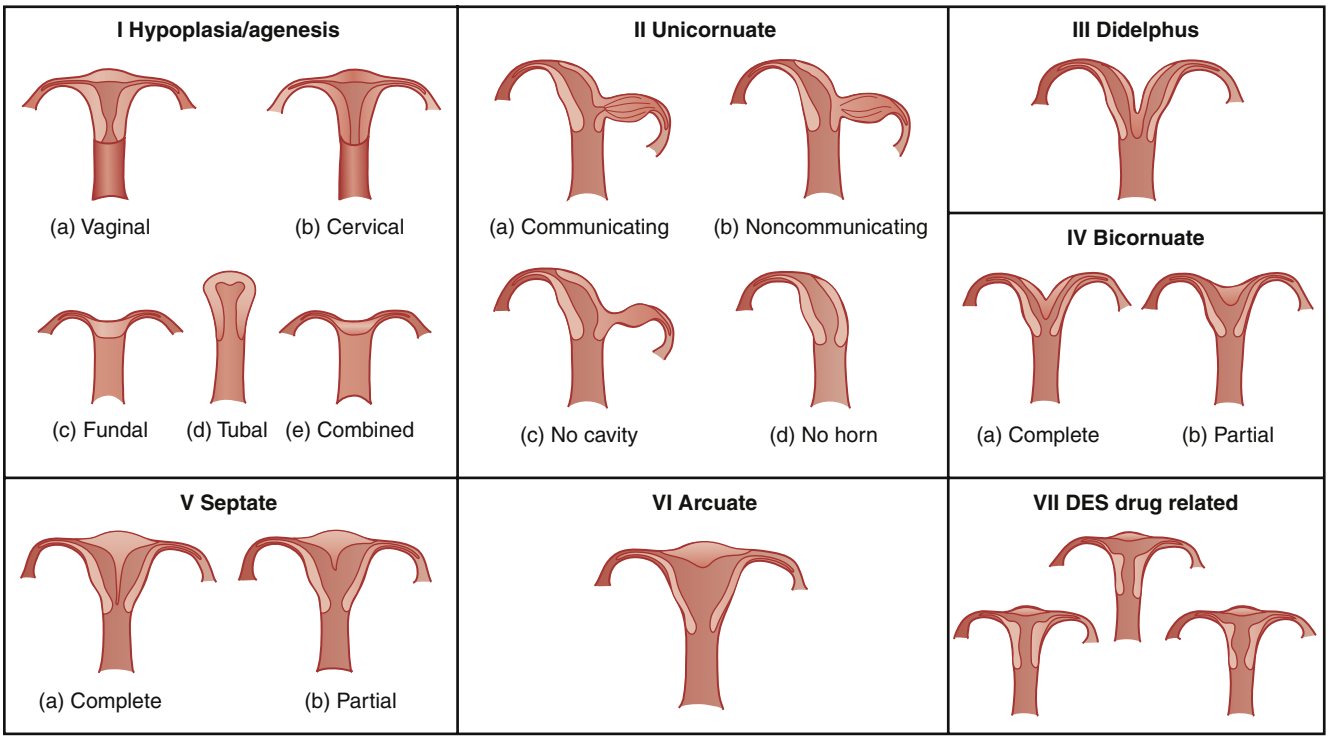
Extending their research on uterine malformations, this team looked at uterine anomalies in 509 women with a history of unexplained recurrent miscarriages and 1976 asymptomatic women in whom a uterine anomaly had been diagnosed fortuitously on ultrasound.[225] There was no difference in relative frequency of various müllerian anomalies or depth of fundal distortion between the two groups. However, in the case of both arcuate and septate uterus, the length of the remaing undisturbed uterine cavity was significantly shorter with more pronounced distortion in the recurrent miscarriage group. This observation is clinically important. It indicates that it is the degree of the müllerian malformation that bears the most important consequences on reproductive outcome rather than the type of malformation encountered.

Uterine Contractility: Direct Visualization on Ultrasound

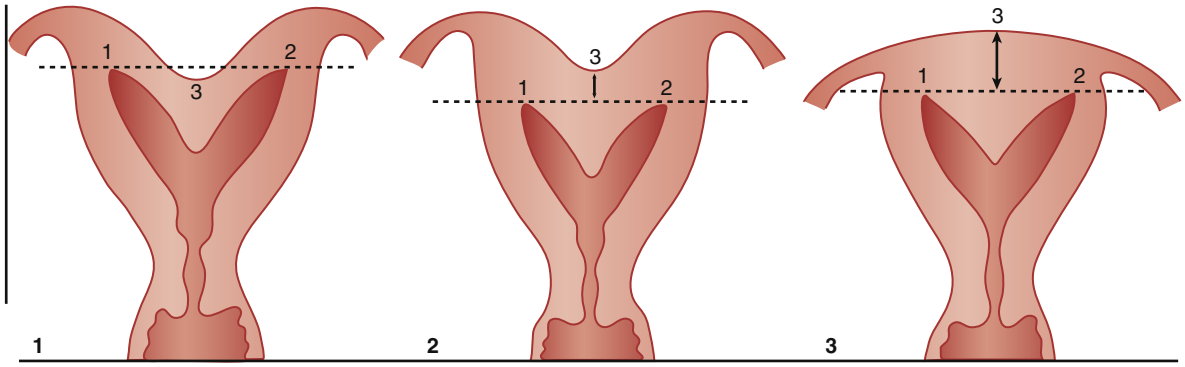
Contractility of the Nonpregnant Uterus: Methodological Issues

Detection of contractions in the nonpregnant uterus previously required the invasive measurement of intra-uterine pressure (IUP). The ability to study uterine contractility by ultrasound has revived interest in this facet of uterine physiology, leading to multiple studies aimed at understanding the role of contractility in reproductive physiology. Uterine contractions (UC) are characterized by three parameters: frequency, amplitude, and direction of contraction. Also pertinent to the contractile status of the uterus is the IUP value that prevails between UCs or *resting tone*.

Identifying UC on ultrasound scans has benefited from various forms of image management, which were necessary because of the relatively slow frequency of UC (maximum of 5 UC per minute in the late follicular phase), making direct UC identification difficult. Most commonly, uterine scans are accelerated by 5 to 10 times for UC identification, using fast plays of VHS recordings[226,227] or digitized image sequences.[83] Some investigators have used custom computer assisted systems for UC recognition and computation of UC frequency. Ayoubi and colleagues[83] used an off-line 3D reconstruction system providing time mode image sequences depicting time-related changes of the myometrial to endometrial interphase (Fig. 35.18). On these sequences, UCs appear as successive waves created by the periodic displacement of the myometrial to endometrial interphase. We demonstrated in a prospective trial that the 3D-based approach and direct identification of UC on fast plays of digitized image sequences provide identical



A



B

FIGURE 35.17 Müllerian anomalies: **A,** American Fertility Society classifiaction of müllerian anomalies. **B,** differentiation of septate from bicornuate uteri. The uterus is bicornuate when apex of the fundal external contour occurs below (1) or within 5 mm above (2) a straight line between the tubal ostia. The uterus is septate when apex is more than 5 mm above the line between the tubal ostia (3). DES, diethylstilbestrol.

UC frequency results.[83] More recently, Pierzynski and colleagues used a system based on image distortion recognition for UC identification.[228] In a prospective trial conducted in estrogenized women, Bulletti and colleagues reported perfect concordance between UC frequency measurements made by IUP analysis and UC recognition on ultrasound scans using a 3D-based approach.[229] This study provided definitive validation of the noninvasive ultrasound-based approach for UC frequency measurement.

The direction of contractions has been assessed by visualizing the displacement of the contractile process on fast plays of uterine scans. Based on direct UC visualization, various types of contractions have been described, ultimately leading to the recognition of two primary types: either retrograde, cervix to fundus; or antegrade, fundus to cervix. It is of note, however, that ultrasound-based analysis of UC displacement have never been validated by comparing ultrasound findings against the actual propagation of the pressure wave, as measured by multiple IUP recorders[230] or displacement of the uterine contents.[231] UC amplitude and uterine resting tone also evade ultrasound-based analyses.[157,232]

Uterine Contractility During the Menstrual Cycle: Three Characteristic Patterns

There are typically three patterns of uterine contractility that are recognized during the menstrual cycle. These are emblematic of the late follicular phase, luteal phase, and intercycle interval (i.e., during menses).[83,233] Each of these patterns is defined by sets of UC characteristic such as frequency, amplitude, and direction of contractions.

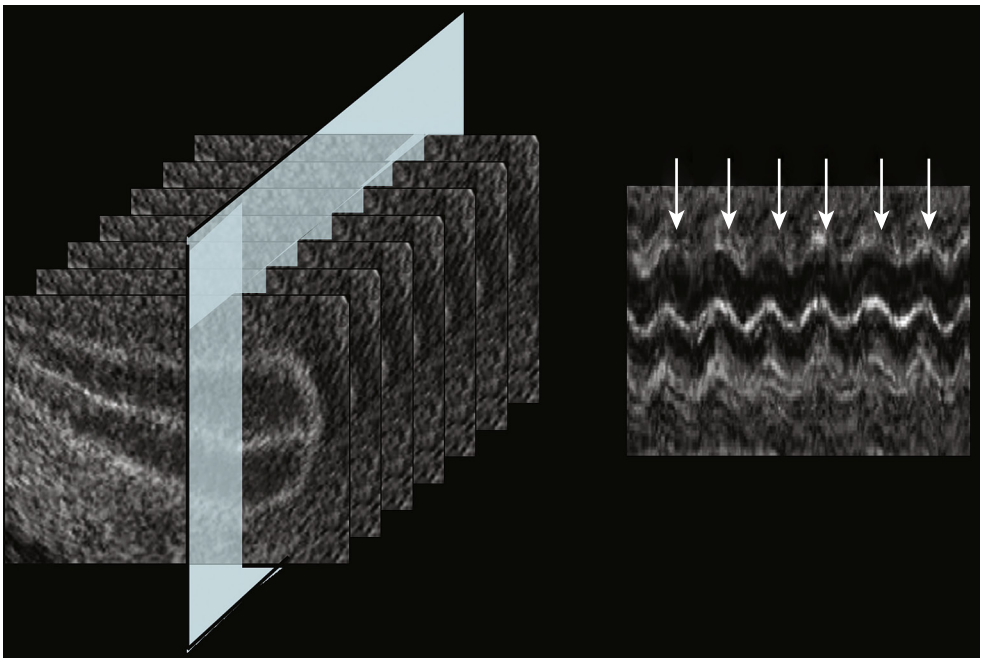


FIGURE 35.18 3D-based approach for uterine contraction (UC) assessment. 2D images are recorded over time without sweeping through a given territory. Hence, the z-axis represents time. On these electronically reconstructed time mode™ scans, displacements of the myometrial-endometrial interphase take the appearance of periodic waves, with each wave individually representing an actual contraction of the uterus.

Follicular Phase

During the follicular phase, there is a progressive increase in uterine contraction (UC) frequency under the influence of rising E_2 levels,[83,234,235] which culminates at approximately 5 UC per minute just prior to ovulation.[73] Characteristically, only the subendometrial layers of the myometrium are involved in these contractile events, which are never perceived by women despite UC frequency being higher at that time than at any other time in the menstrual cycle.[24]

The majority of investigators,[83,236] but not all,[237] have reported a clear predominance of UC dysplaying retrograde cervix-to-fundus displacement during the late follicular phase. In these studies, UC displacement was evaluated on fast plays of ultrasound sequences, or by analyzing the propagation of the increase in the IUP signal.[231] Yet, determining the UC direction is complicated by two unknowns: (1) While the contractile structure that is responsible for UCs is the subendometrial portion of the myometrium, the visualized phenomenon on ultrasound images is the result of the displacement of the endometrium. Hence, the parameter that is analyzed is an indirect reflection of the contractile process. (2) Ultrasound identifies the apparent retrograde displacement of contractile waves without establishing whether this does or does not result in true displacement of the uterine contents. In the case of sperm for example, retrograde transport will also depend on whether an appropriate opening takes place or does not take place at the utero-tubal junction when the contractile event reaches the junction.

Retrograde UCs identified during the late follicular phase are thought to be instrumental in the rapid transport of sperm that takes place from the vagina to the pelvic cavity following intercourse in the late follicular phase.[231,238,239] Thus, UCs and possible disruption of their patterns, may, therefore, play a key role in the reproductive process and in certain forms of infertility. However, as stated above, echographic analyses of UC direction have never been shown to correlate with the actual displacement of uterine contents.

Using ^{99}Te labeled macro-albumin aggregates (MAA), Leyendecker's team showed that contractions with retrograde displacement of uterine contents predominate during the late follicular phase.[235] These authors also observed that transport takes place toward the tube facing the developing follicle.[24,235] In various intervention trials, Leyendecker's team reported evidence for the involvement of oxytocin (OT) in the retrograde contractions encountered during the late follicular phase.[24,231] That OT, and possibly vasopressin (AVP), play a physiological role in late follicular phase UCs, and are instrumental in the control of sperm transport, remains to be determined. Retrograde displacement of uterine contents triggered by late follicular phase UCs appears to be disrupted in women suffering from endometriosis[235] or intramural uterine fibroids.[156,157] This could be responsible for the infertility that accompanies both conditions.

Luteal Phase

During the luteal phase, the UC pattern is characterized by a state of uterine quiescence brought about by the smooth muscle relaxing properties of progesterone. In a prospective trial, Wilcox and colleagues[240] showed that pregnancies never occurred when the single unprotected intercourse took place after the day of ovulation. This finding may be explained in part by the utero-relaxing properties of progesterone and the resulting decrease in uterine contractility, particularly the retrograde UCs that are thought to be involved in sperm transport. In experimental trials, exogenous E_2 and progesterone reproduce the UC patterns encountered in the follicular and luteal phases, respectively (Fig. 35.19). Ayoubi and colleagues observed that physiological E_2 replacement administered to young women suffering from premature ovarian failure (POF) resulted in UC

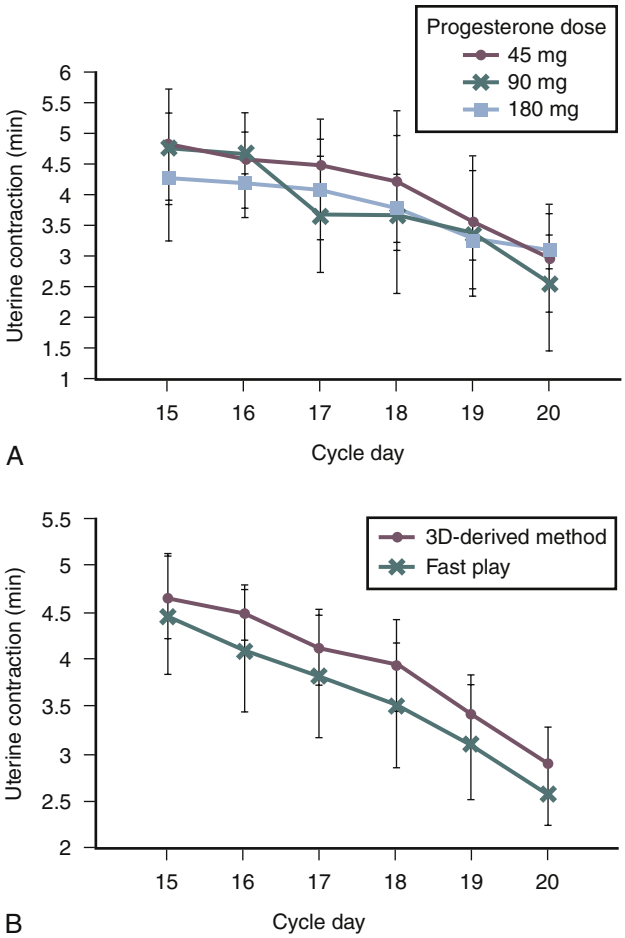


FIGURE 35.19 Effects of exogenous progesterone on uterine contractility. **A,** In estrogenized women, exogenous administration of 45, 90, and 180 mg of progesterone/day resulted in a prompt decrease in uterine contraction (UC) frequency without dose-related differences. **B,** Direct measurements from fast plays of ultrasound sequences or using the 3D-derived method gave similar results. *(Ayoubi JM, Fanchin R, Kaddouz D, et al. Uterorelaxing effects of vaginal progesterone: comparison of two methodologies for assessing uterine contraction frequency on ultrasound scans.* Fertil Steril *76:736, 2001.)*

frequency patterns that were similar to those encountered in the menstrual cycle particularly, during the follicular-luteal transition.[83] Transvaginal administration of 45, 90, and 180 mg of progesterone per day using a sustained release vaginal gel, Crinone, resulted in a prompt decrease in UC frequency with no dosage-related differences. This indicates that following physiological estrogen exposure, minimal amounts of progesterone suffice to induce full utero-quiescence, as seen in the luteal phase of the menstrual cycle.[83]

Intercycle Interval

During the intercycle interval (menses), uterine contractility increases in response to withdrawal from the utero-relaxing properties of progesterone upon demise of the corpus luteum, or discontinuation of exogenous progesterone or progestin administration. The role of concomitant withdrawal from E_2 also produced by the corpus luteum and supplied in most OC preparations remains a matter for debate. In contrast to the late follicular phase, all layers of the myometrium are involved in UC experienced by women at the time of menses. Moreover, UCs are commonly perceived by women at that stage of the cycle. At times, UCs occuring during the inter-cycle interval are painful enough to cause dysmenorrhea, characterized by painful uterine cramping. UC amplitude and resting tone are markedly higher during menses than at any other time during the menstrual cycle.[230,233,241] These are the two parameters that are most increased in dysmenorrhea.[241]

Using MRI-based analyses, Kataoka and colleagues showed an enlargement of the subendometrial layer of the myometrium in dysmenorrheic women on cycle day 1, at a time when these women experienced strong cramping. Uterine images returned to normal on day 3 of the cycle when cramping had subsided (Fig. 35.20).[232] In a prospective trial, Kido and colleagues compared the characteristics of MRI of the endometrium and subendometrial layer of the myometrium in 25 women on OC pills and 23 others having regular menstrual cycles.[157] This trial revealed that endometrial distortion was significantly less prominent, and the subendometrial low intensity area significantly thinner, in the OC group who also presented a lesser degree of cramping as compared to their cycling counterparts.[157] MRI is, therefore, the only imaging method providing noninvasive information on UCs that parallel clinical symptoms in dysmenorrhea.

Effects of Uterine Contractility on ART Outcome

Ayoubi and colleagues[83] were first to report an inverse correlation between UC frequency at the time of embryo transfer (ET) and IVF outcome (Fig. 35.21), a finding supported by data from others,[242] but not all groups.[243] More frequent uterine contractions had negative consequences for implantation and pregnancy rates. As UCs were measured prior to conducting embryo transfers (ETs), the study reflected the state of contractility prevailing prior to ET, not the impact of the ET procedure itself on contractility. In further trials, an inverse correlation between the progesterone levels on the day of ET and UC frequency was observed.[116] Furthermore, an earlier onset of progesterone treatment on the day of oocyte retrieval lowered UC frequency on the day of ET, with a trend toward better pregnancy rates.[83] Similarly, delaying ET to the fifth day after oocyte retrieval resulted in a profound reduction in UC frequency at the time of ET.[83]

Following extensive analyses of UC patterns as observed by ultrasound, Ijland and colleagues[237] reported that women in whom UCs were predomanantly retrograde had higher pregnancy rates. The observation that certain IVF patients have increased UC frequency at the time of ET raises questions about the mechanism(s) at play. In a prospective trial, we studied UC frequency in the same patients during the menstrual cycle that preceded IVF and the IVF cycle itself.[73] In these women, UC frequency reached a similar maximum on the day of the LH surge and hCG administration, despite E_2 levels being markedly higher in the IVF cycle as compared to the menstrual cycle. This suggests that the E_2 levels of the menstrual cycle already exert a maximal effect on UC frequency prior to the LH surge or hCG administration.[73] As illustrated in Figure 35.22, a marked difference in UC frequency exists after ovulation. In the menstrual cycle, uterine quiescence induced by progesterone is observed as early as day 4 after the LH surge, with a mean UC frequency of 1.1 per minute. In contrast, UC

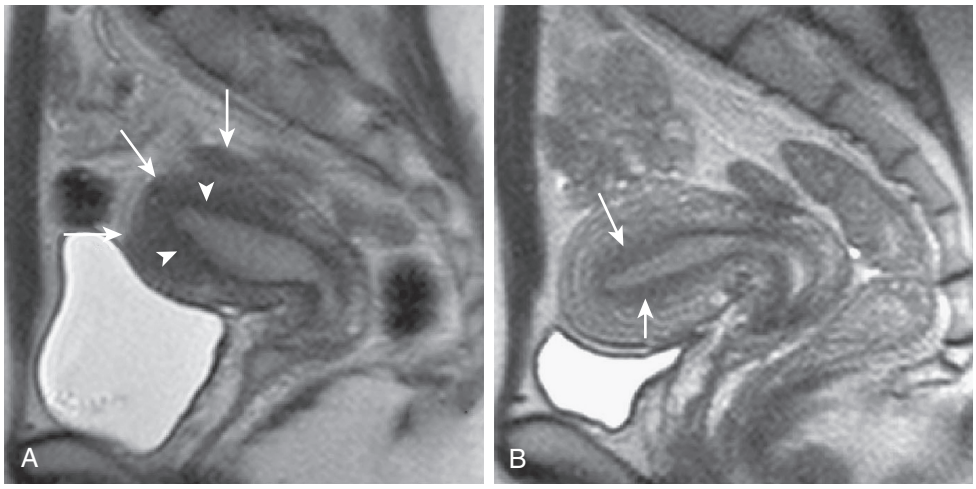


FIGURE 35.20 Using magnetic resonance imaging (MRI), differences were observed in the junctional zone between women experiencing severe dysmenorrhea on day 1 of the menstrual cycle **(A)** and the same women assessed on day 3 when cramping had subsided **(B).** On day 1 a characteristic distortion of the endometrium *(arrows)* and a thickened junctional zone *(arrowheads)* was noticed, a finding that paralleled the intensity of pain perceived. On day 3, when pain had subsided, the endometrium and junctional zone had returned to normal. *(From Kataoka M, Togashi K, Kido A, et al. Dysmenorrhea: evaluation with cine-mode-display MR imaging—initial experience.* Radiology *235:124, 2005.)*

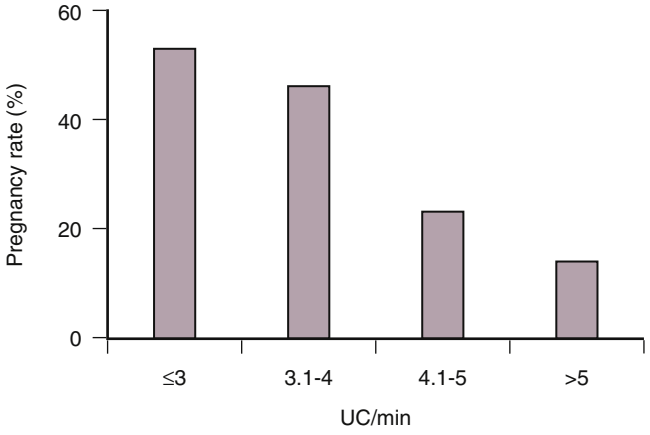


FIGURE 35.21 Uterine contractility (UC) just prior to embryo transfer and pregnancy rates. In women who all had three good embryos available for transfer, an inverse correlation was observed between UC frequency and pregnancy rates. *(From Fanchin R, Righini C, Olivennes F, et al. Uterine contractions at the time of embryo transfer alter pregnancy rates after in-vitro fertilization.* Hum Reprod *13:1968, 1998.)*

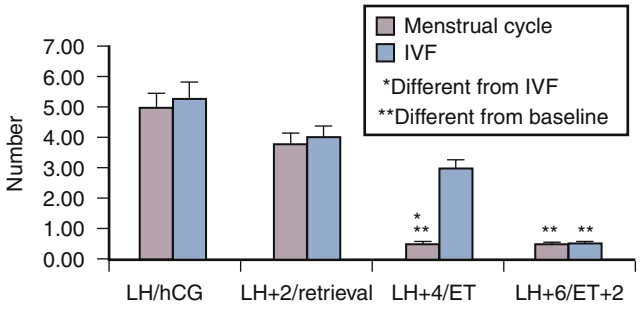


FIGURE 35.22 UC frequency was measured in the same patients, in the menstrual and IVF cycles. UC frequency was similar on the day of LH surge and hCG administration, indicating that the supraphysiological levels of E_2 encountered in ART do not further stimulate UC frequency over what is encountered in the menstrual cycle. Following ovulation, UC frequency decreased earlier in the menstrual cycle than it did in ART, indicating that a relative degree of resistance to the uterorelaxing properties of progesterone exists in ART, most likely as a result of elevated E_2 levels. UC, uterine contractility; E_2, estradiol; ART, artificial reproduction technology; hCG, human chorionic gonadotropin; IVF, *in vitro* fertilization; LH, luteinizing hormone; ET, embryo transfer. *(From Ayoubi JM, Fanchin R, Kaddouz D, et al. Uterorelaxing effects of vaginal progesterone: comparison of two methodologies for assessing uterine contraction frequency on ultrasound scans.* Fertil Steril *76:736, 2001.)*

frequency remained high at 3.6 per minute on the 4th day after hCG administration, ultimately declining on day 6. Of note, however, these women started receiving exogenous progesterone on day 4 after hCG (after the UC measurement). Exogenous progesterone, therefore, may have been instrumental in the decrease in UC frequency observed on the 6th day after hCG in IVF. Taken together, these findings suggest that some degree of resistance to the uterorelaxing properties of progesterone exists in IVF, possibly related to high E_2 levels.

Pierzynski and colleagues[228] provided pilot data suggesting that exogenous administration of the OT antagonist, atosiban, resulted in decreased UC frequency prior to ET. These findings open up new avenues for uterorelaxing therapy in relation to ET using OT and vasopressin receptor antagonists,[244] when other uterorelaxants have failed.[245]

Imaging Markers of Endometrial Receptivity

The endometrial parameters assessed by uterine imaging that have been proposed as markers of endometrial receptivity are endometrial thickness and volume, echogenicity, Doppler perfusion parameters, and contractile patterns. Endometrial thickness reflects endometrial priming by estrogen necessary for an appropriate response to progesterone and, in turn, endometrial receptivity. Rashidi and colleagues[246] reported no correlation between endometrial thickness on the day of hCG administration and pregnancy rate. Endometrial thickness was not different at 10.1 and 10.2 in pregnant and nonpregnant patients, respectively.

Okohue and colleagues reported that an endometrial thickness 7 to 14 mm was associated with better outcome than when the endometrium was either less than 7 mm ($P = 0.004$) or greater than 14 mm ($P < 0.0001$),[247] data in agreement with those of others.[246] The early report by Casper's group of poorer outcome in case of excessively thick endometrium[100] was not confirmed in later studies, such as that of Richter and colleagues on 1294 patients.[89]

The possibility of positive bias towards a thicker endometrium could result from its relationship to heftier responses to COH, a parameter that is associated with better IVF outcome.[248] By extension, a positive bias for Doppler findings of higher perfusion could be the consequence of an association with a more robust ovarian responses to hMG and FSH, and consequently increased oocyte quantity and quality. Whether different ovarian stimulation protocols had different impacts on endometrial thickness was loooked at by Imoedemhe and colleagues.[249] These authors compared the effects of regimens using clomiphene citrate (CC) alone (regimen I) or in combination with 75 IU (regimen II), or 150 IU of hMG (regimen III), and found no differences in endometrial thickness.

Raga and colleagues examined the predictive value of 3D endometrial volume calculations for the establishment of pregnancy in ART.[11] In a study on 72 IVF cycles, pregnancy rates were significantly lower when endometrial volume was less than 2 mL as compared to 2 to 4 mL or greater than 4 mL. Zohav and colleagues found no differences in endometrial volume in 60 women pregnant through IVF, but observed that endometrial volume of less than 2 mL was associated with a significant increase in pregnancy loss.[248] A recent study on E_2 and progesterone cycles for frozen embryo transfers came to similar conclusions that lower volumes are an ominous sign.[75] Ferriani's group reported on endometrial volume measured one week after ET.[250] A significant difference was found between the endometrial volume of pregnant (6.49 ± 1.97 mL) and nonpregnant women (3.4 ± 1.1 mL) and, but to a lesser extent, endometrial thickness. This latter observation of a difference in endometrial volume by a factor of nearly 2, 1 week after ET, between pregnant and nonpregnant women likely reflects a direct early influence of the developing pregnancy on the endometrium rather than preexisting differences. This finding is in agreement with the report of an early increase in E_2 production that even precedes that of hCG in the late luteal phase of conception cycles.[251]

Endometrial echogenicity or 'pattern' has been proposed as a marker of endometrial receptivity.[252] In early work, Casper's team, who observed no differences in endometrial thickness between pregnant and nonpregnant women, reported three different patterns of endometrial echogenicity on the day after hCG administration.[107] In these investigators' hands, a hypoechoic endometrium carried a good prognosis. Numerous publications support the notion that a hypoechoic endometrium with a full three-line pattern is associated with good ART outcomes,[108-113] although some failed to find a good predictive value of endometrial echogenicity.[114,115]

According to Rosenwaks' team, a hyperechoic endometrium is a particularly ominous sign in women exposed to DES.[253] Pregnancy outcome relative to endometrial thickness and pattern was evaluated in 540 cycles of IVF including DES (n = 50) and non-DES-exposed (n = 490) women. Endometrial patterns were designated as p1 = solid; p2 = ring; and p3 = intermediate. DES patients exhibited the 'p1' pattern more often than the majority of the non-DES-exposed group. There was no significant difference in endometrial thickness among the cycles where 'p1' was noted when comparing the DES (10.3 mm) with the non-DES-exposed (10.7 mm) groups. Notably, within the group exhibiting 'p1', no pregnancies occurred in the 18 cycles of DES-exposed women compared with a 39.2% clinical pregnancy and 36.5% delivery rate in the non-DES-exposed controls ($P < 0.0001$ and $P = 0.008$, respectively). The impact of uterine shape on pregnancy outcome was also investigated. A T-shaped uterine configuration was noted in 11 out of 18 (61.1%) cycles of DES-exposed women with pattern 'p1' compared with 9 out of 23 (39.1%) with pattern 'p2'. Of cycles where a T-shaped uterus was demonstrated, none out of 11 (0%) with pattern 'p1' compared with 4 out of 9 (44.4%) with pattern 'p2' resulted in pregnancy ($P = 0.026$). These data suggest that the endometrial pattern is one of the most significant variables for pregnancy outcome in DES-exposed women undergoing IVF.

The two last parameters proposed as markers of endometrial receptivity are Doppler data on endometrial and subendometrial vascularization and uterine contractility. Early Doppler studies that claimed to identify flow parameters that precluded the possibility of embryo implantation were never verified. Instead, all available studies that used either pulsed Doppler data or the more sophisticated and operator independent 3D-PDA failed to find differences between conception and nonconception cycles.[5] These findings showing that Doppler data—even using the newest and most sophisticated techniques—are not predictive of ART outcome were confirmed by recent reports.[21,254] Hence, Doppler data cannot be considered to be a marker of endometrial receptivity. In our experience, increased uterine contractility portends poor embryo implantation rates.[221] These findings have been confirmed by some researchers,[228] but have been challenged by others.[243]

Embryo Transfers

ET should be performed with utmost care. Sloppy practices and imprecise placement of the transferred embryos in the uterine cavity undermine the good clinical and biological work that came together to obtain these embryos.[255] Interestingly, it is the very person who first conceived oocyte retrieval under ultrasound guidance[256] who was the first to advocate in 1987 for ultrasound-directed embryo transfers.[4]

In 1991, a prospective trial reported higher pregnancy rates in 94 women who had an ultrasound-guided transfer as compared to 246 controls who had the classical approach. Similar benefit was found in donor egg recipients. In a retrospective comparison of 137 embryo transfers, Lindheim and colleagues showed that pregnancy and embryo implantation rates were higher in women whose transfers were ultrasound-guided as compared to their counterparts in whom ultrasound was not used,[257] a finding confirmed by Coroleu and colleagues with even greater differences.[258] These pioneering studies were later supported by numerous others, including sets of meta-analyses,[259-262] although a few reports failed to document the superiority of ultrasound-guided transfers.[263-265] Ata and Urman argued against the conclusions

of non-superiority.[266] They noted that no ultrasound-guided trial reported lower results than the classical "clinical touch" method. Ata and Urman also underscored the fact that not all ultrasound-guided trials placed the embryos where their chances of implantation were optimal.[266] Flisser and Grifo, whose own study showed no improvement in pregnancy rates with ultrasound-guided transfers, nonetheless recommended systematic use of ultrasound,[263] since it is impossible to preemptively determine who is likely to benefit from ultrasound-guided ET. A recent meta-analysis that retained 17 trials out of 59 identifed in the literature on ultrasound-guided transfers. In 7 out of 17 of these trials that reported on clinical pregnancy rate, ultrasound-guided ET resulted in improved ART outcomes.[267] Bodri and colleagues suggested that vaginal ultrasound may be used as effectively as the abdominal approach, while providing further comfort for the patient.[268]

With the ability to visualize the tip of the ET catheter, ultrasound-guided transfers beg the question: Which is the optimal location in the uterine cavity for transfer? Data from prospective trials unveiled a counterintuitive scenario: ETs are more successful when performed low rather than high in the uterine cavity. As early as 2002, Coroleu and colleagues reported a prospective trial in which embryos were transferred in 180 consecutive IVF patients at 1, 1.5, and 2 cm from the fundus.[259] Unequivocally, embryo transfers performed 2 mm and 1.5 mm from the fundus resulted in higher pregnancy rates than seen when embryos were placed 1 mm from the fundus. In agreement with these findings, Pope and colleagues observed in a retrospective analysis that the further away the tip of the catheter was from the fundus, the higher the pregnancy rate.[269] A regression analysis in this trial indicated that each millimeter in excess of 1 cm from the fundus translated in an 11% increase in pregnancy rate, a trend later confirmed by others.[5]

As interest in ultrasound-guided transfers gained momentum, industry designed catheters specially equipped with echogenic tips that facilitate visibility on ultrasound. A randomized controlled trial (RCT) comparing a standard ($n = 95$) and a new echogenic soft Wallace catheter ($n = 98$) revealed a trend ($P = 0.08$) for higher pregnancy rates with the echogenic catheter,[270] a finding confirmed by others,[271] but not all studies.[21] It appears, therefore, that while new echogenic catheters are easier to visualize, they do not necessarily bring higher pregnancy rates. Fang and colleagues recommended verifyng the proper positioning of the catheter on 3D-ultrasounds for fear that if the tip deviated from the 2D-plane it might not be properly identified.[272] These authors thoughtfully claim that in such a situation, the tip might be actually more advanced, which would be detriemental. The same argument would favor a catheter having its echogenic mark limited to its tip rather than full length of the catheter for best assurance that the echogenic mark represents the true tip of the catheter. A closing comment regarding the competitive comparisons between various ET catheters is offered by Yao and colleagues, who report that the results of such comparisons are operator-dependent.[273] In 1446 embryo transfers performed in 1155 women undergoing ART, 723 cycles were randomized to 'soft' (Cook) and 723 others to 'semi-soft' catheters (Frydman®). Overall, the odds ratio of clinical pregnancy for the Cook versus the Frydman catheter were not different at 1.11 (95% CI: 0.89 to 1.38). Yet, these differed significantly for two of the three operators, being at 1.19 (95% CI 0.84 t 1.69), 2.35 (95% CI 1.40 to 3.95), and 0.69 (95% CI 0.48 to 0.99) for each of them.[273]

Using ultrasound-guided ET in routine ART practice is a valuable tool for teaching the procedure to new operators.[274] At our institution, new operators have to demonstrate competance at performing mock ETs under ultrasound guidance before moving on to actually performing true ETs.

Ovaries

Unlike the uterus, which reaches a definitive and stable size at the time of puberty (short of developing tumors like fibroids), the ovaries change in size and aspect throughout the different phases of their function. Transvaginal ultrasound provides an unrestricted view of the ovaries with the fluid-filled follicles presenting a characteristic echographic signature to this organ. This makes ultrasound the first choice imaging approach for exploring the ovaries, even in cases of obesity.

Ultrasound identifies two ovarian constituents: the cortex and stroma.[165,275,276] Following a description by Pache and colleagues,[277] the total count of 2 to 10 mm follicles in each ovary constitutes the antral follicle count (AFC).[278] Remarkably, the AFC shows little intracycle variation,[279] and is not affected by hormonal treatments such as the OC pill.[279,280] Granulosa cells of antral follicles are endowed with FSH receptors, rendering these the cohort of follicles capable of responding to rising FSH. They are, therefore, the recruitable follicles.[281] Recruitment by FSH takes place either through the physiological intercycle FSH signal or with exogenous FSH and hMG treatments administered in the context of ART. Therefore, the number of antral follicle—the AFC—will affect the magnitude of the ovarian responses to stimulation in ART or COS.[281,282]

We know from the work of Gougeon's team[135,283] that the cohort of antral follicles identified on ultrasound examination represents a constant fraction of the pool of primordial follicles remaining in the ovary at any given time (Fig. 35.23).[284] Thus, there are grounds for assuming that

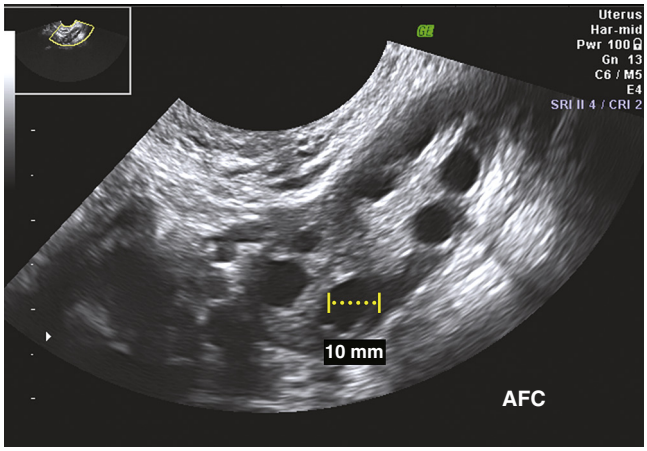


FIGURE 35.23 The antral follicle count (AFC). While the AFC does not fluctuate during the menstrual cycle, computation is classically done at baseline of the menstrual cycle (cycle day 2 to 4), because measurements are not hindered by a growing follicle or a corpus luteum. Traditionally, all follicles of 2 to 10 mm are counted.

the AFC reflects the number of remaining follicles, or ovarian reserve.[168,285] Therefore, the AFC aims at predicting: (1) the amplitude of the ovarian response to exogenous FSH and hMG or COS (for singling out excessive and poor responses and, therefore, adjustment of the doses of FSH and hMG accordingly) and; (2) ART outcome, on the assumption that it depends on the number of oocytes retrieved.[286] The latter point begs a question about whether oocyte quantity and quality are inherently linked or not. As discussed below, the nature of this link between oocyte quantity and quality differs based on whether a reduction in the AFC is age-dependent or independent.

The echographic characteristics of the ovaries (volume and/or AFC) are now taken into account when making the diagnosis of PCOS, per the joint ESHRE/ASRM Rotterdam consensus conference.[82] Ovarian volume and/or AFC represent one of the two of three criteria necessary for making the diagnosis of PCOS.

Large changes in blood flow take place in the ovaries during physiological (from follicular maturation to corpus luteum formation)[132] and abnormal function (ovarian stromal flow in PCOS),[287,288] or in the context of specific pathologies such as cancer.[289,290]

Ovarian Anatomy and Functional Changes

The Ovary Before and After Puberty

Ovarian growth in infants and children reflects functional changes rather than chronological age. In a prospective trial, Buzi and colleagues looked at 117 normal girls between the ages of 1.1 and 15.6 years, and 87 girls presenting with premature sexual maturation.[291] In girls showing no signs of abnormal sexual maturation, ovarian volume was 1.1, 2.3, and 5.3 mL in prepubertal, pubertal, and postmenarchial girls, respectively. These changes correlated better with Tanner stages of sexual development than with chronological age.[292]

In infancy and childhood, follicles begin their maturation and rapidly become atretic in the absence of gonadotropin stimulation, giving the ovary a microcystic pattern.[34,293] Before secondary sexual characteristics become visible, puberty-related changes take place with the ovaries showing changes in their morphology characterized by the presence of several follicles of 4 to 9 mm in size.[34] This appearance, sometimes referred to as megalocystic or multicystic,[294,295] needs to be distinguished from the polycystic ovary.[296-298]

The Ovary in the Menstrual Cycle

The intercycle interval constitutes the baseline state of the ovary in the menstrual cycle. Therefore, ovarian scans have been timed using this reference point for measuring the AFC for assessing the ovarian reserve and for predicting the response to COS in ART (Fig. 35.24). During the intercycle interval, the small 2 to 9 mm follicles, computed in the AFC, are the follicles capable of responding to FSH, whether naturally occurring or part of COS in ART.

Through daily evaluation of individual ovarian follicles on repeated transvaginal scans, Pierson's team identified bursts of synchronous growth in a cohort of follicles, or waves of follicle development.[299] These investigators identified minor waves of follicular growth leading to all the follicles ultimately undergoing atresia and major ones leading to further follicular growth and single follicular dominance.[299] The nature of the mechanisms controlling these successive waves encountered during the menstrual cycle still evades our understanding.

Follicular growth leading to follicular dominance can be ascertained by viewing follicles greater than 10 mm. From repeated measurements, it was determined that follicles reach the 12 to 13 mm mark on average by day 9 of the menstrual cycle.[299] At this stage, granulosa cells acquire LH receptors with evidence that follicular maturation can be promoted with LH only[300] or mini doses of hCG.[301-304] Follicular growth rate appears fairly constant in the menstrual cycle once follicles are greater than 13 mm, with a daily mean increment in diameter of 1.4 to 1.5 mm/day. Follicular growth is accelerated in COH, however, where it reaches 1.6 to 1.8 mm/day.[305]

The side of ovulation in the menstrual cycle and whether this regularly alternates from one cycle to the next has been a matter of enduring debate. Some reports based on histological evidence support the concept of alternating sides,[306] while others claim that it is not more frequent than expected by chance alone.[307] Finally, some propose that the side of follicular growth and ovulation only alternates in cases of short cycles,[308] while the ovulation side is randomly

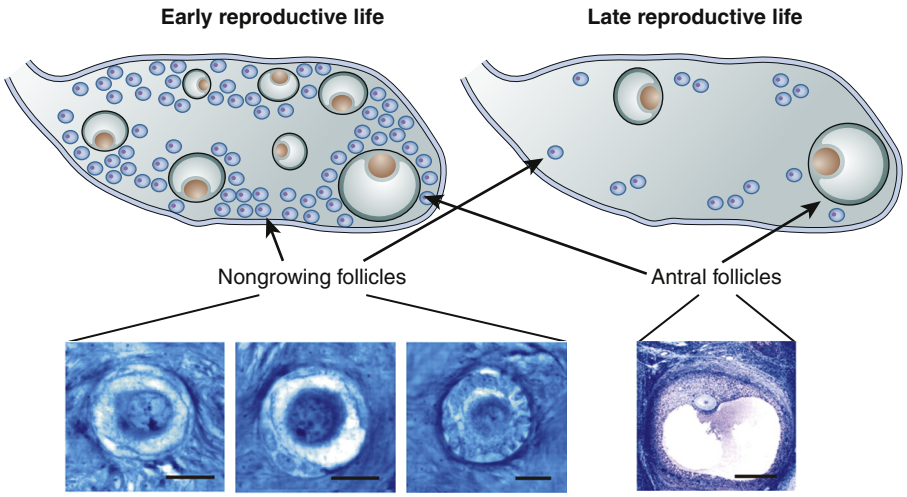


FIGURE 35.24 Nongrowing and antral follicles in early and late reproductive life. In early and late reproductive life, the antral follicle count (AFC) reflects the total number of follicles remaining, or ovarian reserve. *(From Hansen KR, Knowlton NS, Thyer AC, et al. A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause.* Hum Reprod *23:699, 2008.)*

determined in cases of long cycles. Reassessing this latter paradigm, Fukuda and colleagues studied 410 natural cycles of 123 infertile women undergoing 267 IUI and 143 IVF natural-cycle treatments, respectively.[309] In this population, ovulation occurred contralaterally by reference to the preceding cycle in 57% of the 410 cycles, but in 72% of women whose follicular phase was less than 13 days. Conversely, ovulation occurred randomly on either side when the follicular phase was greater than 14 days, thereby giving credence to the concept that the side of ovulation only alternates in short cycles. From these authors' observation, ovulation appeared of better quality when occurring on the contralateral side with reference to the previous ovulation with better outcomes in both IUI and IVF cycles. This supports the concept that ovulation is of better "quality" when the dominant follicle is contralateral to the prior ovulation as compared to ipsilateral. Moreover, significantly higher estradiol to androstenedione and estradiol to testosterone ratios and lower androstenedione levels were observed in contralateral as compared to ipsilateral ovulation cycles. Practically speaking, therefore, the chance of conceiving during a natural cycle may be affected by the site of ovulation in the preceding cycle. The side of ovulation, however, did not influence Doppler blood flow in the ovarian stroma or follicular and uterine arteries.[310]

Pierson's team prospectively assessed imaging characteristics of the corpus luteum (CL) with daily ultrasounds in 50 normo-ovulatory women.[311] The CL was detectable on ultrasound scans in 100% of women during the luteal phase, with persistent structures found in 90% of women during the subsequent follicular phase. Two days after ovulation, a central fluid filled cavity was present in 88% of the cases, declining to 34% after 13 days and 2% after 27 days.[307]

The Ovary during Hormonal Contraception

The impact of OC treatment on ovarian follicles depends on the size of the follicles when OC treatment is initiated. In a prospective trial, Baerwald and colleagues determined that no follicular growth took place when all the follicles were less than 10 mm at the time of starting OC pills.[312] Conversely, approximately 30% of follicles that were greater than 14 mm at the onset of OC treatment went on to ovulate, while the rest regressed and became atretic. In parallel work, these investigators determined that follicular development takes place during the 1-week hormone-free interval of classical OC pill regimens.[313] In a trial looking at normal volunteers on OC pills, they determined that nearly half (47%) of women grew follicles greater than 10 mm, with 53% of them having follicles reaching 14 mm in diameter and an increase in E_2 production. Contrasting with these findings, echographic assessments showed similar rates of follicular growth in women using progestin only or barrier contraception in the postpartum period. Endometrial thickness was markedly reduced, however, in the progestin only contraception group, indicating that this form of contraception acts on the uterus and cervix rather than by blocking ovulation.[314]

Hormonal contraception causes a significant reduction in ovarian volume in normally cycling women[315] and in women suffering from PCOS.[290,316] In cycling women, the effect of hormonal contraception on ovarian volume was more profound in cases of continuous rather than cyclical administration of hormonal contraception.[317] Remarkably, however, AFC and AMH values remained unaltered in PCOS women receiving hormonal contraception, in spite of the reduction in ovarian volume observed.[290]

The Ovary Through Functional Aging and Menopausal Changes

Ovarian volume decreases with age. Early on in the high-resolution ultrasound era, their was optimism about the possible predictive value of ovarian volume measurements for future reproductive performance. Surveys of published data indicated that ovarian volume was a good and easily obtainable index of the primordial follicle population based on comparisons with Faddy's data.[318] Pavlik and colleagues retrospectively reviewed data from 58,673 ovarian scans obtained from 13,963 women 25 to 91 years of age who underwent annual scanning as part of ovarian cancer screening program. Ovarian volume was calculated using the prolate ellipsoid formula ($L \times H \times W \times 0.523$).[123] Mean ovarian volume was 6.6 ± 0.19 cm^3 in women younger than 30 years old with a decrease that reached statistical significance in women between the ages of 30 and 39 years old. Yet, the practicality of ovarian volume measurements for predicting ovarian reserve on an individual basis is somewhat limited, as clinically meaningful changes only materialize when women are at the boundaries of their entrance into the naturally infertile period of life. In Pavlik's analysis, it is in the group of women between 40 and 49 years of age that ovarian volume was found to be markedly reduced at $4.8 \pm 0.3 cm^3$. Ovarian volume decreased to 2.6 ± 0.01 cm^3 in postmenopausal women 50 to 59 years of age, with further decreases observed during the sixth and seventh decades of life.[123] In their extensive study, these authors also looked at relationships between height and ovarian volume. Their findings with no progressive increase with height, but a statistically significant break with higher volumes in women over 5 feet 8 inches tall. There was also a statistically significant reduction in ovarian volume in women younger than 50 years old taking estrogens, but not in postmenopausal women. In a retrospective chart review, Syrop and colleagues looked at possible different predictive values of the volume of the smallest and largest ovaries in individuals.[319] Their results indicated that the volume of the smallest ovary was a better predictor of peak E_2 levels and numbers of oocytes and embryos and clinical pregnancy in IVF.

By current definition, premature ovarian failure (POF) occurs when ovarian functions fail before the age of 40. This implies a complete form of hypergonadotropic hypogonadism with an implicit loss of fecundity. More commonly encountered in everyday ART practice are cases of premature ovarian insufficiency or occult POF (oPOF), which hide behind seemingly normal ovulatory cycles. In oPOF, the menstrual cycle is maintained and seemingly ovulatory, with no anomaly except maybe for a shortening of the cycle duration with a characteristically early ovulation.

Embedded amongst the classical POF and oPOF that results from early ovarian aging, there are sporadic cases of a variant disorder characterized by insensitive ovaries. The difference between oPOF and POF on the one hand, and the rare cases of insensitive ovaries on the other, is more than just an issue of terminology, as pregnancy is much more likely to be achieved in the case of insensitive ovaries. Assessing ovarian volume in POF or oPOF may identify the

subgroup of women suffering from insensitive ovaries for whom therapeutic efforts can be undertaken. In a prospective trial, Mehta and colleagues looked at 17 women with fully expressed POF.[320] Based on the ecographic appearance of their ovaries, these authors sorted POF women into two groups, with (n = 7) and without ovarian follicles (n =10). Hormonal parameters (FSH and E_2) and endometrial thickness were identical in the two groups, whereas ovarian volume was markedly larger at 2.8 ± 0.4 mL than in the no ovarian follicle group (1.4 ± 0.2 mL). On average, two follicles were identified in the "with follicles" group, whereas by definition none were identified in the "no follicle" group. These authors contend that ultrasound imaging of the ovaries allows identification of a subgroup of women presenting with hypergonadotropic hypogonadism who suffer from insensitive ovaries, rather than POF. Pregnancies are more likely to occur in insensitive ovaries than they are in POF following treatments aimed at lowering endgenous gonadotropins in order to resensitize FSH receptors. This is can be achieved by 2- to 3-month treatment with E_2 and progesterone for lowering FSH levels followed by FSH administration. Falsetti and colleagues observed normal ovarian volumes (3.1 ± 0.3 mL) in 14 out of 40 (35%) women with frank POF, whereas ovarian volume was significantly smaller in the remaining 26 (65%) women.[321] In all forms of POF and more importantly oPOF, screening for possible premutations of the *FMR1* gene should be considered. Ignored, these may transform to the full mutation in the next generation, possibly causing Fragile X syndrome in male offspring.[322]

In Turner syndrome, the number of primordial follicles has been estimated to be normal until at least the 18th week of fetal life, with uncertainties as to the time when atresia starts to accelerate.[323] The possibility of transplanting cryopreserved ovaries,[324] including the report of live-births,[325] has sparked interest in cryopreserving ovaries from Turner patients before follicle depletion is completed. In a prospective trial, Hreinsson and colleagues reported ovarian follicles present in eight of nine adolescent girls diagnosed with Turner syndrome, but who all underwent spontaneous onset of puberty.[326] In these adolescent girls, the density of primordial follicles in ovarian samples studied surgically showed an inverse correlation with plasma FSH levels. It would be probably more useful to cryopreserve ovaries in infancy or childhood, when the pool of primordial follicles remaining is likely to be much larger, provided that the diagnosis of Turner syndrome can be made that early. Unfortunately, normative data for infant and child ovary imaging (transvesical) have not been established, which creates challenges in the assessment of subjects who might be candidates for ovarian cryopreservation.

Assessing ovarian volume and particularly, differences in size between the two ovaries is useful when screening for ovarian cancer amongst other echographic criteria.[327] Based on their echographic analyses of postmenopausal ovaries, Campbell and colleagues concluded that the percentage mean difference between the two ovaries was 42.88% ± 32.05%.[328] These authors concluded that any finding showing an ovary with a volume that is greater than twice the size of the other ovary should be considered as suspicious of ovarian cancer.

Antral Follicle Count: A View of the Ovarian Reserve

For each woman, the endowment of primary oocytes is thought to be finite and established during intrauterine life. It progressively decreases to reach approximately 2 million at birth and 400,000 at puberty. Drawing from extensive histological studies of ovaries from women of all ages, Faddy and colleagues described a progressive decay of primordial follicles over time that followed a bimodal pattern (Fig. 35.25).[283] They identified a slow depletion rate that prevails until the age of approximately 37 years old. Then,

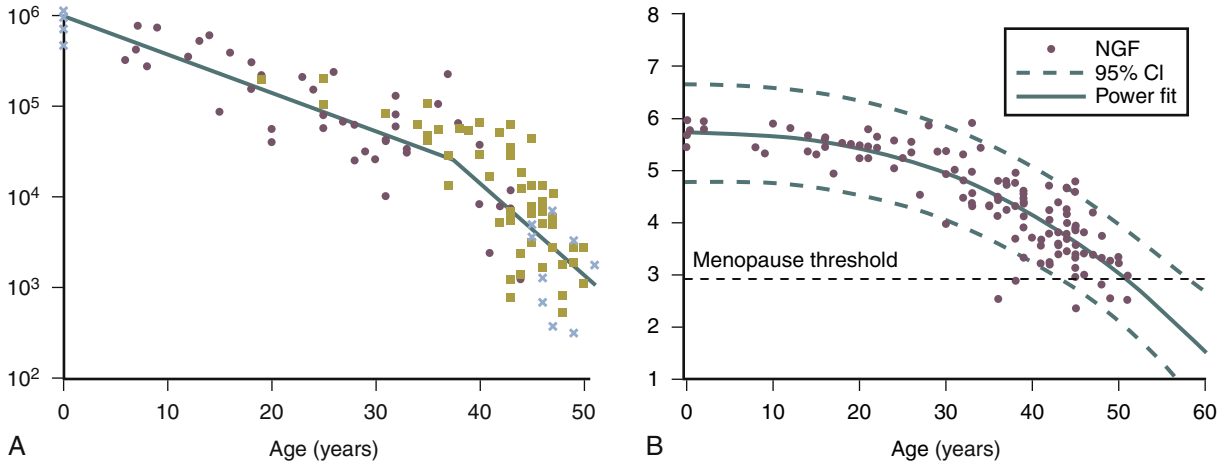


FIGURE 35.25 **A,** Depletion of primordial follicles over time. In their analysis, Faddy and colleagues observed an exponential decline of primordial follicles in a biphasic pattern. The first exponential rate parameter was calculated at –0.097 and the second at –0.237. The change from the first (slow decay) to the second (rapid decay) rate of follicular decline occurs when the number of remaining follicles falls under the critical figure of 25,000. On average this transition from the slow to the rapid follicular decline occurs at age 37.5 years, but occurs earlier in cases of of surgery for ovarian cyst. **B,** In contrast to the bimodal mode of follicular depletion, Hansen and colleagues propose a model with no sudden change in the rate of follicular decay, but rather a constantly increasing pattern. *(**A**, From Faddy MJ, Gosden RG, Gougeon A, et al. Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause.* Hum Reprod *7:1342, 1992.* **B**, *From Hansen KR, Knowlton NS, Thyer AC, et al. A new model of reproductive aging: the decline in ovarian nongrowing follicle number from birth to menopause.* Hum Reprod *23:699, 2008.)*

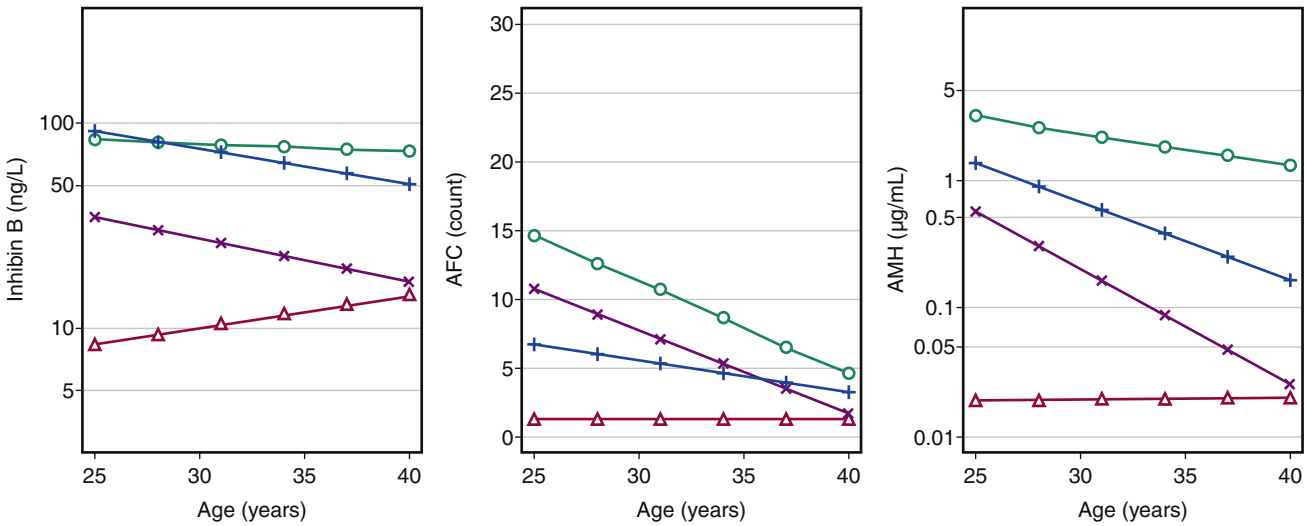


FIGURE 35.26 Depletion of antral follicles over time. Regression lines of inhibin B (log scale), antral follicular count (AFC), and anti-müllerian hormone (AMH) (log scale) values by age for subgroups of young hypergonadotropic women. °-°-°, Controls; +-+-+, incipient ovarian failure (IOF); transient ovarian failure (TOF); Δ-Δ-Δ, premature ovarian failure (POF). *(From Knauff EA, Eijkemans MJ, Lambalk CB, et al. Anti-müllerian hormone, inhibin B, and antral follicle count in young women with ovarian failure.* J Clin Endocrinol Metab *94[3]:786–792, 2009 Mar.)*

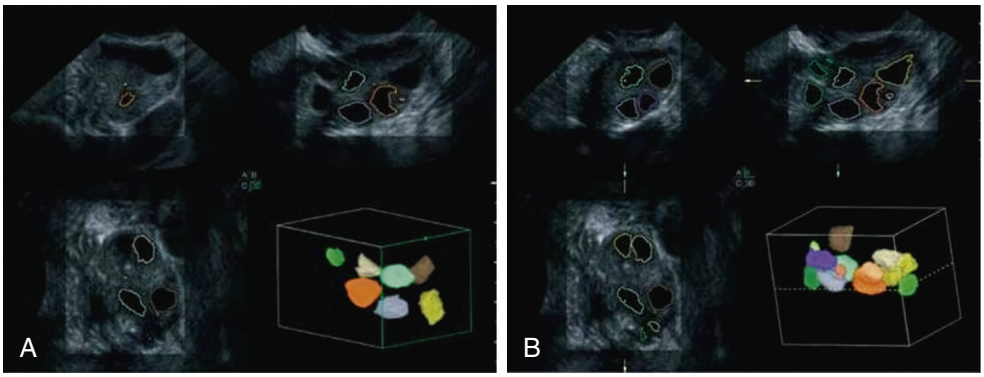


FIGURE 35.27 Method of AFC measurement. The AFC can be computed from 2D or 3D images. In the latter case, antral follicles can be counted using the multiplanar approach, scanning through the ovarian volume, or by automated analysis. The latter relies on an automatic antral follicle recognition system using the Sono-Automatic Volume Count technique based on a 3D-inversion mode rendering method. Using software that individually color codes each identified follicle and provides an objective measurement of its mean and absolute diameters and its volume. **A**, The initial fully automated assessment missed several antral follicles. **B**, The same ovary after these missed follicles have been manually identified through postprocessing. *(From Deb S, Jayaprakasan K, Campbell BK, et al. Intraobserver and interobserver reliability of automated antral follicle counts made using three-dimensional ultrasound and SonoAVC.* Ultrasound Obstet Gynecol *33[4]:477–483, 2009 Apr.)*

when the number of oocytes remaining becomes less than 25,000, an abrupt increase in oocyte depletion rate appears to take place, or fast depletion rate. The change from the slow to the fast depletion rate of primordial follicles takes place at approximately the age of 37 years. These authors suggested, however, that it is the number of remaining follicles—the critical number being 25,000—that triggers the acceleration in the depletion rate, not the age, per se. However, the claim that follicular depletion over time follows a bimodal pattern has been challenged by Hansen and colleagues.[284] These latter authors contend, on mathematical grounds, that follicular depletion over time follows a progressive acceleration pattern described by a power function, rather than the bimodal model (Fig. 35.26).

The methodology for AFC measurement has been the source debate. Both 2D or 3D approaches for AFC measurements showed good overall interobserver and intraobserver reliability.[329] Recently, a novel method of AFC assessment was developed based on automatic isolaton volumes of low echogenicity—the antral follicles—known as the 3D inversion mode rendering or SONO-AVC® method[330] (Fig. 35.27). AFC measurements made by classical 2D- or 3D-based approaches revealed that automated measurements took slightly more time and in general yielded lower values.[331] Jayaprakasan and colleagues determined that the variability AFC was significantly lower with inversion mode, while it was equivalent when measured with the 3D multiplanar and 2D equivalent methods. Yet, the authors determined that interobserver reliability was affected by image quality when using the 3D-rendered inversion method.[332] Moreover, mean time for measurement varied greatly with the methods used, ranging from 34 to 50 to 216 seconds for the three methods.[332]

AFC has been found to correlate with the number of oocytes retrieved in COH[333-335] and risk of developing

OHSS.[336] In a prospective trial, AFC was found superior to basal FSH for predicting ovarian response to COH.[337] Ng and colleagues[336a] compared AFC to serum FSH measured at baseline and post-clomiphene citrate challenge test (CCCT) for their predictive values regarding ART outcome. These authors found that AFC that remained unchanged after stimulation (CCCT) or after GnRH down-regulation, had the best predictive value for the quantitative outcome of COH expressed by the number of oocytes retrieved. This was followed by combined baseline and post-CCCT FSH concentration and age of the woman. Baseline and post-CCCT FSH concentration was a slightly better predictor of E_2 values.

In a prospective trial, Jayaprakasan and colleagues looked at the predictive value of AFC assessed from 3D data and compared their data to baseline FSH levels. One of three methods of measurement was used, including the new 3D-rendered inversion mode.[332] These authors' results indicated that total AFC was the best predictor of the number of oocytes retrieved in ART followed by baseline FSH. Moreover, AFC was significantly lower in the nonpregnant group. AFC less than 7 (less than 6 using 3D-rendered inversion mode) predicted poor ovarian response leading to cycle cancellation with sensitivity of 100% and specificity ranging from 93% to 96%, depending on the method used. Prediction of nonconception was low for all methods of AFC measurement. Data obtained from 3D-rendered inversion mode were the closest to the actual number of oocytes retrieved. More recently, SONO—AVC has been used for an automated assessment of the growing follicles in ART, which was demonstrated to be reproducible and reliable,[338] as well as acruate.[338]

Haadsma and colleagues proposed that amongst the cohort of 2 to 10 mm follicles retained for AFC computation, the subgroups of smaller and larger antral follicles had different predictive values.[339] In a cohort of 474 women, the number of very small follicles (2 to 6 mm) declined with age, while that of larger follicles (7 to 10 mm) remained fairly constant over time. Independent of age, the number of small follicles was related to the results of ovarian reserve tests. These authors claimed that the number of small antral follicle represents the functional ovarian reserve. Along these lines, Scheffer and colleagues observed a steeper yearly decline in the number of small antral follicles (2 to 5 mm) as compared to that seen for larger follicles (6 to 10 mm).[340]

Because a direct correlation has been established between the size of the antral follicle cohort and the number of remaining primordial follicles,[135] interest has been focused on determining the rate of depletion of antral follicles over time. In a prospective trial, Ng and colleagues determined that the rate of AFC decrease per year was of 0.35 follicle per year (95% CI 0.26, 0.45).[340a] These findings are of similar magnitude as the decline rate of 0.52 follicle per year (95% CI 0.26, 0.45) observed by the same group in a subsequent study conducted in a different cohort of women.[82] Yet, both results were obtained in Chinese women, and other publications reported faster AFC decays in Caucasian women where an annual loss of 0.95 follicles was observed,[341] or 8.2%.[342] There are no current explanations for the lower AFC losses observed in Chinese women as compared to their Caucasian counterparts.

Hendriks and colleagues conducted a meta-analysis looking at the respective values of ovarian volume versus AFC for predicting IVF outcome.[343] A total of 17 studies, 11 from prior meta-analysis[337] and 6 new ones were retained for looking at the predictive value of AFC. Comparison of ROC curves showed that the prediction of poor response was significantly better for AFC than for ovarian volume ($P < 0.005$) (Fig. 35.28). The prediction of nonpregnancy was poor for both tests, but the performance of AFC was slightly better than that of ovarian volume. This could have been predicted from the fact that both tests represent the quantitative aspect of ovarian reserve.

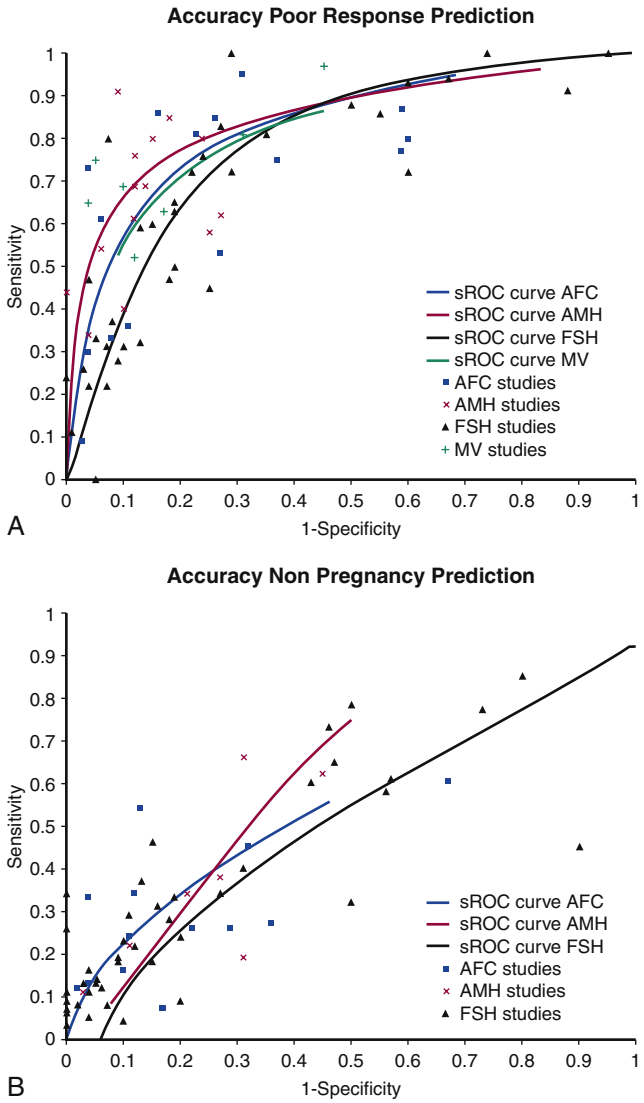


FIGURE 35.28 Prediction of ovarian response to COS and ART outcome. Ovarian reserve parameters (AFC calculation on ultrasound and AMH and FSH levels) predict the ovarian response to COS (data shown for poor response), but not ART outcome (data shown for non-pregnancy prediction). ROC curves of studies reporting on the performance of the AFC, AMH, basal FSH, and multivariate (MV) models to predict poor ovarian response during ovarian stimulation in IVF **(A)** and the occurrence of pregnancy **(B).** sROC, summary receiver operator characteristics; AFC, curves for antral follicle count; AMH, anti-müllerian hormone; FSH, follicle stimulating hormone; MV, mean volume of the ovaries. *(From Broekmans FJ, Soules MR, Fauser BC. Ovarian aging: mechanisms and clinical consequences.* Endocr Rev *30[5]:465–493, 2009 Aug.)*

Sensitive assays for AMH, also referred to as müllerian inhibiting substance (MIS), have revealed that AMH is produced by growing pre-antral follicles.[344] The cohort of AMH-producing follicles (100 to 200) is markedly larger than that of antral follicles (10-20). Yet, both reflect the pool of primordial follicles present at any given time.

From Gougeon's work,[135,283] and that of others,[332] the cohort of antral follicles is believed to remain constant throughout the menstrual cycle, thus providing constant AFC throughout the menstrual cycle and limited intercycle variability as compared to day-3 FSH levels. Likewise, AMH levels show a high degree of correlation with AFC,[345] and remain essentially constant throughout the menstrual cycle[346-348] with limited intercycle variability.[73,349,350] AMH levels are not affected by prolonged treatment with oral contraceptives[348] in spite of the profound decrease observed in ovarian volume,[316] and remain constant in pregnancy and the postpartum period,[351] and following FSH administration in regularly ovulating women[352] and women suffering from PCOS.[353] Possible post-ovulatory fluctuations in AMH levels have been suggested.[354] We concluded that this discrepancy did not result from methodological differences, and that if a slight postovulation decrease truly occurs, it is of lesser magnitude than intercycle variability of AMH measurements and can, therefore, be ignored.[322]

Recent studies have questioned whether AMH measurements add any value over simple AFC determination.[343] AMH does offer practical advantages for identifying PCOS amongst women whose AFC are so elevated that follicles are too numerous to count, or when AFC cannot be obtained.

In conclusion, AFC is a readily available imaging-dependent index of ovarian reserve, which can predict ovarian responses to COS in ART. In the normal ovarian aging process, the decrease in the number of oocytes is paralleled by a decrease in oocyte quality. In this context, a correlation exists between lower AFC and lower oocyte quality. Yet, this link is not an inherent one, but rather reflects the confounding effects of age.

Ovarian Perfusion

Perfusion of the Maturing Follicle and Corpus Luteum (CL)

Power Doppler has revealed the development of vessels in the wall of the dominant follicles in the later stages of the follicular phase[355] and in COH,[356-358] and a possible link with oocyte quality and reproductive outcome. Characteristically, this vascular development gives a colored annular ring that circles the maturing follicle. Typically, this is observed around certain but not all follicles developing in COH, starting on average 1 to 2 days prior to the time when hCG is commonly administered in COH (Fig. 35.29).[359]

Claims have been made that oocytes originating from well-vascularized follicles yield better reproductive outcomes than their counterparts originating from less vascularized follicles.[86,360,361] In line with this notion, a decrease in follicular blood flow in aging ovaries has been reported.[362] However, these results were challenged by Ragni and colleagues.[363] In a prospective trial on 318 women, these authors found that vascularity of the developing follicle did not predict the chance of pregnancy in women undergoing mild COS and IUI cycles. Pregnancy rates in the low-, medium-, and high-grade vascularity groups were 14.1, 10.0, and 11.8%, respectively.

Lozano and colleagues examined how follicular vascularization correlates with oocyte quality.[364] In their study, 61 normally ovulating women were prospectively studied. Their treatment consisted of daily GnRH antagonist (Cetrotid 0.5 mg/day) and 150 IU of hMG, starting when a growing follicle greater than 12 mm was observed. Ovulation was triggered as soon as the single dominant follicle reached 16 mm in diameter. Follicular vascularization was assessed just prior to oocyte retrieval, 34 to 36 hours after hCG (5000 IU) administration. Using 3D-based methodology, the vascularization index (VI) and flow index (FI) were taken as quantitative and qualitative reflections, respectively, of follicular vascularization. VI bore no predictive value to IVF outcome, but FI strongly predicted positive clinical pregnancies. These authors concluded that a qualitative rather than quantitative relationship exists between follicular vascularization and oocyte quality.

After ovulation the developing CL is the site of an intense neovascularization process, rapidly becomes the most vascularized organ of the body on a blood flow per tissue volume basis. On color and power Doppler images, the CL acquires the characteristic "ring of fire" appearance (Fig. 35.30). In a longitudinal study throughout the luteal phase and early pregnancy, Tamura and colleagues showed that the resistance index (luteal RI) was associated with corpus luteum function.[365]

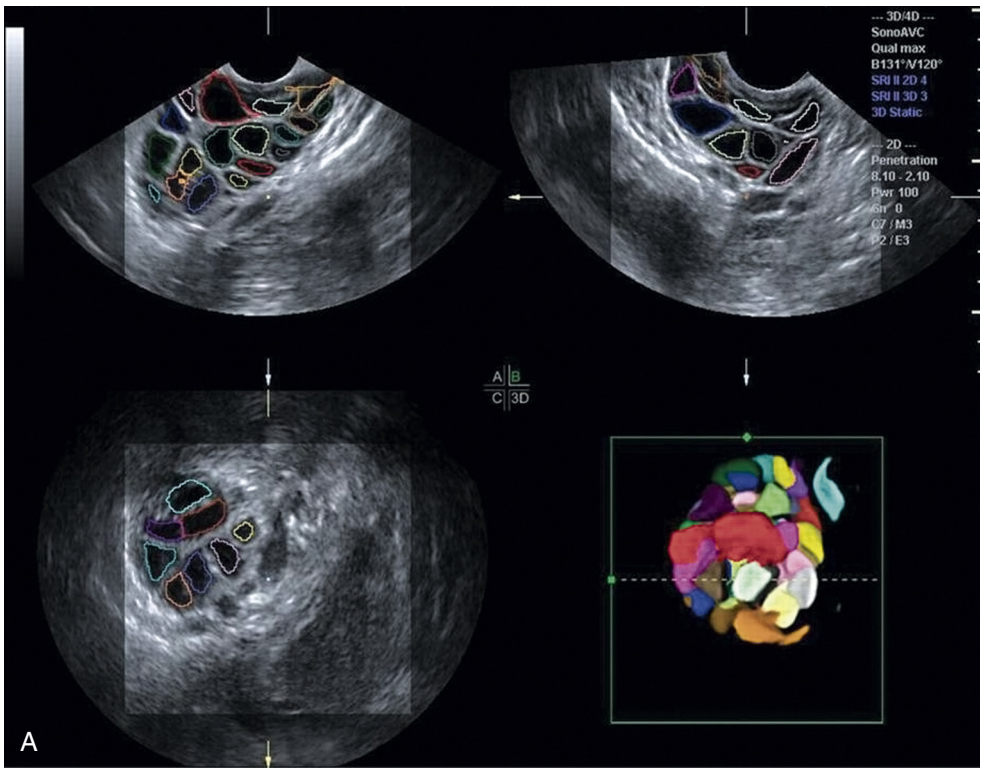
Claims have been made that correlations exist between CL Doppler data and quality of the luteal phase,[366] leading to the proposal that Doppler data could be used for assessing the luteal phase[367] and predicting pregnancy outcome.[368] In spontaneously occurring singleton pregnancies, Frates and colleagues failed to find a correlation between the echographic characteristics of the CL and first trimester pregnancy outcome.[368]

On gray-scale images, we believe that an educated eye can easily identify the fine dual contours of vessels that run around the CL, an impression which is supported by the finding of a correlation between Doppler data and vascular density.[369] It should be noted that the increased vascularity of the CL may at times be confused with the vascular ring that characterizes certain ectopic pregnancies.[370]

Perfusion of the Ovarian Stroma

Color Doppler has revealed the presence of ovarian stromal vascularization. From the outset, it was suspected that a difference in the degree of vascularization exists between normally ovulating and PCOS women. According to Battaglia and colleagues, ovarian stromal vascularization is identified in 50% and 88% of the cases in normally cycling and PCOS women, respectively.[234]

A link has been claimed between the degree of stromal vascularization and the response to gonadotropin stimulation in ART.[371,372] According to Popovic-Todorovic and colleagues, ovarian power Doppler together with AFC are the two most significant predictors of ovarian response amongst 12 possible predictive factors investigated in normally cycling women.[373] The observation of significantly lower 3D power Doppler indices of ovarian stromal blood flow



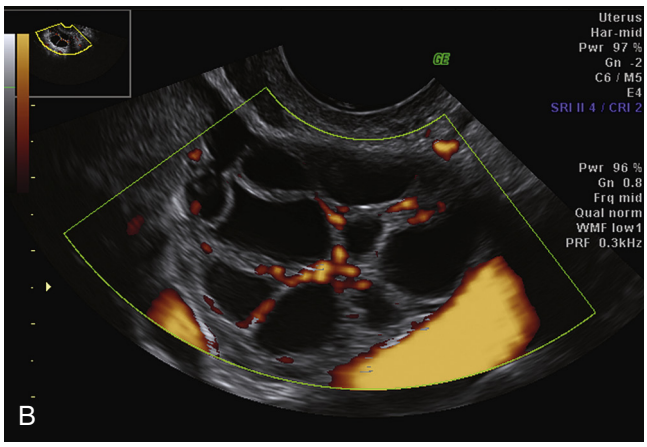


FIG. 35.29 **A**, Monitoring follicular growth in ART using the sonographic automated volume calculation worksheet. Each follicle and its measurements presented in the box at the lower right corner are coded with the same color for ease of interpretation. **B**, Vascularization of maturing ovarian follicles in COS induced for ART. In COS, starting 1 to 2 days prior to follicular maturity and hCG administration, color Doppler mode identifies the development of follicular vascularization. Characteristically, some but not all follicles display the development of end-follicular phase vascularization. *(**A** From Ata B, Seyhan A, Reinblatt SL, et al. Comparison of automated and manual follicle monitoring in an unrestricted population of 100 women undergoing controlled ovarian stimulation for IVF.* Hum Reprod *26[1]:127–133, 2011 Jan.)*

in poor responders as compared to normal responders[128] was taken as supporting evidence for the above. Studying the effect of age on ovarian stromal blood flow, Ng and colleagues observed that an age-related decrease was only observed in women 41 years of age or older.[340a] Using a 3D-based approach, Merce and colleagues found a correlation between ovarian VI and FI and the number of oocytes recovered.[74] Vascular parameters, however, were not independent predictors of the number of follicles developed and oocytes retrieved; ovarian volume and AFC were, a finding consonant with other reports.[5] Ng and colleagues observed that AFC, ovarian volume, and ovarian 3D Power Doppler flow indices did not significantly change after a short-term treatment with a GnRH agonist for pituitary down regulation.[374] Järvelä and colleagues studied the power Doppler signal in the ovaries after pituitary suppression by GnRH agonist. They observed that Doppler data did not provide any additional information beyond that of ovarian volume and AFC in terms of predicting the subsequent response to gonadotropin stimulation during IVF.[310]

The Ovary in Polycystic Ovary Syndrome

Echographic Characteristics of PCOS Ovary

In the original description by Stein and Leventhal, PCOS implied verification of the characteristic ovarian morphology,[375] a parameter which was later dropped.[376,377] In 2003, the Rotterdam conference[82] reinstituted an ovarian parameter in the diagnosis of PCOS when it included specific ovarian echographic criteria in the diagnostic criteria for PCOS. The Rotterdam consensus conference concluded that ovaries needed to hold more than 12 follicles of 2 to 9 mm each or measure more than 10 mL in volume to fulfill the PCOS criteria.

In PCOS, a characteristic feature is an increase in stromal volume[378,379] which in turn translates into an overall

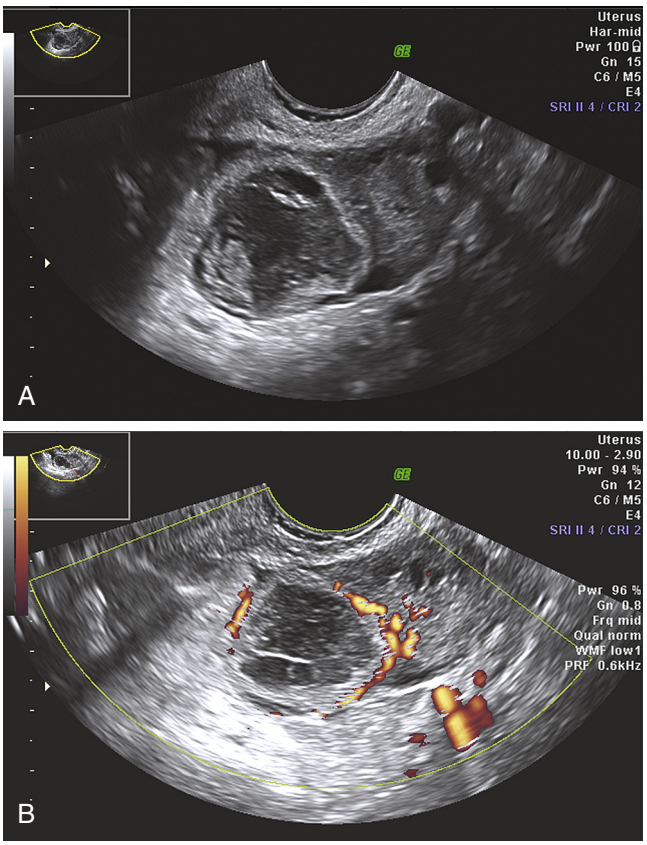


FIGURE 35.30 Corpus luteum appearance in gray-scale imaging **(A)** and color Doppler mode **(B).** On color Doppler mode, the characteristic intense vascularization of the corpus luteum confers the typical "ring-of-fire" appearance.

increase in ovarian volume.[378] Ovarian suppression achieved with the OC pill results in reduced ovarian volume,[315]—a finding not shared by everyone[380]—but without changes in either AFC and AMH levels.[353]

Ovarian volume measurement raises methodological issues. Originally, 2D measurements of ovarian volume used the simplified formula for the prolate ellipsoid (0.5 × width × length × thickness), which assumes a regular ovoid shape of the ovary.[381,382] Direct 3D measurements using the VOCAL-based rotation approach[12,383] surpass the classical 3-diameter-and-ellipse-formula approaches.[371]

The novelty of the Rotterdam criteria is the introduction of echographic criteria in the definition of PCOS.[384,385] However, the Rotterdam criteria have implicitly defined a category of women who do not qualify for PCOS diagnosis but have *PCO-like* ovaries, yet without androgen excess and the ovulatory dysfunction.[386] If exposed to COH for ART, these women are at risk of ovarian hyperstimulation syndrome (OHSS), just like true PCOS women.[174]

Franks argued that the Rotterdam conference did not settle the controversy concerning the diagnosis of PCOS.[387] He asserted that hyperandrogenism and anovulation are the mainstays of the definition of PCOS, while the Rotterdam criteria allowed for identification of PCOS women without clinical or biochemical androgen excess. Azziz stressed the fact that little is known about the degree of insulin resistance and long-term metabolical risk of oligo-anovulatory women with PCO ovaries, but without hyperandrogenism.[388] Allemand and colleagues[389] questioned the number of small follicles that had been retained in the Rotterdam citeria for diagnosing PCOS, opting for a higher number of follicles or ovarian volume as cut off criteria. Using ROC curves, these authors proposed to set the diagnostic cut off at more than 20 follicles per-ovary, offering a predictive value of 100% and negative predictive value of 91%. Dewailly and colleagues contend that the small 2 to 5 mm follicles found in PCOS are actually atretic follicles that reflect follicular arrest,[390] probably the result of an exaggerated inhibition of the terminal follicle growth.[391]

In PCOS, as in normally ovulating women, there is a strong correlation between AFC and AMH levels. Chen and colleagues observed that a significant correlation existed between the number of antral follicles and the ovarian volume on one hand and AMH on the other. AMH levels also correlated with total testosterone and the free androgen index.[392] AMH levels were inversely correlated with body mass index (BMI), fueling the argument that the ovarian expression of PCOS is more profound in lean individuals.[174] All findings indicate, however, that AMH levels parallel AFC and may do a better job defining PCOS and the degree of expression of this trait. AMH levels help when ultrasonographic data are not available.[393]

The Ovarian Stroma in PCOS

The description of the echographic characteristics of the PCOS ovary goes beyond the cardinal features of an increase in size and number of small 2 to 10 mm follicles.[394] An accessory echographic feature often put forth, yet more rarely objectively studied, is the increase in ovarian stromal echogenicity.[395,396] Buckett and colleagues[379] looked at the echographic appearance of 67 unselected women on no medication who were scheduled to undergo IVF. These authors measured total and stromal ovarian echogenicity, computing a stromal index as the ratio of stromal echogenicity over total echogenicity. Criteria for PCOS, AFC greater than 10, were found in 37% of the cases. Total ovarian volume and stromal volume were higher in PCOS ovaries, but contrary to expectation, there were no differences in mean stromal echogenicity between PCOS women and their normal cycling counterparts. There was, however, a reduction in mean echogenicity resulting from the increase in ovarian follicles, which accounted for a significant increase in stromal index in spite of the lack of change in stromal echogenicity per se. These results were confirmed with the most recent VOCAL-based volume measurements.[397] Lam and colleagues compared 40 Caucasian women identified as having PCOS by the Rotterdam criteria and 40 cycling controls having normal ovaries on ultrasound.[397a] Ovarian and stromal volumes were markedly larger in the PCO group (12.56 and 10.79 mL) as compared to controls (5.66 and 4.69 mL), but there was no significant difference in stromal echogenicity between PCOS and controls, respectively. Likewise, Jarvela and colleagues[398] failed to find a difference in echogenicity between 14 PCOS and 28 women with echographically normal ovaries. In subgroup analyses, Lam and colleagues observed that hirsute PCOS patients had larger stromal volume than the nonhirsute counterparts,[397a] but found no differences in ovarian stroma echogenicity between these subgroups. It can be concluded, therefore, that the impression of denser ovarian stroma in

PCOS results from a visual illusion linked to the increased stromal volume and a decreased echogenicity of the periphery of PCOS ovaries due to the increase in follicles.

Interestingly, AFC remains constant in PCOS women taking OC pills in spite of the profound decrease in ovarian volume.[316] This indicates that the effect of hormonal contraception is on ovaran stroma not follicles.[399]

The availability of pulsed and color Doppler functions on vaginal ultrasound probes led to the study of intra-ovarian stromal flow as a distinct phenomenon from changes in flow taking place in the follicular walls,[400] and after ovulation in the CL.[355,401,402] Stromal flow was found to be increased in women suffering from PCOS[371] as compared to findings in cycling controls.[130,136] Zaidi and colleagues compared ovarian stromal flow status measured by Doppler (peak systolic blood flow velocity) to ovarian responsiveness to FSH and hMG in ART cycles. Their observation found a positive correlation between blood flow at baseline measured by Doppler and ovarian response to COH with higher values in 13 POCS patients as compared to 63 cycling controls.[403] Conversely, ovarian blood flow was decreased at baseline in women found to have a poor response as compared to normal responders.[371]

In 2D-based studies, Balen and colleagues concluded that there is an increase in ovarian blood flow in PCOS,[386] a finding shared by some[288] but not by other investigators. Using 3D PDA, Jarvela and colleagues failed to find an increase in stromal vascularity in 14 women with PCOS as compared to 28 control women.[398] Lam and colleagues[88] compared ovarian flow assessed by 3D ultrasound in 40 women fulfilling the Rotterdam criteria for PCOS with those of 40 age-matched cycling controls. Ultrasound data were obtained on day 3 to 5 after spontaneous or induced bleeding. A 3D-based data set was acquired using a sweep angle set to 90 degrees and a slow-sweep mode. Per ovary AFC was significantly higher at 16.3; 9 to 35 (median; range) in the polycystic ovary group as compared to controls (5.5; 2 to 10). The results showed an increase in some (VI) but not in all 3D-PDA indices (FI). Interestingly, differences were observed among various phenotypes of PCOS. Ovarian blood flow was increased in a subgroup of 30 of 40 hirsute PCOS women as compared to their 10 nonhirsute PCOS counterparts. Likewise, blood flow was also higher in 14 lean PCOS women as compared to their overweight counterparts. Yet, there were no differences in AFC between lean and obese PCOS and hirsute and nonhirsute women. Ng and colleagues[403a] confirmed the differences in ovarian blood flow between different PCOS phenotypes. This team also found a significant increase in ovarian blood flow in women whose BMI was less than 25 as compared to their overweight counterparts with a significant negative correlation between total ovarian indices (VI, FI, and VFI) and BMI.[174] There was also a strong trend toward higher LH levels in lean PCOS women. Hirsute women with PCOS had increased stromal volume compared to nonhirsute women with PCOS.[404]

Early Diagnosis of PCOS in Adolescents and Prepubertal Girls

Chang and Coffler stressed the importance of diagnosing PCOS early in adolescent girls in order to offer treatments for the hormonal (hyperandrogenism and hirsutism) and metabolical expression of the disease.[298] Based on studying AMH levels through the pubertal process, Crisosto and colleagues observed that the increase in follicular mass is established during early development and persists during puberty.[405] First amongst individuals in whom an early diagnosis of PCOS is important are daughters of PCOS women. Sir-Petermann and colleagues determined that the daughters of women with PCOS are at increased risk of suffering from PCOS themselves.[406] These authors compared 14 female infants (2 to 3 months old) and 25 prepubertal girls (4 to 7 years old) born to PCOS mothers to a control group of 21 female infants and 24 prepubertal girls born to mothers with regular menses and without hyperandrogenism. Serum AMH levels were significantly higher in PCOS as compared to the control group during early infancy (20.4 ± 15.6 versus 9.16 ± 8.6 pmol/L; $P = 0.024$) and during childhood (14.8 ± 7.7 versus 9.61 ± 4.4 pmol/L; $P = 0.007$). Yet, gonadotropin and serum sex steroid concentrations were similar in both groups during the two study periods, except for FSH, which was lower during childhood in girls born to PCOS mothers. The discrepancy between the alteration of the follicular cohort, as expressed by the increase in AMH levels, and the lack of hormonal alteration in these infants and prepubertal girls suggests that the ovarian facet of PCOS precedes the altered gonadotropin pattern. That there is evidence of an altered follicular development during infancy and childhood speaks for the need of screening ovarian patterns either directly by ultrasound or through the surrogate for the follicular cohort, AMH levels.

Adnexal Cysts and Tumors

Ovarian versus Extra Ovarian Pathologies

Vaginal ultrasound has become the primary approach used for investigating adnexal masses. As stressed in a review by Brown[290] the primary question that pelvic imaging needs to answer is whether the adnexial mass is of ovarian or extraovarian origin. Most adnexal masses arise from the ovary. When the mass is of ovarian origin, malignancy is a concern. Once established that the mass is of extraovarian origin, short of rare cases of tubal cancer,[407] the mass is not likely of cancerous nature. The primary clinical presentation of tubal cancer is most commonly one of pelvic pain rather than an asymptomatic pelvic mass.[408] If the ovary and the tube are both involved with tumor, the bulk of the tumor should be in the tube.[409]

Identifying small antral follicles can help determine whether a mass is ovarian or not. Likewise, recognizing an ipsilateral ovary independent from the adnexal mass allows for recognition that the mass is nonovarian in nature. In postmenopausal women, the absence of visible ovarian follicles complicates ascertainment. Helpful tips for determining the ovarian or nonovarian nature of the mass include the possibility of mobilizing the structure that is being investigated by pressure applied with the vaginal probe. In case of a cystic mass, the impact of the pressure applied with the probe on the cystic dilatation should be followed. In case of an ovarian cyst, the probe usually bounces off the nondeformable cyst wall. When the cystic structure is a hydrosalpynx, the pressure of the probe will modify the shape of the cystic dilatation as fluid displaced by the probe will move elsewhere along the length of the tube.

The vascular supply of the lesion may also help in delineating the ovarian or extra-ovarian origin. In the case of pedunculated fibroids, one might identify vessels bridging with the uterus. The peripheral development of vessels can also help in recognizing the fibroid nature of an adnexal mass.

Extra-Ovarian Pathology

Paraovarian (or paratubal) cysts appear as simple cysts indistinguishable from ovarian cysts, if it were not for their nonovarian nature, which can normally be determined in premenopausal women because ovarian tissue can easily be identified,[410,411] including identification of an ipsilateral ovary.[412]

Hydrosalpinges are the most important para-ovarian structures encountered in clinical reproductive endocrinology. Typically, they appear as an elongated cystic mass.[413] Other typical features of hydrosalpinges include the waist sign and images evoking beads on a string. In a review of 67 cystic adnexal masses, Patel and colleagues observed hydrosalpinges in 26 (39%). These authors concluded that an elongated aspect, together with the waist sign, carried the highest probability for hydrosalpinx.[414] Ultrasound scans aimed at excluding the presence of hydrosalpinges in ART need to be performed at midcycle because fluid tends to accumulate during the follicular phase or after ovarian stimulation,[415] including in women with PCOS.[416] Peritoneal inclusion or pseudocysts are believed to result from fluid trapped within pelvic adhesions which may create the impression that the ovary lies within the cyst itself.[290,417]

Ovarian Cysts and Tumors

Brown described five primary findings for ovarian masses, each having their echographic characteristics, which most often permit a positive diagnosis.[290]

Simple Cysts

In premenopausal ovaries, a simple cyst is a structure greater than 3 cm in diameter with a thin anechoic wall free of protrusion and showing characteristic distal enhancement. Structures of lesser diameter are best described as follicles, which may have stopped developing or failed to regress because of isolated or recurrent ovarian dysfunction. Simple cysts most often disappear spontaneously, a process that may be hastened by suppressing ovarian function with the OC pills. The majority of persisting cysts treated surgically turned out to be serous cystadenomas.

The Corpus Luteum

The imaging characteristics of the CL may be so dramatic that they can easily be mistaken for cancer because of the intense low impedance blood flow. Baerwald and colleagues followed luteal images and serum E_2 and progesterone in 50 women daily from one ovulatory episode to the next.[311] The day of ovulation was defined as the day of follicle disappearance. The CL was detected on the day of ovulation and later in 100% of women, and during the subsequent follicular phase in 90% of them. Some CL presented as a central fluid filled cavity (identified in 88%), and some without (12%). A progressive decrease in echogenicity was observed throughout the luteal phase. The CL is intensely vascularized with an annular arrangement of vessels around the CL itself, which can be recognized on gray-scale images by a circular dual contour line.[132]

Hemorrhagic Cysts

Hemorrhagic cysts are common occurrences in premenopausal women. They can be associated with acute pain or they can constitute a fortuitous finding in routine scans done prior to ART. The internal echo pattern varies depending on the stage of the hemorrhage and the amount of fluid present. The acutely hemorrhagic cyst is typically more echogenic than the surrounding ovarian tissue. Characteristically, findings will evolve first to a sponge-like appearance, later followed by the development of irregular internal echos after the clot starts to retract after a few days, leaving a mesh-like maze of intertwined fine structures that are the echographic expression of fibrin strands.[418,419] These rarely run totally across the cyst. A gentle brisk pressure stroke applied with the vaginal probe may generate a disruption wave that propagates through the mesh-like network of internal echos with a characteristic jello-like effect where the wave rebounds backwards after hitting the cyst wall.

The retracted blood clot may mimic a mural nodule. The sharp angles of the retracting clot are different from solid tumors that do not tend to have acute angles.[419] To distinguish the clot from more ominous internal echos, it is most helpful to document the absence of vessels entering the solid area. MRI is classically of little help for diagnosing hemorrhagic cysts.[420] Most commonly, hemorrhagic cysts rapidly change their echographic appearance and ultimately disappear, a process that may be hastened by suppressing ovarian function with OC pills.[421,422] The pill is commonly started on day 2 of the menstrual cycle or arbitrarily, provided that endogenous progesterone is low (less than 1.5 ng/mL), which precludes the possibility of pregnancy. The differential diagnoses include endometrioma and dermoid cysts. Typically, the former has a homogeneous mixed echogenicity (gray) appearance, whereas the latter may add to that grayish appearance the finding of brightly echogenic calcifications, and on occasion, a denser mesh image when the dermoid cyst contains an accumulation of hair. Occassionally, a hemorrhagic CL may have a clinical course suggestive of ectopic pregnancy.

Endometriomas

Unlike other locations of endometrisosis which are difficult to identify, ultrasound examinations are efficient for diagnosing ovarian endometriosis, the endometriotic cyst or endometrioma. Out of 1170 scans yielding 252 adnexal masses the diagnosis of endometrioma was made in 40 cases (prevalence 16%).[423] Taken in isolation, low level internal echoes have a sensitivity of 93% and a specificity of 83%. At the other end of the spectrum, identifying all characteristic findings of endometriomas, namely low-level internal echoes, no neoplastic features, and hyperechoic wall foci or multilocularity, led to a specificity of 99%, whereas, sensitivity dropped to 45%. These data demonstrate that gray-scale vaginal ultrasounds can achieve a high degree of accuracy for diagnosing endometriomas. Hence, an adnexal mass with diffuse low-level internal echoes and no neoplastic features is most likely to be an endometrioma.

Mature Cystic Terratomas or Dermoid Cysts

Typical features of dermoid cysts include shadowing echodensity, regional diffuse bright hyperechoic areas with an attenuating effect, hyperechoic lines and dots and fat-fluid levels.[424] Because of the great variability in the appearance of cystic terratomas has been emphasized, it has been suggested that MRI is needed to rule out the possibility of ovarian cancer, except when the most typical echographic are observed. Suspecting that echographic findings are sufficiently specific, Patel and colleagues demonstrated in a prospective study that 55 of 74 cystic terratomas diagnosed amongst 252 adnexal masses had two or more sonographic features associated with dermoids.[425] These authors observed a positive predictive value of 80% for shadowing echogenicity, 75% for regionally bright echoes, 50% for hyperechoic lines and dots, and 20% for fat-fluid level. Taking these signs together, each reviewer had 98% positive predictive value, which reached 100% when an adnexal mass had two or more sonographic findings associated with dermoids.

Most ovarian cancers are epithelial neoplasms, which include serous, mucinous, endometrioid, and clear cell neoplasms. Additionally there are borderline-malignancy tumors or tumors of low malignant potential of all epithelial cell types with the mucinous and serous cell types being by far the most common. The presence of a solid component is the most common feature that reveals the cancerous nature of an ovarian tumor. In spite of the initial interest in color and power Doppler, there is a consensus that Doppler indices do not provide much more information than grey scale morphological assessment of suspected ovarian tumors.

Contrast Imaging of the Fallopian Tubes

The fallopian tubes are usually not identified on pelvic ultrasound, nor with other imaging approaches such as MRI or CT scans. Dilated tubes in case of hydrosalpinges are easily seen, however, by the contrast effect generated by fluid contained in the tubes. Exploring the fallopian tubes, therefore, requires some sort of contrast. Classically, the fallopian tubes are visualized on x-ray images by transcervical infusion of an opaque dye in HSG. Positive *(white)* contrast media are routinely used.[426]

Hysterosalpingography

Indications

Hysterosalpingography (HSG) is the ancestral imaging tool used for investigating infertility.[427,428]

The primacy of HSG for exploration of the uterine cavity is challenged by transvaginal ultrasound and HySo on one hand, and hysteroscopy on the other. The former offers the advantage of depicting both the cavity and the myometrial contour. This made HySo the undisputed primary imaging tool for investigating uterine malformations. The miniaturization of endoscopic instruments has allowed diagnostic hysteroscopy to become an office procedure, thereby leading to marked increases in its use. Diagnostic hysteroscopy offers the advantage of direct visualization of the pathological structures. Sonography using positive contrast products allows delineation of the tubal lumen, which appears white on grey scale images. Positive contrast agents used include Echovist®, Levovist®, and Albumex®, none of which is approved for use as fallopian tube contrast medium by the Food and Drug Administration (FDA).[429]

The need for HSG, traditionally included in all infertility workups, has been questioned for women whose serology for Chlamydia is negative. In these women, it has been claimed that HSG has a yield of tubal pathology that is so low that the routine use of HSG is not cost-effective.[430,431]

Technical Considerations

Classically, HSG is performed during a relatively short interval between cycle day 5 and days 12 to 14. This time limit achieves two objectives: (1) menses must be finished in order to minimize the risk of enhancing retrograde bleeding by the procedure; (2) The x-ray procedure is performed before ovulation in order to minimize the risk of irradiating a developing pregnancy. It may be handy to place the patient on a short course of OC pills for the sake of programming.

Several thorough reviews on HSG have been published which describe in detail practical aspects of this procedure.[432-434] Many groups routinely prescribe 600 mg of ibuprofen or other nonsteroidal anti-inflammatory drugs (NSAIDs) 30 minutes to 1 hour before the procedure in a effort to ease the uterine cramping that may be generated by the procedure.[433] Frishman and colleagues randomly compared the effects of intrauterine instillation of 2% lidocaine solution or saline before performing an HSG.[435] Moreover, Zhu and colleagues reported less pain after warming the contrast medium.[436] The issue of routine antibiotic prophylaxis has been much debated, with infectious complications being less than 1%.

With the patient in the dorsal lithotomy position and a speculum in place, the instillation device is positioned in order to provide a sealed connection with the cervix. The instillation instrument is either a Jarcho canula,[437] which is held to the cervix with tenaculums, or plastic catheters equipped with inflatable balloons, such as the 5-F HSG catheter (Cooper Surgical, Trumbull CT). The balloon is inflated once it has passed the internal os of the cervix. The balloon must be filled with the same contrast material as used for the examination. While the latter are probably easier to handle, they may create pain because of the balloon,[438] and in certain circumstances, may hinder the vision of the cervical canal. The speculum needs to be removed once the instillation instruments are in place. Air needs to be meticulously expelled from the instilling device so that only the contrast medium is pushed into the uterine cavity. Failing to do so could lead to a false diagnoses of filling defects.

Prior to instilling contrast medium, and after a mark identifies sides, a scout x-ray of the pelvic cavity is examined, looking for possible calcifications. Water-soluble contrast material is slowly instilled under intermittent control on fluoroscopic vision. A minimum of four spot radiographs are recommended.[434] The first image is obtained during early filling of the uterine cavity in search for filling defects, which may later be obscured when the uterus is totally opacified. The second image is obtained when the uterus appears fully distended. The third image aims at depicting the fallopian tubes. A fourth image is obtained to detect free spillage of dye into the pelvic cavity and proper mixing. Additional spot radiographs are obtained as necessary, including oblique views to avoid the superimposition of images.

Choice of Contrast Medium: Oil-Based or Water Soluble?

The original contrast media used in HSG was lipid based, with Lipiodol being used since it became available in the early 1920s.[439] In a pre-IVF era study, Mackey and colleagues compared the occurrence of spontaneous pregnancies when women had an HSG or not, sorting out results for when the oil-based Ethiodol or a water-soluble contrast medium was used.[440] In their study population of 460 women, the spontaneous pregnancy rate over 1 year following oil-based dye HSG was 58%, whereas it was 38% in women in whom a water-soluble dye was used. These puzzling results, which speak in favor of a fertility enhancing effect of HSG using lipid-based dye as compared to findings observed following HSG using water-soluble dyes, were confirmed in a number of other studies.[441-443] Oil-based contrast medium come with the risk of granulomas,[441] or the serious complications of a pulmonary or cerebral oil embolism.[444] A sequential combination of water-soluble dye for documenting tubal permeability followed later by instillation of oil-based Ethiodol has been proposed to benefit from the fertility enhancing effects of HSG using oil-based dyes while avoiding its risk. Alternatively, the use of the oil-based dye has been proposed in women whose tubal patency had been demonstrated by laparoscopy.[445] Water-soluble dyes offer better visualization of the distal regions of the fallopian tubes, allowing identification of striae.[443,434]

Attempting to provide an explanation for the pregnancy promoting properties of oil-based dye HSG, Goodman and colleagues proposed that the lipid dye inhibited the activity of peritoneal lymphocytes and macrophages.[446] That HSG was found to enhance fecundity in women whose infertility was of unknown etiology,[441] or due to endometriosis,[374] suggests that the inhibiting effect of the lipid dye on macrophages might be most significant in this subset of patients.[447]

The Uterine Cavity

Filling defects constitute a common finding in HSG. Once artifacts such as air bubbles or endometrial folds have been excluded, the differential diagnosis is synechiae as a result of post-traumatic or infectious endometrial scarring. Full fledge development of endometrial scarring leads to Asherman syndrome, classically associated with multiple scarring of the endometrium and clinically, amenorrhea.

Myomas may indent the uterine cavity. For practical purposes, the degree of distortion must be assessed to determine whether surgery is indicated in the absence of symptoms, and whether an intracavitary approach is feasible. HySo allows for simultaneous visualization of the whole fibroid and the portion that extends into the uterine cavity.

Assessment of the uterine cavity includes a search for congenital malformation of the organ. 3D sonography—with or without HySo—has taken the lead for diagnosing uterine malformations by offering simultaneous vision of the uterine cavity dilated by saline and the organ itself identified on gray-scale imaging.[448] The 3D reconstruction of the uterus in the frontal plane is of great practical help,[449] and even lends itself to virtual hysteroscopy exploration[450] (Fig. 35.31). Finally, the assessment of the uterus must include the cervical canal, notably to rule out cervical insufficiency.

Clues regarding the possibility of adenomyosis can arise from HSG images.[451] The characteristic image is one of diverticula filled with contrast medium that extends into the myometrium. This may exist in the context of an overall irregular contour with multiple outpouchings. The ultimate expression is the classical honeycomb pattern.[452,453] Although it is held as being diagnostic of adenomyosis, it is now recognized as not being specific.[451,454]

In cases of large adenomyomas, the image may be one of a mass-like filling defect, with contrast material filling the mass. Adenomyosis is more accurately diagnosed by MRI where it is characterized by a thickening of the junctional zone.[455] This is the only feature that has been fully validated against histological identification of adenomayosis in women undergoing hysterectomy.

The Fallopian Tubes

In spite of emerging approaches based on positive contrast enhanced ultrasound imaging, HSG remains the best method for visualizing and evaluating the fallopian tubes. Normal tubes appear thin, with smooth outlines and a characteristic distension in the ampullary area. Proximal disease that prevents the visualization of the tubes may be the reflection of salpingitis isthmica nodosa (SIN) or the result of a simple spasm. In the case of SIN, the penetration of the contrast medium into the thickened fallopian tube wall produces the typical honeycomb appearance (Fig. 35.32). SIN is usually the result of pelvic inflammatory disease that leads to fibrosis of the proximal tubal section with stricture of the lumen and tubal occlusion.[456] We now know that SIN may also result from a tubal form of endometriosis.[456] In this case, temporary treatment with a GnRH agonist has been documented to be effective,[457] offering alternatives to the surgical approach for SIN.[458] Our experience showed that 3 months of treatment with GnRH antagonist resulted in repermeabilization of 15 of 18 women who had bilateral proximal occlusion, and in whom a laparoscopy showed some endometriosis and no evidence of distal tubal disease.[73] We believe that medical treatment of SIN should be considered each time endometriosis is present and there is no evidence of distal tubal disease. Spastic constriction of the tube may result in complete tubal occlusion, opening the door to an erroneous diagnosis of proximal tubal disease. Timely administration of glucagon promotes uterine muscle relaxation and can be used to reverse spasms.[459]

Distal disease is usually associated with dilatation of the tube as a consequence of hydrosalpinges or the incomplete form, sactosalpinges. Pelvic inflammatory disease is the most common cause of distal tubal disease, but it may at times result from endometriosis.[460,461]

We believe that the diagnosis of hydrosalpinx should be made on visualizing dilated tubes on ultrasound at midcycle. This nuance is important, as it is commonly held that hydrosalpinges must be removed (salpingectomy) to optimize IVF outcome.[44] From the clinical trials that exist, there is now a consensus for recognizing that pregnancy rates in IVF are approximately halved when a hydrosalpynx is visible on ultrasound.[336,462] Taylor's group found that hydrosalpinges, probably by the release of fluid into the uterine cavity, interferes with the role of *HOXA10* in receptivity and implantation.[44,463-465]

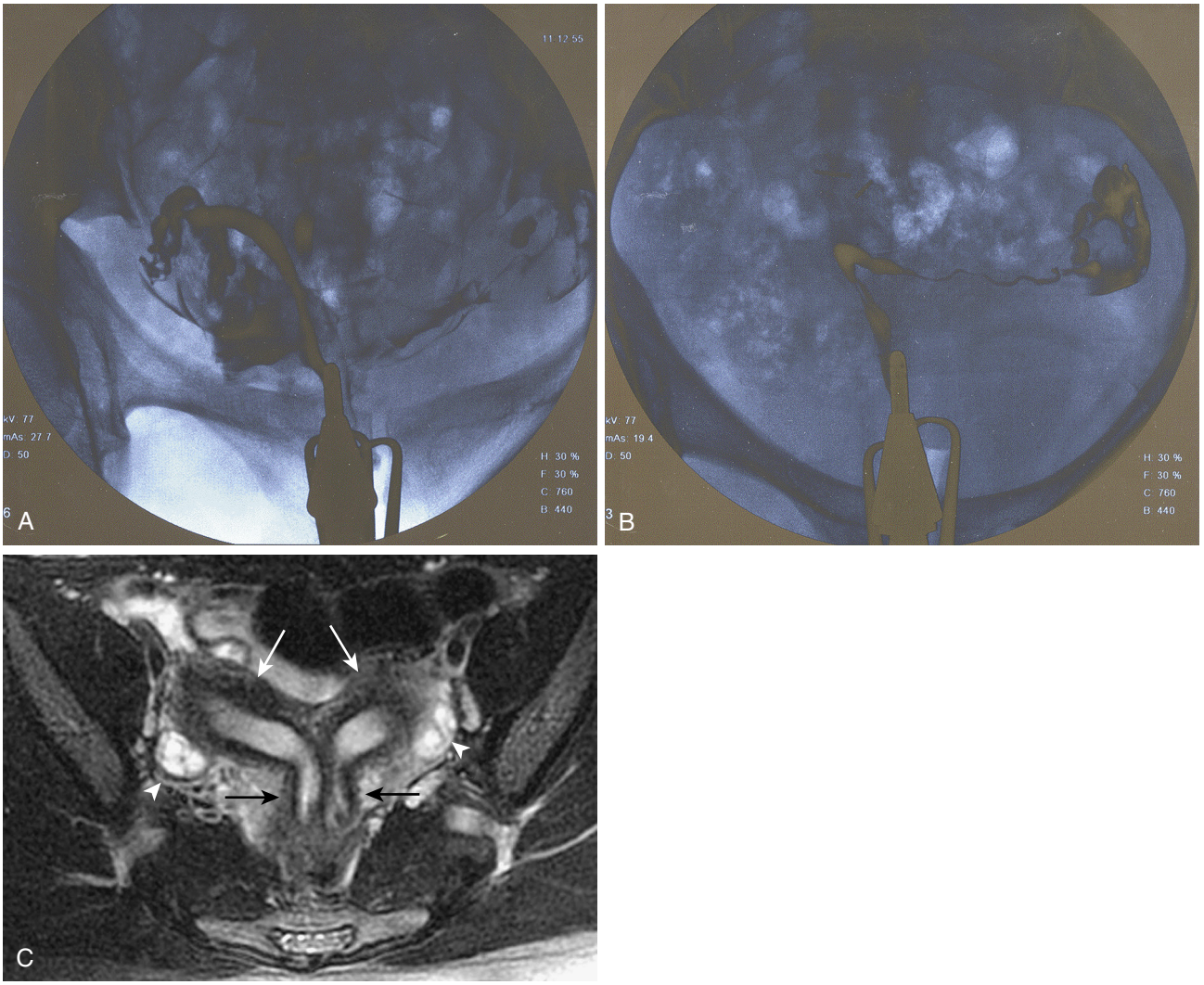


FIGURE 35.31 Hysterosalpingography (HSG) showing a completely unfused uterus with two cervices and two corpora uteri. Note *right* **(A)** and *left* **(B)** uterus and tube. Corresponding appearance on magnetic resonance imaging (MRI) **(C).** *(From Imaoka I, Wada A, Matsuo M, et al. MR imaging of disorders associated with female infertility: use in diagnosis, treatment, and management.* RadioGraphics *23:1401, 2003.)*

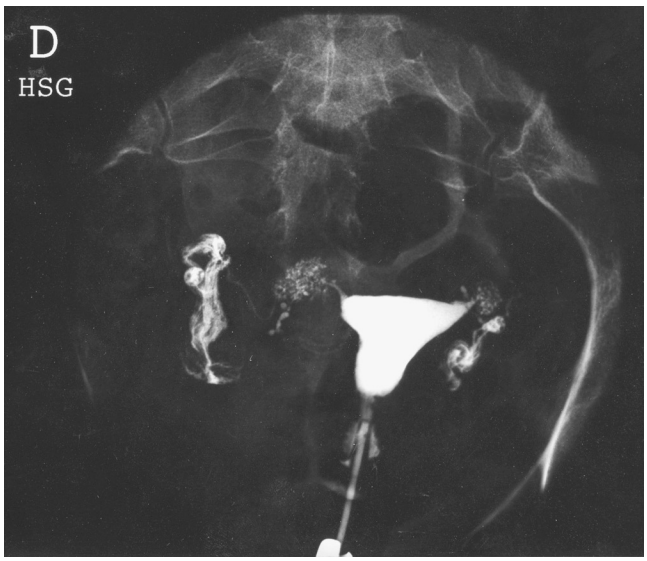


FIGURE 35.32 Characteristic honeycomb appearance in the proximal segment of the fallopian tube seen in case of salpingitis isthmica nodosa (SIN).

Complications

Minor complications of HSG include pain and cramping, which is usually mild to moderate and subsides within hours of the procedure. The most serious complication is a pelvic infection. Albeit rare (less than 1%), the risk exists, particularly in cases with a past history of pelvic inflammatory disease.[466] Some have advocated for antibiotic prophylaxis, either systematically or in selected cases in which there is a higher risk of infection.[467] The most common regimen has been doxycycline 100 mg, twice a day, administered for 1.5 to 5 days. We commonly administer 100 mg immediately prior to the procedure, on the evening of the procedure, and the morning after the procedure in women at higher risk of infection by history or findings on HSG.

Other infrequent complications include allergic reaction to iodine based contrast agents. In the latter case, gadolinium can be used as a replacement.[468] Perforation of the uterus or fallopian tube has been reported with HSG, but it is extremely rare.

Infusion Sonography

Numerous publications have described the possibility of documenting tubal patency by infusion sonography.[469] A consensus has been reached over the fact that positive contrast solutions providing white visualization offer better and easier visualization of the tubes, particularly of the proximal tubal sections.[429,470] Solutions such as Echovist® or Levovist®, while offering unquestionable value for assessing tubal patency suffer from: (1) Not providing an image of the fallopian tubes that matches that of HSG, particularly of the distal tubal segment; (2) the not insignificant cost of the contrast product. 3D reconstruction capability is likely to facilitate tubal assessment by sonography, but without inherent changes to imaging capabilities.[471] It is our impression that while the proximal section of the fallopian tube can easily be identified, HSG images remain largely superior for assessing the distal ends of the tubes.

A key issue when attempting to assess tubal physiology is to determine whether retrograde transport of sperm takes place normally during the late follicular phase. Using a ^{99}Tc-labeled macroalbumin aggregates in a procedure referred as hysterosalpingo scintigraphy, Kissler and colleagues reported evidence of retrograde transport alterations in endometriosis and adenomyosis.[472,473] This approach is cumbersome, however, and does not lend itself to repeated measurements, nor to studies conducted in mock reproductive conditions.

An advantage of HySo over x-ray-based methods for assessing tubal function is the possibility of repeating examinations. Unfortunately, the ideal infusion preparation for assessing tubal function does not yet exist. An ideal preparation capable of fulfilling the expectations of infertility specialists would be a product that would have the following characteristics: (1) offer positive contrast imaging for identification of the fallopian tubes, (2) a consistency that permits lasting contrast properties for durations sufficient for studying whether actual transport takes place through the tubes, (3) nontoxicity to gametes. This latter characteristic would be helpful for studying retrograde tubal transport under intercourse-like conditions, when the uterotubal unit is exposed to prostaglandins and other constituents of semen. This is deemed necessary as constituents of semen may exert clinically relevant effects on retrograde contractility[474] or other uterine functions.[475] Uterine and tubal influences exerted by semen constituents may be facilitated by a direct vagina-to-uterus transport through the functional portal system, or *first uterine pass effect*.[476]

Endometriosis

The clinical expression of endometriosis is infertility[477] and pelvic pain,[478] which is notoriously exacerbated in cases with endometriomas.[479,480] One can distinguish the manifestations of endometriosis that are readily identifiable on ultrasound (i.e., endometriomas) from others that are known to evade conventional vaginal ultrasound scrutiny.

Identifying and staging endometriosis, and its possible deep pelvic infiltration, has been the primary basis for reverting to MRI in benign gynecology imaging.[481,482] In certain circumstances, rectal ultrasounds or special procedures such as sonovaginography[199] have been proposed for evaluating the presence and extension of deep infiltrating endometriosis. Preoperative staging is challenging, and requires exploring all the possible locations of endometriosis. Vaginal ultrasound, put in expert hands, is now competing with MRI to the point that it may become the first-line diagnostic tool.[483]

While endometriosis can affect all organs and structures of the pelvic cavity, its extension commonly follows known paths. It is widely accepted the ovary constitutes the most common site for the development of endometriosis, where endometriotic cysts or endometrioma are formed. In general, peritoneal lesions are classified as either superficial or deep, depending on whether penetration exceeds 5 mm or not. Anterior endometriosis includes invasion of the bladder, and particularly its detrusor muscle.[484] Posterior endometriosis includes the extension of the disease to the uterosacral ligament, the upper and posterior portion of the cervix (torus) and finally, the various forms of vaginal, bowel, and uretral endometriosis.[485,486]

Endometriotic Cysts (Endometriomas)

Endometriomas are best diagnosed and described on ultrasound, where they present as isolated or multiple cystic structures filled with homogenous low-level echos.[418,487] This gives what is commonly referred to as grayish fairly homogenous aspect to the cystic structure. The majority of endometriomas range from 30 to 60 mm.[487] Color Doppler helps establish the endometriotic nature of newly identified cysts, and can document the absence of blood vessels within the walls of the cyst, or penetrating inside the cyst.[485] Differential diagnosis includes benign teratomas[425] and hemorrhagic cysts, particularly CL cysts.[488] Benign teratomas because of their sebum content, which has the general consistency of the old blood found in endometriomas, may provide echographic characteristics that are fairly similar to those of endometriomas. The presence of echos of hairballs, or shadowing effects from calcified structures (bones), strongly speaks for a dermoid cyst.[489]

Hemorrhagic CL cysts vary in size (2.5 to 10 cm). They differ from endometriomas by the presence of complex internal architecture that reflects the presence of a retracted clot and its fibrin content,[419,490] a characteristic that can be appreciated with new automatic texture assessment.[491] Moreover, a sponge-like reticular pattern is often identified within the cyst.[365] These two findings are not encountered in endometriomas. The ultimate hemorrhagic nature of cysts is confirmed by witnessing their disappearance (or profound change) on repeat ultrasounds performed 2 to 4 weeks later, a measure mandatory in our eyes.[492]

Endometriomas are often multiple. The choice of surgical management or ART will be influenced by the unilateral or bilateral nature of the lesions.[493-495] They characteristically have hyperchoic wall foci that are clearly identified in nearly a third of the cases.[423] In a recent study conducted on 65 women suspected of having an endometrioma, Alcazar and colleagues observed that peripheral vascularization of the endometrioma's wall was more important when pelvic pain was present than when it was not.[496] Although ultrasound is the diagnostic tool of choice for exploring endometriomas, in certain circumstances, MRI may prove to be more helpful, let alone necessary when ultrasound findings are inconclusive or their interpretation is hampered by the presence of other disorders, notably uterine fibroids.[497,498]

Endometriosis of Pelvic Areas Other Than the Ovary

Deep endometriosis is defined by lesions that extend ≥5 mm beneath the surface of the endometrium[499] and are associated with increased nerve density.[500] Chapron and colleagues stressed the fact that pelvic examination does not suffice for reliably diagnosing and locating deep endometriosis prior to surgery.[288] While elements in the patient's history,[500] particularly at the time of adolescence,[500] such as an early use of OC pills for managing severe dysmenorrhea,[500] or phenotypic characteristics,[501] increase the risk of finding endometriosis, these alone or collectively do not suffice for making the diagnosis preoperatively.[502] Taken together, these elements mandate deploying an image-based workup aimed at diagnosing and mapping deep endometriosis,[288] using diagnostic criteria,[503,504] before undertaking surgery. While MRI remains the gold standard for diagnosing deep endometriosis preoperatively, vaginal ultrasound is becoming ever more efficient.[502,505,506] Deep endometriosis lesions are described as hypoechoic linear thickenings or nodular masses with and without regular contour in the cul-de-sac, retro-cervical region, and recto-vaginal septum, involving or not involving the vaginal wall.[502,505] Several groups stress the fact that vaginal ultrasound allows the visual identification of lesions to be correlated with pain generated by exerting pressure on the suspected lesions.[507,508] Abrao and colleagues found that transvaginal ultrasound offered better sensitivity and specificity for identifying deep endometriosis than pelvic examination and even MRI.[509] The fact that all patients had an enema prior to the ultrasound examination might have contributed to the positive findings. Dessole and colleagues underscore that saline placed in the vagina can improve the visualization of the vaginal wall.[199]

Anterior and bladder involvement of endometriosis is an extension that is found in nearly 20% of endometriosis cases.[510,511] Bladder endometriosis commonly declares itself by localized bladder wall thickening on ultrasound.[512,513] At times infiltration may be recognized by the presence of deep irregular hypoechogenic infiltration of the bladder wall, particularly in the area of the uterobladder flap, with protrusions that may extend inside the bladder.[288] At MRI, bladder lesions are identified as heterogeneous T_2 isointense thickening of the bladder wall,[485] with lesions varying from 10 to 40 mm.[504] In a study involving 195 patients, MRI had a sensitivity and specificity of 88% and 99%, respectively, for the diagnosis of bladder endometriosis.[481] Extension of endometriosis to the ureter can be identified on T_2-weighted sequences.[485,514]

Endometriosis of the uterosacral ligament and posterior aspect of the cervix could be assessed by transvaginal ultrasound in 64% of cases, a diagnosis confirmed surgically in 88% of cases.[481] MRI provides higher sensitivity, however, with reported values ranging from 76% to 86%.[481,504] Retrocervical endometriosis is identified when thick irregular nodular formations of hypoechoic nature are found in this area, with possible extension to one or both uterosacral ligaments. Signs of adhesion are looked for by sliding loops of bowel along the uterosacral ligament and retrocervical area. The sensitivity of ultrasound is notoriously weak, however, reaching 64% in Bazot's hands[481] for diagnosing retrocervical endometriosis. In contrast, MRI offers an improved sensitivity for diagnosing deep endometriosis of the retrocervix.[504] An elegant MRI-based study demonstrated that deep infiltrating endometriosis does not originate from the rectovaginal septum, but rather from the posterior of the cervix at the height of insertion of the uterosacral ligaments.[288]

Endometriosis of the bowel most often, but not exclusively, affects the rectosigmoid colon,[515] a finding in agreement with the intriguing, yet generally recognized assymetry of pelvic endometriosis.[516-518] It is characterized by long, nodular, predominantly solid hypoechogenic lesions with varying degrees of infiltration from the serosal layer down to the muscularis propria identified as two hypoechoic lines separated by a fine hyperechoic line.[502,509,514,519] On MRI, the reported sensitivity and specificity is of 84% and 99%, respectively, in women with documented intestinal involvement.[481] Diagnostic criteria for rectal invasion include rectal wall thickening and a characteristic triangular attraction of the rectum toward the posterior aspect of the cervix or torus uteri.[481,485,512,520] Preoperative staging of endometriosis extension, and particularly, identifying the presence and extent of deep lesions, is considered an obligation today.[521]

Adenomyosis

A variant of endometriosis, adenomyosis, is characterized by the heterotopic development of glands and stroma in the subendometrial myometrium with various degree of hyperplasia present amongst adjacent smooth muscle cells.[522] Adenomyosis can be either diffuse or focal.[523] In the diffuse form, glandular and stromal constituents of the endometrium extend throughout the sub-endometrial layers of the myometrium, resulting in an overall enlargement of the whole uterus.[522] In the localized variant, the affected area is isolated in the frontal or posterior wall of the uterus, taking on an appearance that may be difficult to distinguish from a fibroid.[524] While adenomyosis has been claimed to cause infertility, its contribution is debated.[455,525]

Various contemporary transvaginal ultrasound approaches are being tested and developed to identify and delineate the territorial extension of adenomyosis. The most commonly identified marker of diffuse adenomyosis on ultrasound is a poorly marginated hypoechoic extension in the subendometrial layers of the myometrium.[526] According to Devlieger and colleagues, localized adenomyosis can be distinguished from fibroids based on the following criteria: (1) absence of circular vascularization at the border of the lesion, which may be replaced by vessels actually penetrating into the lesion; (2) presence of a shaggy limit between the lesion and the outer myometrium that typically lacks the characteristic shell common to fibroids with its acoustic shadowing effect (Fig 35.33).[524]

MRI, especially through T_2-weighted images, provides excellent soft tissue differentiation. In the uterus, MRI delineates a subendometrial band of low-signal referred to as the junctional zone (JZ). Histological analyses have demonstrated that JZ corresponds to the subendometrial layer of the myometrium.[527,528] Yet, these studies failed to provide clues as to the nature of the histological basis that accounts for the low-signal on MRI. In contrast to the outer layers of the myometrium, the subendometrial layer of the myometrium is of müllerian origin and the site of changes

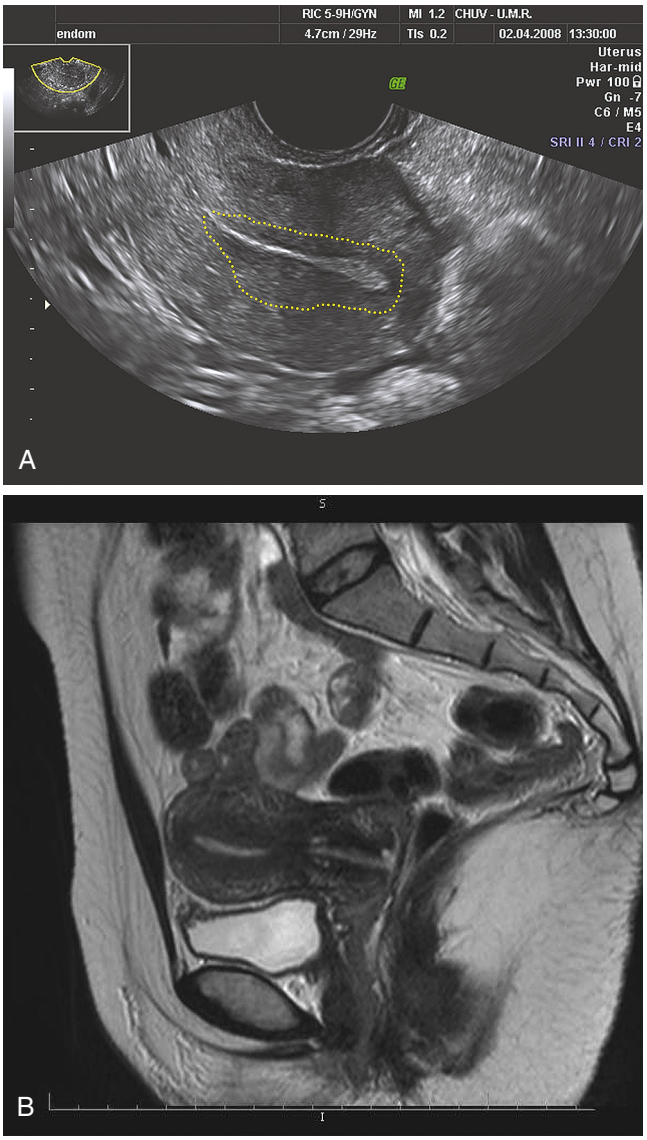


FIGURE 35.33 Diffuse adenomyosis. **A**, The ultrasound appearance is characterized by a thickened subendometrial sonoluscent area. **B**, Magnetic resonance imaging (MRI) is characterized by a diffuse and fairly regular thickened subendometrial transitional zone.

in E_2 and progesterone receptors that follows the pattern of changes encountered in the menstrual cycle.[26] Considerable variation in JZ thickness has been observed, with reported values that range from 2 to 8 mm.[529,530] In one report, transvaginal ultrasound was as effective as MRI in delineating the subendometrial layer of the myometrium.[531] Excessive local or diffuse thickening of the low-signal JZ with ill-defined boundaries has been the hallmark of adenomyosis, with many authors proposing 8 mm as the cutoff value between normal and overt diffuse adenomyosis.[532] In a study of 119 patients undergoing hysterectomy, the JZ was 7.7 mm in 91 women without adenomyosis, and 15 mm in 28 women with adenomyosis.[531] In women whose JZ was measured between 8 and 12 mm, associated findings such as focal thickening of the JZ or high-signal foci within areas of low signal on T_2-weighted sequences may represent island of ectopic endometrium.

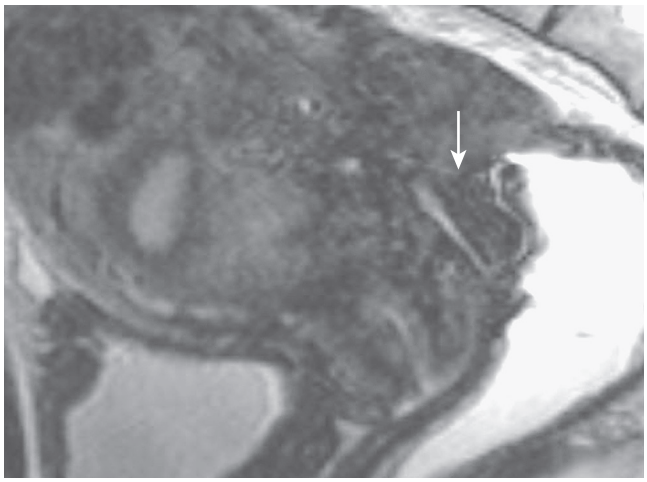


FIGURE 35.34 Endometriotic lesion: Sagittal T_2-weighted fast spin-echo magnetic resonance imaging (MRI) of the pelvis showing an infiltrative lesion (typical fibromuscular lesions of endometriosis) extending to the anterior rectal wall and posterior wall of the uterus *(arrow)*.

Focal adenomyosis in areas of low-signal intensity on T_2-weighted images was found to consist of smooth muscle hyperplasia associated with heterotopic endometrial tissue (Fig. 35.34).[454,533] It is important to distinguish focal adenomyosis from leiomyomas. In 21 women suspected to have focal adenomyosis, MRI adequately diagnosed signs of focal adenomyosis in 12 women. In 10 of these women, the diagnosis was ultimately confirmed by histological analysis of the pathological specimen.[534] According to Reinhold and colleagues,[454] features that favor the diagnosis of adenomyosis on MRI include: (1) lesions with poorly defined borders; (2) lesions that extend along the endometrium; (3) minimal mass effect on the endometrium relative to the size of the lesion; (4) linear striation radiating out of the endometrium into the myometrium; and (5) absence of large vessels at the margins of lesions contrary to the typical findings of leiomyomas. Furthermore, these authors stress the possibility that uterine contractions and the resulting deformation of the endometrial-myometrial interphase may generate images evocative of focal adenomyosis.[454] There is general agreement about the high sensitivity of MRI for detecting focal adenomyosis, with sensitivities of 88% and 86%, reported respectively, in studies by Ascher and colleagues[532] and Reinhold and colleagues.[531] These authors disagreed, however, about the ability of endovaginal ultrasound to detect focal adenomyosis, with the former and the latter groups reporting 53% and 89%, respectively.

Endometriosis and Uterine Contractility

Adenomyosis is likely associated with myometrial dysfunction,[535,536] possibly associated with endometriosis-types of alterations of the eutopic endometrium[537] and the JZ.[27] In a provocative series of publications, Leyendecker's team promoted the concept that endometriosis is associated with a hyperkinetic-dyskinetic condition of the uterus[473,538] as a result of structural abnormalities of the uterine wall,[539]

which impairs the retrograde transport of sperm during the follicular phase,[24,231,540] and the proper antegrade emptying of uterine content at the time of menses.[236] From their studies using the uterine displacement of ^{99}Tc-labeled MAA, these authors observed two pathological findings in endometriosis: (1) a pathological increase in retrograde transport at the time of menses; and (2) the loss of retrograde transport targeted toward the tube that faces the developing follicle during the late follicular phase of natural cycles.[24,235] Both phenomena could contribute to the infertility that accompanies even the milder forms of endometriosis. The original report of activation of the aromatase gene in the eutopic endometrium in case of endometriosis[541] that has been amply confirmed[542] offers a possible explanation for the local state of hyperestrogenism purported to be the cause for the relative progesterone resistance[542,543] and the dyskinetic alterations encountered in endometriosis.[241,544] That COH-IUI cycles have markedly lower pregnancy rates in endometriosis as compared to unaffected controls[545-547] is consistent with the concept that endometriosis is accompanied by a sperm transport problem stemming from uterine dyskinesia.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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CHAPTER 36

Contraception

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The use of modern contraceptives has contributed substantially to the reduction of maternal and infant morbidity and mortality and to the ability of women to contribute to society. However, evidence-based utilization of the methods has not been fully realized since approximately 50% of all pregnancies, 3 million pregnancies per year in the United States, are unintended.[1] Unintended pregnancy has negative effects on women's health and education and the health of newborns, and requires significant personal financial resources from individuals and society.[2] According to the U.S. census bureau, 62 million American women are in the childbearing years (15 to 44 years old). The vast majority (7 out of 10) of women age 18 to 44 are sexually active and trying to avoid pregnancy. The two contributors to unintended pregnancy are contraceptive non-use and contraceptive failure.[1] The importance of maximizing use of effective contraceptive methods cannot be overstated.

Table 36.1 demonstrates the changes over time in American contraceptive use and underscores that although long-term effective contraceptive methods are increasing in use, these methods still make up a slim minority of contraceptive technologies utilized by American couples. The oral contraceptive pill is the most widely used method by women in their teens and twenties, women who have never been married, and women who have at least a college degree. Poor and low income women are twice as likely as higher income women to use the 3-month injectable method, with depo-medroxyprogesterone acetate. Fifty percent of all women age 40 to 44 years old who practice contraception have been sterilized. The proportion of women who use a contraceptive method at first intercourse has doubled in recent times to 79% at present.[2-5] The majority of these couples use condoms at first intercourse. Teenagers who do not use a contraceptive at first intercourse are twice as likely to become teen mothers as teenagers who do use a contraceptive method. As demonstrated by Table 36.1, contraceptive use has increased overall among American women. Recently, after years of under-utilization, intrauterine contraceptive use has increased among American women. Since long-acting reversible contraception is superior to the most commonly used oral contraceptive pills, continued uptake of these methods should ultimately decrease the unintended pregnancy rate in the U.S.[6]

Contraceptive Effectiveness versus Efficacy

Contraceptive clinical trials typically report their failure rates either by the Pearl Index or Life Table Analysis. The Pearl Index is defined as the number of contraceptive failures per 100 women-years of exposure, and uses as the denominator the total months or cycles of exposure from the initiation of the product to the end of the study or the discontinuation of the product. Life Table Analysis, on the other hand, provides the contraceptive failure rate for each month of use and can provide a cumulative failure rate for any specific length of exposure. A recent Food and Drug Administration (FDA) briefing reviewed the actual utility of the Pearl Index, noting that it does not take into account the effect of failures over time (www.fda.gov/ohrms/dockets/ac/07/briefing/2007-4274b1-01-FDA.pdf). The Life Table Analysis method of reporting effectiveness is considered an actuarial method. Effectiveness can be difficult to compute because there are many factors that influence contraceptive use. The term "effectiveness" is defined as the ability of a contraceptive to prevent pregnancy in a clinical trial setting, meaning a failure or success that occurs when a product is used perfectly in accordance with the recommended dose regimen. "Efficacy" is a term used to define the ability of a contraceptive to prevent pregnancy in typical use, for example, when the subject does not completely follow the recommended dosing regimen. The terms "typical use failure rate" and "perfect use failure rate" are also used to describe these differences. Table 36.2 demonstrates the differences in the typical use failure rate from the perfect use failure rate in the different contraceptive methods. It is critical to note that typical use applies to the population in general whereas perfect use refers to the clinical trial setting.

Intrauterine Contraception

Intrauterine contraception is a widely used method of reversible birth control globally. Benefits of intrauterine devices (IUDs) include a high level of effectiveness, lack of associated systemic metabolic effects, and the need for only a single act of motivation for long-term use, as well as its ability to be used over several years. The United States has seen a recent uptick in the prevalence of IUD use.[7] If this continues, it is likely that the increased use of IUDs will lower the unplanned pregnancy rate in the U.S.[6] Historically, a major barrier to uptake has been cost.

Currently there are two intrauterine contraceptives available on the United States market: The copper ParaGard IUD and the levonorgestrel-containing Mirena intrauterine system. Over the past 35 years, however, many intrauterine contraceptives have been designed and used clinically throughout the world. The design of IUDs has evolved since

Table 36.1 Percentage Distribution and Number of Contraceptive Users (in Thousands) Aged 15 to 44 in the United States, by Current Method, 1982-2008

	1982		1988		1995		2008	
Method	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*	*No.*
Sterilization	34.1	10,295	39.2	13,686	38.6	14,942	37.0	14,200
Female	23.2	6998	27.5	9614	27.7	10,727	27.1	10,400
Male	10.9	3298	11.7	4069	10.9	4215	9.9	3800
Pill	28.0	8431	30.7	10,734	26.9	10,410	28.0	10,700
Implant	NA	NA	NA	NA	1.3	515	400	1.1
Injectable*	NA	NA	NA	NA	3.0	1146	3.2	1,200
Intrauterine device	7.1	2153	2.0	703	0.8	310	5.5	2,100
Diaphragm	8.1	2436	5.7	2000	1.9	720	—	—
Male condom	12.0	3608	14.6	5093	20.4	7889	16.1	6200
Foam	2.4	711	1.1	371	0.4	161	—	—
Periodic abstinence	3.9	1166	2.3	806	2.3	883	300	0.9
Withdrawal	2.0	588	2.2	778	3.0	1178	2,000	5.2
Other†	2.5	754	2.1	733	1.3	508	200	0.4
Total	100.0	30,142	100.0	34,912	100.0	38,663	38.241	100

*Ring and Patch included in this row for 2008 data.
†Other includes emergency contraception, female condom or vaginal pouch, foam, cervical cap, Today sponge, suppository or insert, jelly or cream (without diaphragm), and other methods.
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their original use in the 1960s. IUDs were initially developed in the 1980s as T-shaped plastic devices covered with copper wire. The amount of copper in the IUD accounts for the length of time of efficacy. Each IUD does have a certain rate of copper dissolution, which accounts for its need to be replaced over time. In the United States, the T380A IUD is the only copper-bearing IUD. The multi-load CU375 is widely used in Europe. The T380A has a frame of polyethylene with a vertical length of 36 mm and a horizontal length of 32 mm. The exposed surface area of copper is 380 mm^2, hence its name. The amount of copper dissolution daily amounts to less than what the ingested amount of copper is in the normal diet. The copper T380A is currently approved in the United States for 10 years, but evidence has shown that it is effective for up to at least 12 years.[8] All copper-containing IUDs have a number as a part of their name and this indicates the surface area of copper that the IUD provides.

Copper Intrauterine Devices

Copper T IUDs have failure rates of less than 1% per year and cumulative 10-year failure rates of approximately 2% to 6%. A large World Health Organization (WHO) trial[8] reported a cumulative 12-year failure rate of 2.2% for the T380A (ParaGard). This is an average of 0.18% per year over 12 years of use. This failure rate is equivalent to the failure rate seen with female sterilization methods. The mechanism of action is not fully understood for the copper IUD. The presence of in intrauterine device has been shown to prompt the release of leukocytes and prostaglandins by the endometrium.[9] This creates a hostile milieu to both sperm and eggs. The presence of copper increases the spermicidal effect.

Hormonal Intrauterine Devices

A reservoir of progestin in the vertical arm of the T-shaped IUD increases its efficacy. The progestin-releasing IUD was marketed initially as a 1-year device but is no longer manufactured. Currently, a T-shaped IUD containing a levonorgestrel in the vertical arm has been developed and has undergone extensive clinical testing. It is available in the United States and other countries as the levonorgestrel intrauterine system (LNG-IUS), or Mirena. A large comparative trial of the copper T380A and the levonorgestrel-releasing IUD found that the efficacy of the two methods is similar, as are the continuation rates. The LNG-IUS measures 32 mm both horizontally and vertically. It is made with a silicone reservoir containing 52 mg of levonorgestrel in the vertical stem. There is an initial burst release of levonorgestrel of 20 μg/day, resulting in plasma levels of 150 to 200 pg/mL.[10] The plasma concentrations of levonorgestrel decrease over the number of years it is used. There is sufficient levonorgestrel in the intrauterine system to last for the estimated duration of 5 years.[11] The levonorgestrel-releasing intrauterine system produces high concentrations of progestins in the endometrium. The progestin-dominant effect allows the endometrium to remain thin and not proliferate. In addition to the contraceptive efficacy that is obtained, bleeding patterns change. Approximately 20% of women who use this device become amenorrheic at 1 year.[11]

A new progestin-releasing IUD that contains 13.5 mg of levonorgestrel has recently become available (Skyla). This device provides 3 years of contraceptive efficacy. The device releases about 14 μg of levonorgestrel per day shortly after insertion. After 3 years, the device releases about 5 μg of levonorgestrel per day. The inserter has a diameter of 3.8 mm, which is about 1 mm smaller than the Mirena inserter. This device may be of use for women who do not need 5 years of contraception and for some nulliparous women where a narrower inserter may facilitate the placement of the device.

The mechanism of action of the levonorgestrel intrauterine system is better understood than is that of the copper intrauterine device. The LNG-IUS works primarily by changing the endometrial lining and thickening the cervical mucus. This thick cervical mucus obstructs the passage of

Table 36.2 Percentage of Women Experiencing an Unintended Pregnancy During the First Year of Typical Use and the First Year of Perfect Use of Contraception and the Percentage Continuing Use at the End of the First Year: United States

Method	Women Experiencing an Unintended Pregnancy within the First Year of Use (%)		Women Continuing Use at 1 Year[c] (%)
	Typical Use[a]	Perfect Use[b]	
No method[d]	85	85	42
Spermicides[e]	29	15	43
Withdrawal	27	4	51
Periodic abstinence	25	—	—
Calendar	—	9	—
Ovulation method	—	3	—
Sympto-thermal[f]	—	2	—
Postovulation	—	1	—
Cap[g]			
Parous women	32	26	46
Nulliparous women	16	9	57
Sponge			
Parous women	32	20	46
Nulliparous women	16	9	57
Diaphragm[g]	16	6	57
Condom[h]			
Female (reality)	21	5	49
Male	15	2	53
Combined pill and minipill	8	0.3	68
Evra patch	8	0.3	68
NuvaRing	8	0.3	68
Depo-Provera	3	0.3	56
Lunelle	3	0.05	56
Intrauterine device (IUD)			
Progestasert (progesterone T)	2	1.5	81
ParaGard (copper T)	0.8	0.6	78
Mirena (LNG-IUS)	0.1	0.1	81
Norplant and Norplant-2	0.05	0.05	84
Female sterilization	0.5	0.5	100
Male sterilization	0.15	0.1	100
Emergency contraceptive pills: Treatment initiated within 72 hours after unprotected intercourse reduces the risk of pregnancy by at least 75%.[i] Lactational amenorrhea method (LAM) is a highly effective, *temporary* method of contraception.[j]			

[a]Among *typical* couples who initiate use of a method (not necessarily for the first time), the percentage who experience an accidental pregnancy during the first year if they do not stop use for any other reason.

[b]Among couples who initiate use of a method (not necessarily for the first time) and who use it *perfectly* (both consistently and correctly), the percentage who experience an accidental pregnancy during the first year if they do not stop use for any other reason.

[c]Among couples attempting to avoid pregnancy, the percentage who continue to use a method for 1 year.

[d]The percentages becoming pregnant in columns 2 and 3 are based on data from populations where contraception is not used and from women who cease using contraception in order to become pregnant. Among such populations, about 89% become pregnant within 1 year. This estimate was lowered slightly (to 85%) to represent the percentage who would become pregnant within 1 year among women now relying on reversible methods of contraception if they abandoned contraception altogether.

[e]Foams, creams, gels, vaginal suppositories, and vaginal film.

[f]Cervical mucus (ovulation) method supplemented by calendar in the preovulatory phase and basal body temperature in the postovulatory phase.

[g]With spermicidal cream or jelly.

[h]Without spermicides.

[i]The treatment schedule is one dose within 120 hours after unprotected intercourse, and a second dose 12 hours after the first dose. Both doses of Plan B can be taken at the same time. Plan B (1 dose is 1 white pill) and Preven (1 dose is 2 blue pills) are the only dedicated products specifically marketed for emergency contraception. The Food and Drug Administration has in addition declared the following 17 brands of oral contraceptives to be safe and effective for emergency contraception: Ogestrel or Ovral (1 dose is 2 white pills), Alesse, Lessina, or Levlite (1 dose is 5 pink pills), Levlen or Nordette (1 dose is 4 light-orange pills), Cryselle, Levora, Low-Ogestrel, or Lo/Ovral (1 dose is 4 white pills), Tri-Levlen or Triphasil (1 dose is 4 yellow pills), Portia or Trivora (1 dose is 4 pink pills), Aviane (1 dose is 5 orange pills), and Empresse (1 dose is 4 orange pills).

[j]However, to maintain effective protection against pregnancy, another method of contraception must be used as soon as menstruation resumes, the frequency or duration of breastfeeding is reduced, bottle feeds are introduced, or the baby reaches 6 months of age.

From Trussell J. Chapter 13. *In* Hatcher RA, Trussell J, Stewart F, et al (eds). *Contraceptive Technology,* 18th rev. New York, Ardent Media, 2004.

sperm through the cervix. The foreign body reaction also makes the environment hostile to sperm and eggs. The frequency of ovulation is reduced with the LNG-IUS overall. However, this is not the primary mechanism of action. Women may ovulate variably with the LNG-IUS in place. All the effects that render both intrauterine contraceptives effective are reversed promptly upon their removal.

Resumption of fertility seems to occur at the same rate as discontinuation of barrier methods of contraception,[12,13] although the data on the contemporary IUDs are limited. Pregnancy outcomes including term delivery, spontaneous abortion, and ectopic pregnancies after IUD removal is unchanged from the baseline incidence of these events in the population.

Considerations for IUD Placement

Considerations that should be reviewed with patients before IUD placement include the timing of insertion, the bleeding patterns to be expected, the side effects of the method, and the expected interval of use. There are very few contraindications to IUD use; appropriate candidates for the IUD include women who desire long-term contraception regardless of age of parity.[6] The insertion of an IUD during the menstrual period does lend some benefits which include lubrication and slight opening of the cervical os from the menstrual flow, a decrease in bleeding symptoms after IUD placement due to the pre-existing bleeding from the menses, and the assurance that the patient is not pregnant. However, IUDs may be inserted at any time during the cycle so long as pregnancy has been excluded. The copper IUD is an effective post-coital contraceptive as well so it can be placed after unprotected intercourse and then used long-term.[14,15]

Bleeding patterns may change with both intrauterine contraceptive methods. The copper-T 380A is associated with approximately a 55% increase in menstrual blood loss.[16] In contrast, the amount of blood loss is significantly reduced to about 5 mL per cycle with the LNG-IUS.[17] The bleeding patterns with the T380A IUD may be most increased during the first few months of use, and some patients may require supplemental iron and prostaglandin synthetase inhibitors during menses to help counteract these effects. Bleeding patterns with the LNG-IUS are variable. The general picture of bleeding patterns is unscheduled bleeding during the first few months of use due to the erratic thinning of the endometrium. Over time, bleeding days decrease and approximately 20% of women are amenorrheic at 1 year of use. Women who do experience menstrual periods with the LNG-IUS in place generally have light periods or perhaps even spotting as opposed to longer and full menstrual flow.

Rare but potential complications of IUD insertion include expulsion, infection, and perforation. Expulsion is the most common risk of IUD placement, and while not particularly dangerous, the detection of the expiation is important so that the woman can use a back-up method of pregnancy prevention. Risk factors for expulsion include: nulliparity, menorrhagia, prior expulsion, age less than 20 years, insertion immediately after a second trimester abortion, or postpartum.[7,18,19] Patients can be quoted a 3% to 10% annual risk of this occurrence. Monthly string checks and warning signs should be reviewed so that early detection of expulsion is maximized. There has been a documented transient increase in the risk of infection within the first 20 days of intrauterine device placement.[12] This is likely due to the introduction of bacteria into the upper genital tract with the placement of the IUD or with the sounding of the uterus. However, over time Pelvic Inflammatory Disease (PID) prevalence is the same with IUD users as in the general population. One study has shown a decreased risk of PID among LNG-IUS users when compared to those who use the T380A. For this reason, some have advocated the levonorgestrel intrauterine system as an optimal method for younger women who may be at a baseline increased risk of PID. As with all nonbarrier methods of contraception, patients should be counseled that the intrauterine system does not protect against sexually transmitted infections, and if such a risk is present, condoms should be used.

Perforation is another uncommon risk of IUD insertion, but it is potentially serious. Perforation occurs at the time of insertion, and perforation rates are approximately 1 in 3000 insertions.[20] This risk is due to the blind introduction of the device into the uterus. At any time, if an IUD is found to be outside the uterus, even in asymptomatic patients, it should be removed from the peritoneal cavity. The presence of the copper IUD in the peritoneal cavity has been associated with complications, including severe adhesions and bowel obstruction. Such data are not available for the levonorgestrel intrauterine system, but until there is reason to believe otherwise, this IUD should also be removed, usually via laparoscopic approach.

Pregnancies are rare with an IUD in place given their efficacy. However, if a woman conceives while using an IUD and the device is not subsequently removed, the incidence of spontaneous abortion is about 55%, about three times greater than would occur in pregnancies without an IUD. The current recommendations include removal of an IUD should a woman conceive with the IUD in place and wishes to continue the pregnancy. There is difficulty with removal of the IUD at the time of pregnancy diagnosis. Ultrasound guidance and other techniques may improve the ability to safely remove the IUD without repeated prodding.[20,21]

The overall safety of intrauterine contraceptives demonstrate that they are not associated with an increased risk of endometrial or cervical carcinoma and may actually be associated with a reduced risk in these neoplasms during use and after removal.[22,23] The IUD is a very useful method of contraception for women who have completed their families and do not wish to have permanent sterilization. The LNG-IUS has many noncontraceptive benefits such as a decrease in menstrual symptoms and bleeding, and it is also a treatment for endometrial hyperplasia. Studies have also shown that the LNG-IUS is useful for treatment of women with adenomyosis and endometriosis. Women in the United States who use an IUD have a higher level of satisfaction with their method of contraception than do women using any other method of reversible contraception. IUD use is very common among female gynecologists but is underutilized by a large proportion of candidate women in the United States.

Contraceptive Implants

Implants allow for long-acting reversible contraception without a pelvic exam and regardless of shape and size of the uterus. Subdermal implants were approved by the FDA in 1990. Norplant is a sustained release progestin-only system that consists of six capsules, each measuring 34 mm in length, with a 2.4 mm outer diameter. Each capsule contains 36 mg of levonorgestrel for a total of 216 mg of steroid hormone that remains unchanged within capsules over 9 years of use. Norplant is no longer available on the U.S. market. However, it is widely used in other parts of the world. Implanon/Nexplanon is a progestin-only method that contains 68 mg of 3-ketodesogestrel (etonogestrel) within a single 4-cm flexible rod. The major mechanism of action of subdermal implants is ovulation inhibition via inhibition of luteinizing hormone.[24-26] Insertion is accomplished in the outpatient setting. The capsules are implanted in the subcutaneous tissue through a trocar that is enclosed in the contraceptive implant

kit. As with all progestin-only methods, the cervical mucus is thickened and the endometrial lining becomes decidualized. The effects on the endometrial lining produce the clinical symptoms of initial irregular unscheduled bleeding, followed by scant bleeding or amenorrhea.[25,26] Amenorrhea rates with Implanon in clinical trials run about 50% of women. Frequent and prolonged bleeding occurred in 20%.[25,26]

In one multicenter study in Europe and Canada, the discontinuation of this method for abnormal bleeding reached a rate of 23% within the first 2 years of use.[27] However, in a comparative study of the single implant (Implanon) with the six capsules of levonorgestrel (Norplant), there were fewer bleeding episodes and less blood loss per episode with the single implant. The most common adverse effects with the implant were acne, breast pain, and headache.[26] The etonogestrel implant (Implanon) lasts for 3 years. It releases 60 to 70 mg of etonogestrel per day and this gradually decreases over time.[28] Levels of etonogestrel in the serum are therapeutic within 8 hours of insertion. In clinical use, etonogestrel implant (Implanon) can be inserted within 5 days of the onset of the menstrual cycle in a cycling woman and in the setting of a negative hCG in a noncycling woman. Etonogestrel implant has not yet been FDA approved for postpartum use, but it appears to be safe for immediate postpartum insertion[29-31] with improved long-term pregnancy prevention when compared with other postpartum methods.[32] Implanon, or Nexplanon, is also approved for use immediately after miscarriage and after abortion.

Steroid Contraceptives

Hormonal contraception was initially marketed in the United States in pill form in 1960. The original formulation included 50 μg of estrogen. Such dosing is rarely used today, and the majority of hormonal contraceptives on the U.S. and European markets include doses of 35 μg of estrogen or less, all of which qualify as "low-dose" hormonal contraceptives. The oral contraceptive pill is still the most widely used hormonal contraceptive delivery system. However, steroid contraceptives also take the form of a transdermal patch, a combined hormonal vaginal ring, and progestin-only methods include a 3-month injectable, a subdermal implant, and an intrauterine contraceptive. The oral contraceptive pill is perhaps the most widely studied medication in history. Although it is biologically plausible that other delivery systems, such as the transdermal patch and the vaginal ring, carry with them similar contraceptive benefits, side effects, and contraindications as the combined contraceptive pill, verification of this biological plausibility has not yet been done in large clinical trials. For this reason, we will focus on the combined hormonal contraceptive pill when discussing the details of the mechanism of action, the side effects, the noncontraceptive benefits, and the contraindications to combined hormonal contraceptive use. The specific issues related to the other delivery systems will be addressed in the respective sections.

Hormonal Contraception

The history of hormonal contraception dates back into the early 1900s when it was observed that ovulation is linked to pregnancy and the presence of a corpus luteum. The history of hormonal contraception is not glamorous, but instead exemplifies the dedicated, multidisciplinary efforts required to make scientific advances that may be politically challenging. The chemist Russell Marker purified progesterone from both animal and plant sources. Carl Djerassi, another chemist at the company Syntax, found that the progestational activity of yam-derived progesterone could be enhanced by removing the 19-carbon from this plant progesterone. A reproductive health activist named Margaret Sanger brought the scientist Gregory Pincus together with the philanthropist Catherine McCormick in the 1950s. Pincus, together with other scientists from Boston, worked with the funding supplied by Catherine McCormick to develop a progestational agent in pill form to act as a contraceptive. The clinical trials of the early oral contraceptive pill were conducted in Puerto Rico. The initial compound consisted of 10 to 40 mg of synthetic progestin. The initial products, however, were contaminated with 1% mestranol, a synthetic estrogen. When the mestranol was removed from the compound for a more pure progestational agent, clinical trial results demonstrated more breakthrough bleeding and the decision was made to retain the estrogen for cycle control. This is the history behind the combined hormonal contraceptive as we know it today.

Pharmacology

There are three main formulations for combined hormonal contraception delivered orally. These include monophasic combination pills, multiphasic combination pills, and progestin-only pills. The progestin component of the combined hormonal contraceptive is most commonly a 19-nortestosterone progestin. These progestins more closely resemble testosterone than their alternative, the 21-carbon acetoxyprogesterone derivative. Medroxyprogesterone acetate and megestrol acetate are 21-carbon progestins. The two classes most commonly used are the estranes and gonanes. Those derived from 17α-acetoxyprogesterone are referred to as the pregnanes. 19-Nortestosterone derivatives are orally active, but they do carry some androgenic and progestational effects. The estrane family of progestin, norethindrone derivatives (Fig. 36.1), include norethindrone, norethynodrel, norethindrone acetate, ethynodiol

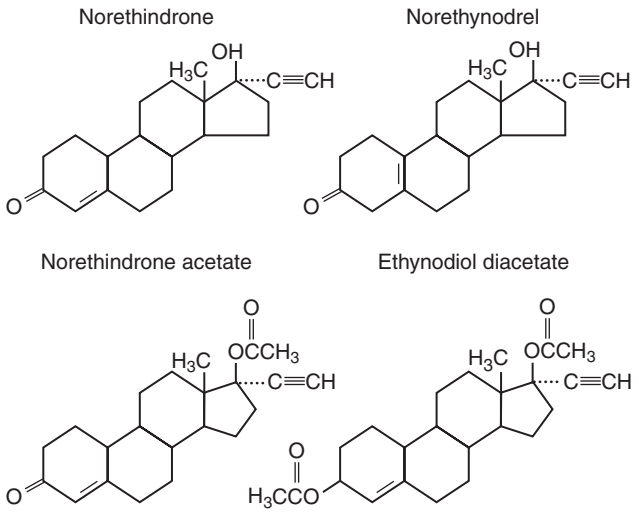


FIGURE 36.1 Chemical structures of the estrane progestins used in oral contraceptives.

diacetate, lynestreneol, norgestrel, norgestimate, desogestrel, and desogen. Regardless of the nomenclature, most estrane progestins are converted to norethindrone when they are metabolized. The norgestrel family of progestins, the gonane progestins (Fig. 36.2), include desogestrel, gestodene, norgestimate, and etonogestrel, and are utilized in the combination vaginal ring. Drospirenone is one progestin that is used in oral combination hormonal contraception but is not a member of the estrane or gonane families. Drospirenone is considered to have less androgenicity than the 19-nortestosterone-derived progestins. Drospirenone is derived from spironolactone and it has anti-androgenic and antimineralcorticoid activity. This formulation has not been shown to have decreased progestin associated side effects in randomized control trials at a population based level. However, it is an option for women using combined hormonal contraception today. Because the progestin component of combined hormonal contraception is what is responsible for the contraceptive efficacy, most dosages of oral contraceptive pills are dependent upon the amount of progestin required to achieve contraceptive efficacy. The gonane class has greater progestational activity per unit weight than the estrane class, which accounts for the smaller amount required in these oral contraceptive formulations.

Combination hormonal contraceptive pills by definition combine the progestin with one of two types of estrogens, most commonly ethinyl estradiol and less commonly ethinyl estradiol 3-methyl ether, which is otherwise known as mestranol (Fig. 36.3). The original oral contraceptive formulations contain mestranol, which was utilized in the 50-μg formulation. These 50-μg formulations are termed first-generation combined hormonal contraceptives. These forms are rarely utilized today, and most oral contraceptives contain estrogen doses of 35 μg or less and are referred to as low-dose estrogen oral contraceptives. Oral contraceptive pills with 20 to 35 μg of ethinyl estradiol are termed second-generation products. The third-generation formulation refers to those with the newer versions of the levonorgestrel derivatives, the newer versions of the gonane progestins, and they include desogestryl, norgestimate, and gestodene. Combination oral contraceptives are given continuously for 3 weeks, and the traditional formulation allows for a placebo, medication-free week, in the fourth week. The withdrawal of the estrogen in the combined hormonal contraceptives results in endometrial sloughing. This sloughing results in a withdrawal bleed that usually lasts 3 to 4 days. Uterine blood loss is about 25 mL and this is less than the average 35 mL that is lost with menses in an ovulatory cycle.

Mechanism of Action

The traditional contraceptive pills are given for 3 out of 4 weeks in the month. Newer formulations have focused on diminishing or eliminating the pill-free week in order to allow for a decreased number of bleeding days or elimination of bleeding days entirely. The progestin component of the combination pills prevents ovulation by inhibiting gonadotropin secretion. This effect comes directly from the progestational agent's suppression of luteinizing hormone (LH) secretion, thereby preventing ovulation. The estrogenic agent suppresses follicle-stimulating hormone (FSH). However, it is important to note that even if follicles do develop, which is commonly seen with combination pills of 20 μg or less, these follicles do not develop sufficiently and the progestational component still inhibits the luteinizing hormone surge that is necessary for ovulation. The progestin component also works by thickening the cervical mucus and altering the endometrium such that it is not receptive to implantation. The endometrium in a progestin-dominant milieu is decidualized and atrophic. These lower genital tract effects are sufficient in and of themselves to afford

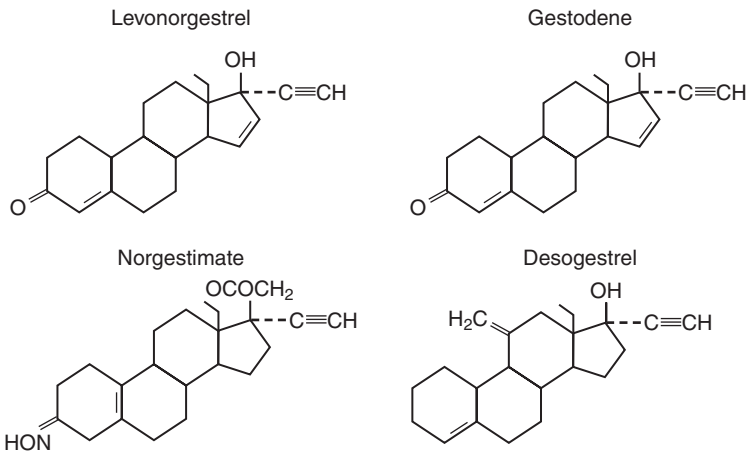


FIGURE 36.2 Chemical structure of the gonane progestins used in oral contraceptives.

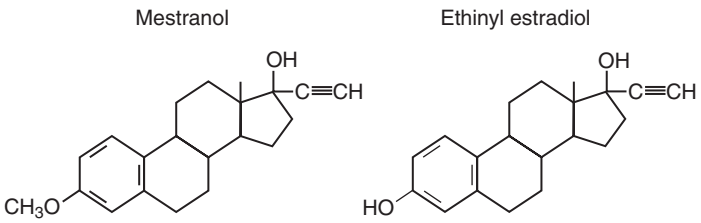


FIGURE 36.3 Structures of the two estrogens used in combination oral contraceptives.

good contraception. In fact, many progestin-only methods work primarily by cervical mucus thickening and endometrial lining decidualization.

The FDA has approved three new contraceptive formulations for continuous use. These methods are called Lybrel, Seasonale, and Seasonique. The efficacy of these regimens appears to be similar to the 21/7-day efficacy period. The clinical trials that were done initially for the approval of these formulations demonstrate that continuous use of hormonal contraception suppresses folliculogenesis at improved rates over the traditional 21/7-day regimens. This choice should be considered in the clinical scenario when patients may have ovarian cysts and are looking for suppression of follicle growth as a noncontraceptive benefit of their hormonal contraceptive. Studies have demonstrated that these new formulations are acceptable to women of reproductive age. They are highly convenient with fewer bleeding days over time. Noncontraceptive benefits include decreased dysmenorrhea, decreased endometriosis pain, and fewer instances of headaches, bloating, and mood swings. Good candidates for continuous hormonal contraceptives are all women but specifically women who have symptoms in their pill-free week. Breakthrough bleeding is, however, more common than during the first 3 months of use in the conventional 21/7-day regimen. Over time, this breakthrough bleeding reaches the usual rate.

Long-term safety data have not yet been amassed with continuous hormonal contraceptives, but markers of safety and pregnancy after discontinuation appear to be similar to the traditional 21/7-day oral contraceptive pill. Lybrel is a 20-μg ethinyl estradiol pill with 0.09 mg of levonorgestrel. It can be taken 365 days per year. There is no placebo or pill-free interval. The Pearl Index for efficacy is 1.55 per 100 user-years.[33] There appears to be no differences in pregnancy rates between Lybrel and a 20-μg ethinyl estradiol per 0.1 mg levonorgestrel pill.[34] Sixty percent of women are amenorrheic by the year's end. Breakthrough bleeding is common, with 40% of women complaining of breakthrough bleeding at the third pack, decreasing over time to 21% at pack 13.[33] So, a large percentage of women on Lybrel are amenorrheic, and those who are not will likely have unscheduled bleeding. Once Lybrel is discontinued, 99% of women will resume regular menses within 90 days.

Seasonale was FDA approved in 2003 as a 30-μg ethinyl estradiol and 0.15 mg levonorgestrel 84-day pill regimen with 7 days of placebo every 3 months. Patients have four withdrawal bleeds per year with 91-day cycles. A randomized control trial[35] comparing Nordette, which is the same hormonal dosing but in a traditional 21/7-day regimen, to Seasonale showed that Nordette had a higher continuation rate at 1 year, and an improved acceptability of bleeding patterns. Both have high compliance, however, and the Pearl Index is 0.6 for Seasonale and 1.78 for Nordette, rendering Seasonale a more effective contraceptive. Total number of bleeding days is decreased with Seasonale but there is increased breakthrough bleeding in the first 3 months. Its safety profile approximates that of other hormonal contraceptive pills with no unexpected laboratory changes, no lipid changes, and no thromboembolic events in greater than 1000 cycles of Seasonale use. Seasonique[36] is a 30-μg ethinyl estradiol pill with 0.15 mg of levonorgestrel for 84 days, followed by 7 days of 10 μg of ethinyl estradiol. This pill allows for four withdrawal bleeds per year and has a Pearl Index of 0.78. Unscheduled bleeding decreased with time in clinical trials, and scheduled bleeding occurs for about 2 to 3 days in duration. The reasoning behind these newer formulations is to increase efficacy and theoretically decrease side effects associated with the pill-free period. Shorter hormone-free intervals suppress the hypothalamic-pituitary axis more consistently.[37] Studies have demonstrated less folliculogenesis in the extended cycle regimen.[38] A randomized control trial[39] was done to compare a 21/7-day pill consisting of 20 μg of ethinyl estradiol and 100 μg of levonorgestrel versus a 20-μg ethinyl estradiol per 150-μg desogestrel regimen, followed by two placebo pills, followed by five pills of 20 μg of ethinyl estradiol versus 28 solid days of 20 μg ethinyl estradiol and 150 μg desogestrel. This trial had three arms and examined the traditional pill-free interval versus estrogen alone in place of a pill-free interval and demonstrated a statistically significant decrease in folliculogenesis on ultrasound and lower FSH and LH levels during the pill-free interval. A Cochrane review[40] of traditional 21/7-day administration versus continuous administration showed no difference in efficacy, no difference in safety profiles, high compliance rate, and high satisfaction rate. Continuous administration was in this review associated with a decrease in headaches, genital irritation, feelings of fatigue, bloating, and menstrual pain. Mood, headache, and pelvic pain scores improved with continuous hormonal contraception. In women who had undergone ablation for endometriosis and then failed traditional 21/7-day low-dose monophasic contraception for suppression of pain, there was significantly reduced frequency and severity of pain on continuous low-dose monophasic contraceptives for up to 24 months. Continuous oral contraceptive pills (OCPs) suppress both the ovary and the endometrium to a greater extent than cyclic OCPs and have been shown to have beneficial effects on both pain and mood symptoms related to menstruation.[41] It is important to note that although the FDA has approved these three formulations for extended use, many physicians have been using extended cycle regimens for their patients for years. Most monophasic hormonal contraceptive pills will produce acceptable results when used continuously.

Side Effects of Continuous Cycle Oral Contraceptives

There are limited long-term data to answer the question of adverse outcomes in continuous hormonal contraception. The longest study is a 2-year study on Seasonale.[42] What these data do show are that unexpected bleeding and spotting decrease with length of use: this is an important counseling point for any woman considering use of extended cycle OCPs. The incidence of thromboembolism is not well known. There has only been one reported case.[35] No endometrial hyperplasia or cancers were reported in these clinical trials.[35,42] In terms of lipid profiles, triglycerides and low-density lipoproteins (LDLs) were similar in range for women on continuous hormonal contraception as those who were cycling.[35,36] In one study, there were five cases of women with cholecystitis or cholelithiasis, but it is unclear if this was due to other risk factors.[38] Return to fertility appears to be fairly prompt after continuous hormonal oral contraception is discontinued.[34,38,42] The study of Lybrel[34] demonstrated that 99% of women were ovulating within

90 days of discontinuing the 365-day per year pill. Ninety-two percent ovulated within 60 days, and 38.5% ovulated within 30 days of discontinuing the oral contraceptive.

Metabolical Effects

The use of synthetic steroids and combination hormonal contraceptive formulations has multiple metabolical effects. The vast majority of these effects, including effects on lipids, carbohydrate metabolism, and other hormonally responsive organs, such as the breasts, are generally transient and self-limited and minor. Rarely, however, these other effects can result in serious consequences. The majority of the serious side effects associated with hormonal contraception are cardiovascular events that are related to the effects that estrogen has on the coagulation system.

Venous Thromoembolism

The increased cardiovascular events including venous thromboembolism and arterial thromboembolism appear to be due to coagulation-mediated effects, not atherosclerosis. Epidemiological data have shown us that the risk of venous thromboembolism (VTE) is dose-dependent with the amount of estrogen in the oral contraceptive formulation.[43] The background rate of venous thromboembolism in women of reproductive age is thought to be about 0.8 per 10,000 women-years. Recent data,[44] however, have demonstrated that the true prevalence of VTE in the population of women is difficult to elucidate. Deep venous thromboembolism (DVT) in particular is often silent and self-limited. Given that symptomatic venous thromboembolism is clearly a rare event, but potentially also asymptomatic and therefore underreported, background risk is hard to discern. This should be kept in mind at all times given the reporting bias that may be present when venous thromboembolism is attributed to the use of oral contraceptives.

Several observational studies have been performed to determine the occurrence of VTE in users of oral contraceptive containing formulations with less than 50 μg of estrogen[45-47] These studies were consistent and showed that the risk of DVT was increased approximately three- to fourfold among women using oral contraceptives compared with women not using oral contraceptives. Thus, the use of oral contraceptives with less than 50 μg of estrogen is still associated with about a threefold to fourfold increased risk of VTE compared with a nonpregnant population who are not taking oral contraceptives, but about a 50% reduction in risk of VTE compared with a pregnant or recently postpartum population.[47]

Several epidemiological papers have similarly considered the impact of the different progestin preparations on VTE risk.[48-52] Many of these use insurance-claims databases to address the questions, and one used a prospective study design. In young healthy women overall, the risk of VTE is low at 1 to 13 events per 10,000 women. When compared with pregnancy and the postpartum period, the benefits of combined oral contraceptive (COCs) outweigh the risks. In the 1980s, disproportionate concern about the risk of VTE associated with COCs caused many women in the United Kingdom to discontinue their COCs and was associated with an increase in unintended pregnancy and induced abortions. While there may be debates about the very small potential differences in risk among hormonal contraceptive preparations, the clear message should be that as a class, hormonal contraception is safe and promotes health.[53]

Stroke and Myocardial Infarction

The use of 50-μg oral contraceptive pills did appear to have an impact on the coagulation system. This was due to an increase in clotting factors, such as factors V, VIII, X, and fibrinogen. Studies that addressed this question in the group taking COCs of 35 μg and less have not demonstrated clearly an increased risk of clotting related events in women using pills at this dose.[54,55] The incidence of a cardiovascular event is not associated with duration of oral contraceptive (OC) use.[56] There is no increased risk of cardiovascular morbidity among past-users of OCs.[57] Most of the studies that have shown an increased risk of myocardial infarction have been observational studies in which the women at risk for myocardial infarction (MI) also had other risk factors that cause arterial narrowing, such as preexisting hypercholesterolemia, hypertension, diabetes mellitus, or smoking more than 15 cigarettes per day. Smoking is clearly thrombogenic in and of itself,[58] and smoking is an independent risk factor for MI.[54] It appears, however, that the use of low-dose oral contraceptives by heavy cigarette smokers significantly enhances their risk of experiencing an MI because these two factors act synergistically.[54] Prior oral contraceptive use in a smoker and prior smoking in an oral contraceptive user does not seem to be associated with an increased risk of MI. Both the Royal College and the WHO studies report that the risk of MI in oral contraceptive users was severalfold greater if they had hypertension than if they did not. Two large case control studies in the United States have shown no significantly increased risk of MI in oral contraceptive users.[55,59] A recent World Health Organization (WHO) technical report stated that women who do not smoke and have their blood pressure checked and who do not have hypertension or diabetes have no increased risk of MI if they use combined oral contraceptives, regardless of their age.[60]

Exogenous estrogen does increase the synthesis of several coagulation factors that promote thrombosis in a dose-dependent manner. The small increased risk of VTE exists in all women using combined oral contraceptives (COCs). The risk of MI or stroke in nonsmoking, normotensive women is not increased with COC users. However, in women with preexisting medical problems, the risk of thrombosis may be further increased by simultaneous use of combined hormonal contraceptives. The potential risk of stroke with combined hormonal contraceptives is even more difficult to assess than the risk of MI or DVT. Early studies in the 1970s of higher dose (50 μg) combined hormonal contraceptives did suggest a potential relationship between this dose of pill and stroke. However, even these data were conflicting. Data on early formulations of pills do not apply to the contemporary pills. Women without other risk factors do not appear to have an increased risk of stroke while on oral contraceptive pills.[61,62] The safety of pills with doses under 35 μg of estrogen in women who smoke or have other thrombogenic medical problems, such as hypertension with end-organ disease, is not known because women with these risk factors are rarely on combined hormonal contraceptives today.

Other Metabolical Effects

Although combined hormonal contraceptives do have metabolical effects that are detected at the laboratory level, such effects are very rarely associated with statistically significant or concerning outcomes. Table 36.3 shows the chemical and clinical effects that estrogens and progestins have on the human metabolical system.

Mood Changes

The 19-nortestosterone derivatives do have central nervous system effects and estrogen does as well. It was postulated that high doses of synthetic estrogens would replace changes in luteal depression. No study to date has demonstrated an increase in depression due to estrogen at the dosing currently used in combined oral contraceptives.[63,64] However, the progestin component may be associated with an increase in irritability, fatigue, and depression.

Hepatic Proteins

Synthetic estrogens in oral contraceptives cause an increase in hepatic production of several proteins and these include sex hormone–binding globulin (SHBG) and globulins that are part of the coagulation cascade, such as factors V, VIII, and X and fibrinogen.[65] Progesterone and progestins decrease the synthesis of SHBG. The balance between these effects make one pill more appropriate for, for example, a woman with hyperandrogenicity due to polycystic ovary syndrome over another.

Increased angiotensin may be the mechanism by which blood pressure can become elevated. This seems to be an estrogen-driven effect, because there an increase in mean blood pressure in women who ingest oral contraceptive pills levels, but this is lower in women who ingest formulations with 30 to 35 μg of ethinyl estradiol. Women who use oral contraceptive pills are slightly more likely to develop hypertension and about 0.4% of current low-dose oral contraceptive users became hypertensive in one study. Women who receive progestins without an estrogen component do not have an increase in blood pressure over time.[66]

Obesity

Obesity and its accompanying comorbidities are rapidly increasing worldwide. Obesity alone does not appear to be protective against unintended pregnancy. Furthermore, pregnancy in obese women increases risks for obstetrical and neonatal complications. For this reason, appropriate contraception is important in the obese population in order to help reduce unnecessary risk of stillbirth neonatal death, cesarean delivery, endometritis, and thromboembolism—all of which appear to be increased in pregnancy in obese women.

Obesity may decrease the effectiveness of some contraceptives. It has been shown that obesity can affect how a person metabolizes any medication, including hormonal contraceptives.[67-69] It remains unclear if these pharmacokinetic differences are substantial enough to result in unwanted pregnancies from contraceptive failure. A recent systematic review demonstrated that there was a higher failure rate among women with increased body weight and/or increased BMI using the following methods: certain combined oral formulations, the transdermal patch, a progestin-only vaginal ring, and the six-rod levonorgestrel implant.[70]

Table 36.3 Metabolical Effect of Contraceptive Steroids

Steroid	Chemical Effects	Clinical Effects
Estrogen: Ethinyl Estradiol		
Proteins		
Albumin	↓	None
Amino acids	↓	None
Globulins	↑	
Angiotensinogen		↑ Blood pressure
Clotting factors		Hypercoagulability
Carrier proteins (CBG, SHBG, TBG, transferrin, ceruloplasmin)		None
Carbohydrate		
Plasma insulin	↑	None
Glucose tolerance	↓	None
Lipids		
Cholesterol	↑	None
Triglyceride	↑	None
HDL cholesterol	↑	? ↓ Cardiovascular disease
LDL cholesterol	↓	? ↓ Cardiovascular disease
Electrolytes		
Sodium excretion	↓	Fluid retention
Vitamins		Edema
Vitamin B complex	↓	None
Ascorbic acid (vitamin C)	↓	None
Vitamin A	↑	None
Other		
Breast	↑	Breast tenderness
Endometrial steroid receptors	↑	Endometrial hyperplasia
Skin	↓	↓ Sebum production
		↑ Facial pigmentation
Progestins: 19-Nortestosterone Derivatives		
Proteins (SHBG)	↓	None
Carbohydrate		
Plasma insulin	↑	None
Glucose tolerance	↓	None
Lipids		
Cholesterol	↓	None
Triglyceride	↓	None
HDL cholesterol	↓	? ↑ Cardiovascular disease
LDL cholesterol	↑	? ↑ Cardiovascular disease
Other		
Nitrogen retention	↑	↑ Body weight
Skin—sebum production	↑	↑Acne
CNS effects	↑	Nervousness, fatigue, depression
Endometrial steroid receptors	↓	No withdrawal bleeding

↑, increases; ↓, decreases; ?, questionable; CBG, corticosteroid-binding globulin; CNS, central nervous system; HDL, high-density lipoprotein; LDL, low density lipoprotein; SHBG, sex hormone–binding globulin; TBG, thyroid-binding globulin.

A case control study of 454 patients age 15 to 45 with a first episode of venous thromboembolism (VTE) revealed that the combined effect of obesity and oral contraceptive pills incurred a tenfold increased risk of VTE among women with a BMI greater than 25 kg/M^2. This risk increase was 2 to 4.6 times higher than in patients with only one risk

factor. On these data, some organizations, specifically the Royal College of Obstetricians and Gynecologists, advised that the risk of oral contraceptives generally outweigh the benefits for women with BMI of 35 to 39 kg/M^2 and that oral contraceptive pills that are combined should not be used in women with a BMI of 40 kg/M^2 or more.[71,72]

Oral Contraceptives and Malignancies

Given the extensive history now of oral contraceptive pills and our ability to follow populations of women who used these pharmaceuticals in their reproductive years, both prospective cohort and retrospective case control designs have been used to assess the relationship between OCs and cancer risk. In general, hormones are considered promoters but not initiators of cancer, so the oncological effects, if any, of steroids should be seen as a dose response and may or may not be related to duration of use.

A study examining cancer incidence in relation to OC use in the Oxford Family Planning Association contraceptive study included 17,032 women recruited at family planning clinics ages 25 to 39 years old between 1968 and 1974, who were using OCs, a diaphragm, or an intrauterine device. Breast cancer findings (844 cases) were very reassuring (relative risk ratio [RR][45] comparing women ever using OCs with those never doing so 1.0, 95% confidence interval [CI] 0.8 to 1.1). There was a strong positive relationship between cervical cancer incidence (59 cases) and duration of OC use (RR comparing users for 97+ months with nonusers 6.1, 95% CI 2.5 to 17.9). Uterine body cancer (77 cases) and ovarian cancer (106 cases) showed strong negative associations with duration of OC use: RRs for 97+ months of use were 0.1 (95% CI 0 to 0.4) and 0.3 (95% CI 0.1 to 0.5), respectively. This apparent protective effect for both cancers persisted more than 20 years after stopping OCs.[73]

Ovarian Cancer and Benign Ovarian Neoplasms

Over 20 reports of oral contraceptives and the subsequent evolvement of ovarian cancer have been published. Eighteen of these have found a reduction in risk in the most common of epithelial ovarian cancers (Fig. 36.4). The magnitude of the risk reduction appears to be linked to the duration of time of use of the oral contraceptive pill.[74] A large case control study has demonstrated that not only is the duration of oral contraceptive use related to the protective effect, but that this risk reduction lasts for years after the cessation of oral contraceptive use.[73] Oral contraceptives appear to also significantly reduce the incidence of benign ovarian neoplasms, including serous and mucinous cystadenomas, benign cystic teratomas, fibromas, and endometriomas.[75] Endometrial cancer has similarly been well studied in relation to oral contraceptive use. Twelve retrospective studies have suggested an association between oral contraceptive use and endometrial cancer risk reduction.[76] One study has shown that oral contraceptive users have one-third the risk of endometrial cancers as nonusers and this likewise persists after cessation of use, and the duration of oral contraceptive use seems to correlate with the amount of risk reduction found.

Breast Cancer

Multiple studies have tried to address the concern that estrogen might stimulate the growth of neoplastic breast tissue. One study of over 53,000[77] women did show that there is a slightly increased risk (RR 1.24, 95% CI 1.15 to 1.3) of women who are actively using oral contraceptives developing breast cancer. This association appears to decline after stopping OCs and was no longer present after 10 years of use. These cancers appear to be less advanced clinically than the cancers that occur in non-OC users. It is possible that breast cancer is diagnosed earlier in OC users than in nonusers, but this could also be due to a biological effect of the OCs in terms of tumor promotion. New data demonstrate that OCs do not further increase risk for women with a family history of breast cancer and may be associated with a reduction in risk of breast cancer.[78,79]

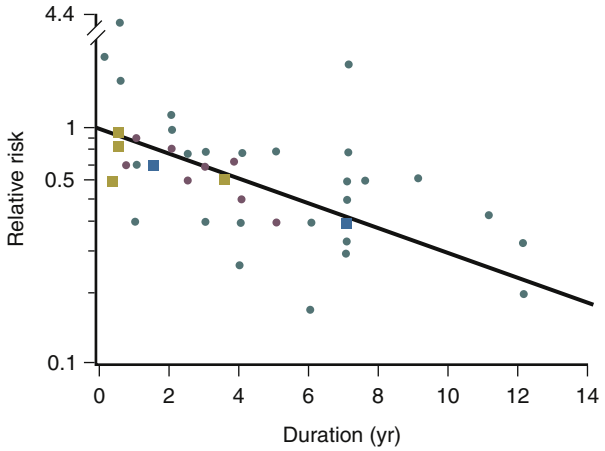


FIGURE 36.4 Relative risk of ovarian cancer associated with different durations of oral contraceptive use: Findings of 15 studies. Study categories, indicating category weights ranging from smallest (weight in bottom 25% of range) to largest (weight in top 25% of range): tan squares, 1 (smallest); blue squares, 2; purple dots, 3; green dots, 4 (largest). *(From Hankinson SE, Colditz CA, Hunter DJ, et al. A quantitative assessment of oral contraceptive use and risk of ovarian cancer.* Obstet Gynecol *80:708–714, 1992.)*

Postcoital Contraception

Postcoital contraception, also known as emergency contraception or the "morning after pill," is the use of a contraceptive method after unprotected acts of intercourse. For women who have had known unprotected intercourse, meaning they were not using a primary method of contraception or in whom a barrier method may have failed, postcoital contraception provides a second opportunity to decrease the risk of pregnancy from a midcycle act of intercourse. Both hormonal methods and the ParaGard IUD can be used as effective postcoital contraception. The original methods of emergency or postcoital contraception were developed by using high doses of estrogen compounds. Examples of such compounds include diethylstilbestrol 25 to 50 mg per day, ethinyl estradiol 5 mg per day, or conjugated estrogens 30 mg per day. With these agents, treatment was continued for 5 days. If treatment was initiated within 72 hours of an isolated midcycle act of intercourse, the efficacy was estimated at 75%.[80] If more than one act of intercourse had occurred, or if the treatment was delayed, the efficacy was decreased.

The side effects associated with these high doses of estrogenic compounds led to the development of an alternative approach tested in Canada in the 1970s and now known as the Yuzpe method. This is a combination oral contraceptive method of postcoital contraception that initially consisted of using the Ovral oral contraceptive pill, using a total of four tablets of ethanyl estradiol (0.05 mg) and DL-norgestrel (0.5 mg). These are given in two doses of two pills each, 12 hours apart. Studies[81,82] demonstrated that this regimen had a similar degree of efficacy and decreased adverse side effects when compared with the 5 day estrogen regimens. Pooled data from studies on the Yuzpe method from 1977 until 1993 that include 5226 women demonstrated a failure rate of about 1.5%.[83] Demographers estimated that the Yuzpe regimen prevents about 75% of expected pregnancies when used within 72 hours of unprotected intercourse. A combined method of estrogen and progestins called Preven was then FDA approved as a dedicated product for postcoital contraception. This product, however, is no longer on the market in the United States.

More recently, a progestin-only method of postcoital contraception has been developed and is now available in a dedicated product called Plan B. The initial Plan B product consisted of two tablets of 0.75 mg of levonorgestrel. The product labeling indicates that the first pill should be taken at time 0 and the second 12 hours later. The use of levonorgestrel alone has been compared directly to levonorgestrel plus estradiol in a randomized control trial.[81] Failure rates were similar when these two regimens were ingested within 48 hours of unprotected intercourse, each about 2%. Substantially less nausea and vomiting was noted with the levonorgestrel-alone method.[82,84] Given the comparable efficacy and improved side effect profile, progestin-only methods of postcoital contraception are now preferable. A randomized control trial performed by the World Health Organization[84] that included 2000 women in 21 centers evaluated the efficacy of levonorgestrel alone within 72 hours after a single act of unprotected intercourse. In this trial, the 1.1% pregnancy rate demonstrated superior efficacy over the Yuzpe method, which was associated with a 3.2% pregnancy rate. The estimates calculated by the authors demonstrated that 85% of pregnancies were prevented with levonorgestrel alone compared to 57% of pregnancies prevented with the combined regimen. Decreased nausea and vomiting were once again demonstrated with levonorgestrel alone.

Effectiveness is greatest with any method of postcoital contraception when it is given within 24 hours of sexual intercourse than in the subsequent 48 hours. Randomized trials have shown that taking both the 0.75 mg tablets of levonorgestrel as a single dose offers the same efficacy without increasing side effects as taking each 12 hours apart. Thus, Plan B One-Step was developed and marketed as a dedicated product containing 1.5 mg of levonorgestrel in a single pill. In addition, though original studies all tested the efficacy of emergency contraceptive regimens within 72 hours of unprotected intercourse, more recent studies have demonstrated efficacy up to 120 hours after intercourse.[85,86] As stated above, the efficacy declines with increasing time from the unprotected intercourse event. Patients should thus be counseled that emergency contraception can be taken within 120 hours of unprotected intercourse but that it is a time-sensitive drug with maximum efficacy resulting when the intervention is used as early as possible.

Advanced prescription or advanced supply of Plan B has been shown to increase its use among various subgroups of women, including adolescents, and has not been shown to decrease use of the primary method of contraception.[87] Advance prescription of Plan B should be standard of care given its potential to help prevent unintended pregnancy as a backup method. Multiple studies have found no adverse effects in terms of either increased risk taking or decreased contraceptive use. In the United States, Plan B was first made available over the counter for women above the age of 18 in August 2006. In April of 2009, the over the counter access was expanded to include women above the age of 17. There is no reason to suspect that levonorgestrel is unsafe for women younger than the age of 17.[88] As teens are at particularly high risk for unintended pregnancy, advance prescription of emergency contraception should be encouraged for all young women using barrier method contraception or daily methods of contraception. Reproductive health care providers should discuss the availability of postcoital contraception with all patients seeking pregnancy prevention as its use may be necessary in any woman not using a long-term method of contraception (intrauterine device, implant, or sterilization).

The exact mode of action of emergency contraception has not yet been established. Initially it was thought that the estrogen and progestin combination alter the endometrium to prevent implantation of the fertilized ovum.[89,90] More recent studies, however, have suggested that emergency contraception may work through delaying ovulation, or, if taken post ovulation, through the interference of ovum transport or through impairment of corpus luteum function.[91,92] However, the data to support any single mechanism of action with postcoital contraception is inadequate at this point; thus, it is likely a combination of effects that accounts for its efficacy.

The most recent addition to the postcoital contraception armamentarium is ulipristal acetate, marketed in the United States as Ella. Ulipristal is a selective progesterone receptor modulator with primarily antiprogestin activity that is highly effective for use as emergency contraception. Antiprogestins may be more effective than progestins due to their ability to both delay ovulation and inhibit implantation.[93] Ulipristal can delay ovulation by as much as 5 days; of note, ulipristal appears to be effective in the advanced follicular phase even after LH levels have begun to rise, a period when levonorgestrel is no longer effective in inhibiting ovulation. A meta-analysis of two trials of ulipristal versus levonorgestrel for emergency contraception found that ulipristal was at least or more effective at all time intervals (0 to 24 hours, 0 to 72 hours, and 0 to 120 hours).[94] For each time interval, the rate of pregnancy in patients receiving ulipristal ranged from 0.9% to 1.4% versus 2.2% to 2.5% in patients receiving levonorgestrel; these differences were statistically significant. There was no difference in the side effect profile of the two drugs. Ella is given as a single dose of 30 mg ulipristal acetate and can be taken within 120 hours of unprotected intercourse. Currently, Ella is only available by prescription in the United States.

Several studies have found that the insertion of a copper IUD within 5 to 10 days of midcycle unprotected

intercourse is an effective method to prevent pregnancy.[95,96] One such study included nine different trials involving almost 900 women who had received an IUD in the postcoital midcycle. Only one pregnancy occurred in this aggregated group, for a pregnancy rate of approximately 0.1%. Thus, based on studies to date, the insertion of a copper IUD is the most efficacious method of postcoital contraception. Benefit can occur up to 10 days after unprotected midcycle intercourse. The copper IUD is a good option for women who also desire an IUD for long-term contraception after an episode of unprotected intercourse.

Transdermal Contraceptive System

Approved for use in the United States in 2001, the transdermal contraceptive system (TCS) consists of a 20-cm^2, beige-colored, three-layered matrix patch that releases 150 μg of norelgestromin (the active metabolite of norgestimate) and 20 μg of ethinyl estradiol at a constant rate each day into the systemic circulation.[97] The three layers of the thin matrix consist of an outer protective layer of polyester, a medicated adhesive middle layer, and a polyester inner liner that is removed just prior to application to the skin.

Circulating blood levels of each steroid remain relatively constant during the 7 days the patch is scheduled to be in place, as well as for an additional 2 days, in contrast to the peaks and troughs that occur after ingestion of an oral contraceptive pill (Figs. 36.5 and 36.6).[98] In a pharmacokinetic study,[97] steady-state conditions of each steroid were achieved in the serum of women using the patch for three cycles. Steady-state serum concentrations increased only slightly from the first week of the first treatment cycle until the third week of the third treatment cycle, indicating minimal accumulation. The circulating levels of norelgestromin are sufficient to inhibit ovulation while the patch is in place.

The patches are applied to the skin of the abdomen, buttocks, upper arm, or upper torso (except for the breasts) for 1 week and then removed. A patch is applied to a different site for 3 consecutive weeks; no patch is applied during the fourth week to allow withdrawal bleeding to occur. Withdrawal bleeding similar in duration to that which occurs with OC use usually begins 2 to 3 days after the last patch is removed.

Contraceptive effectiveness of the TCS is similar to that for OCs. In a pooled analysis from three large clinical studies, the overall pregnancy rate from more than 22,000 treatment cycles was 0.88 pregnancies per 100 woman-years.[99] In these studies the pregnancies were uniformly distributed among the 3236 subjects with body weight less than 198 pounds, but 5 pregnancies occurred among the 83 subjects weighing 198 pounds or more. Thus, the TCS appears to have a lower level of effectiveness in women with body weight of 198 pounds or more.

A randomized trial demonstrated that the incidence of perfect compliance with the TCS, 88.2% of cycles, was significantly better than with the OC, 77.7% of cycles.[100] The convenient dosing schedule of the patch likely enhances compliance and may result in greater effectiveness than the OC in actual use. In the clinical trials during which 70,552 patches were worn, the rate of replacement for complete detachment of the patch was 1.8%, and for partial detachment was 2.9%.[101] Various conditions of exercise, heat, and humidity did not increase the incidence of detachment or the force required to remove the patch.[102]

Adverse effects were analyzed in the comparative study with the OC. The patch had a higher incidence of intermenstrual spotting and breast tenderness than the OC in the first two cycles of use but not thereafter.[100] About 20% of women using the TCS noted intermenstrual spotting and breast discomfort in the first cycle of use. Application site reaction also occurred in 20% of the subjects using the TCS. Other adverse effects, such as headache and nausea, were similar with these two methods of steroid contraception.[100] Thus, the TCS is an effective reversible alternative method of steroid contraceptive.

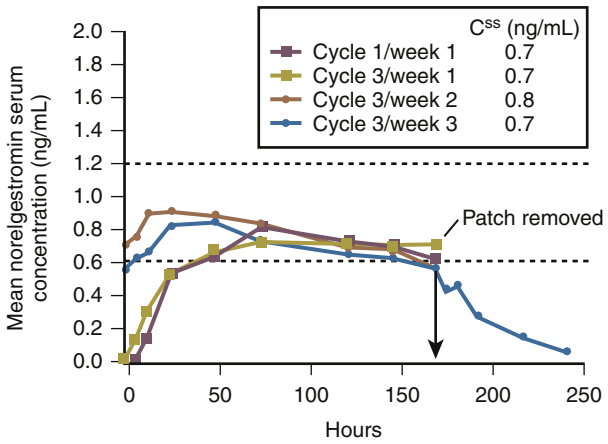


FIGURE 36.5 Mean serum concentration-versus-time profile of norelgestromin after patch application on the buttock for three consecutive cycles. *Dashed horizontal lines* indicate the reference range. Cˢˢ, steady-state concentrations. *(From Abrams LS, Skee DM, Natarajan J, et al. Multiple-dose pharmacokinetics of a contraceptive patch in healthy women participants.* Contraception *64:287–294, 2001.)*

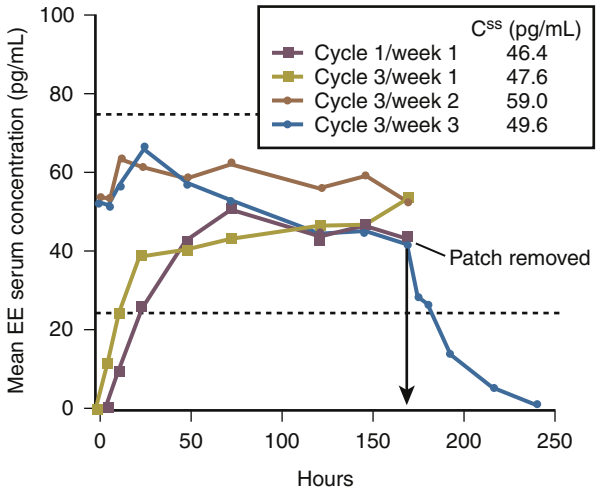


FIGURE 36.6 Mean serum concentration-versus-time profile of ethinyl estradiol (EE) after patch application on the buttock for three consecutive cycles. *Dashed horizontal lines* indicate the reference range. Cˢˢ, steady-state concentrations. *(From Abrams LS, Skee DM, Natarajan J, et al. Multiple-dose pharmacokinetics of a contraceptive patch in healthy women participants.* Contraception *64:287–294, 2001.)*

Venous Thromboembolism Risk

In 2006, the U.S. product labeling for the TCS was revised to include a warning that the TCS provides a higher steady-state concentration of ethinyl estradiol than OCs and lower peak concentrations. Pharmacokinetic studies reveal that steady-state levels are approximately 60% higher than an OC containing 35 μg of ethinyl estradiol while the peak concentration on the TCS is 25% less than that in women taking OCs (Ortho Evra [norelgestromin/ethinyl estradiol transdermal system], Raritan, NJ; Ortho-McNeil Pharmaceuticals, Inc, 2006).

It is not clear whether this difference in pharmacokinetics translates to a change in the risk of serious adverse events, such as venous thromboembolism. Two studies on this subject have been reported to date. The first was a case-control study with data abstracted from a managed-care database; 68 new cases of idiopathic VTE in TCS users were identified, and when compared to control subjects, the odds ratio (OR) was 0.9 (95% CI 0.5 to 1.6), demonstrating a similar risk for both methods.[103] A second study performed from insurance claim outcomes from a major national health insurer reported a more than twofold increase in the VTE rate (adjusted incidence rate ratio 2.2, 95% CI 1.3 to 3.8) among TCS users compared to norgestimate-containing OC users. Acute myocardial infarction and ischemic stroke were also investigated in this study, but each had too few outcomes to provide conclusive results.[104] The story here is similar to that of the studies on VTE risk with differing progestins. The counselor should keep in mind that the risk of VTE on either TCS or OCs remains lower than the risk of VTE during pregnancy, and if the patient and health care provider believe that the TCS is the best way to prevent pregnancy, it use is certainly warranted.

Continuous Regimens or Extended Use

The TCS had also been studied to evaluate for extended use to delay or prevent menses. A study performed to evaluate the bleeding profile and satisfaction on a continuous regimen compared 158 women using an extended regimen (weekly application for 12 weeks, followed by 1 week off, then 3 more weeks) to 81 women using the usual cyclic regimen (weekly application for 3 weeks followed by 1 week off, continued for four cycles). Extended use resulted in fewer bleeding days overall, with delayed median time to bleeding of 54 days. Women were equally satisfied with both regimens.[105] Recent data suggest that continuous use may result in fewer contraceptive failures that traditional 21 to 7 day use.[106]

Contraceptive Vaginal Ring

The vaginal contraceptive ring contains ethinyl vinyl acetate that releases ethinyl estradiol and the third-generation progestin, etonorgestrel. The ring currently on the market has an outer diameter of 54 mm and a cross-sectional diameter of 4 mm. It was approved by the FDA in 2001. The vaginal contraceptive ring is highly efficacious with a Pearl Index of 1.18 and an efficacy of 99.1%. The vaginal ring is a flexible, soft device and is available in one size only and does not need to be fitted. If desired, the ring can be removed for up to 3 hours without altering the contraceptive effectiveness. In a review of 12 randomized controlled trials comparing the contraceptive vaginal ring and combined oral contraceptives, both forms achieved the same contraceptive efficacy and adequate ovarian suppression with comparable incidence of adverse effects, such as breast tenderness, headache, and nausea. Vaginal administration was associated with higher local adverse events such as leukorrhea, vaginal discomfort, vaginitis, foreign body sensation, expulsions, and coital problems, leading to higher discontinuation rates. Overall, both contraceptives improved overall sexual function and were highly acceptable. Advantages to the ring included lower systemic estradiol exposure (50% compared to a 30-μg ethinyl estradiol pill) with a lower incidence of irregular bleeding and spotting.[107]

Injectable Contraceptives

Injectable contraceptives are used throughout the world. These include depo-medroxyprogesterone acetate (DMPA), norethisterone enanthate (NET-EN), and combined injectable contraceptives of different combinations of estrogens and progestins that are given monthly. DMPA is the only currently available injectable contraceptive in the United States. It is used by approximately 3% of women of reproductive age.

Medroxyprogesterone Acetate

Medroxyprogesterone acetate (MPA) is a 17-acetoxyprogesterone compound. Unlike most other synthetic progestins, MPA is structurally most closely related to progesterone, instead of testosterone, and possesses less androgenic activity. MPA is rapidly metabolized in humans to progesterone. The 3-month injectable dosage is 150 mg. The drug is delivered by intramuscular injection into the deltoid or gluteus muscle and is released slowly into the circulation.

MPA is an extremely effective contraceptive with a 1-year pregnancy rate of 0.1% and a 2-year accumulative pregnancy rate of 0.4%. Its primary mechanism of contraceptive action is ovulation inhibition, but it works by increasing the cervical mucus viscosity and creating progestational changes in the endometrium as well.

Mechanisms of Action

With consistent use, DMPA is highly effective, with a failure rate of approximately 0.4% per WHO clinical trial.[108] DMPA requires re-dosing every 3 months intramuscularly. The contraceptive levels are maintained for at least 14 weeks and may be metabolized differently in different women. There is a margin of safety built into the dosing regimen of intramuscular DMPA. Unlike other lower dose progestin-only methods, DMPA works primarily by blocking the LH surge and ovulation. FSH is not systematically suppressed with progestin-only methods. In addition to LH blockade, the progestin-dominant hormonal milieu results in thickening of the cervical mucus and alteration of the endometrium. Estrogen levels are comparable to those in early follicular phase of noncontracepting women. Because of this, symptoms of estrogen deficiency, such as vasomotor symptoms or vaginal atrophy, are not present with this method of contraception. In order to obtain suppression of ovulation in the initial injection cycle, DMPA must be administered within several days after the onset of menses.[109] Studies show that if DMPA is administered as late as

9 days after the onset of menses, 2 out of 13 women showed evidence of ovulation.[110] It is recommended in the package labeling that the first dose be given within the first 5 days of the onset of menses and evidence supports giving DMPA no later than 7 days after the onset of menses.

Pharmacokinetics

DMPA reaches therapeutic levels within 20 minutes of intramuscular injection.[111] Effective blood levels are found at greater than 0.5 ng/mL and this occurs within 24 hours after injection. MPA appears to be cleared slightly differently depending on the assay used in the study. MPA levels plateau at about 1 to 1.5 ng/mL and remain at that level for approximately 3 months, after which they begin to decline. In some subjects it appears that MPA levels remain detectable in the circulation for 7 to 9 months after initial injection. Estradiol levels in these subjects are found to be in the range of the early follicular phase during these times. However, other studies demonstrated that MPA levels decreased to less than 0.5 ng/mL after 4 to 6 months of use and one study demonstrated a decrease in circulating levels at 13 weeks of use.[112]

The pharmacodynamics of MPA may be variable based on individual metabolism or based on the study assay used, and further studies are needed to improve our understanding of this. However, no study has shown metabolical clearance of therapeutic levels of the drug before 12 weeks after the initial dose, and this supports the current product labeling.

Return to Fertility

The lag time in the clearing of MPA from the circulation results in delayed resumption of ovulation for women in the population.[113] This quality is a by-product of the injectable delivery system. Women who use MPA should be counseled that there will be a delay in the resumption of fertility. This, however, is not a permanent effect. Ninety percent of MPA users become pregnant by 18 months after the last injection.

Disadvantages of MPA Use

Women who use MPA will experience unscheduled bleeding during the early months of use. Perhaps most important, women who experience breakthrough bleeding on MPA should be reassured that this is a well-understood side effect of use of MPA. Weight changes are another disadvantage of MPA use. Other studies have shown longitudinally that MPA users may gain between 3.3 and 8.8 lbs (1.5 and 4 kg) in the first year of use.[24] The weight gain associated with MPA may also have some individual risk factors such as tendency to gain weight and ethnic background. One study demonstrated a statistically significant increase in weight gain among Navajo women randomized to MPA versus combined hormonal contraceptives. The metabolical effects of MPA on body weight are unclear, and weight and exercise should be evaluated in all women who are concerned about weight gain on MPA before the method is discontinued.

There have been no randomized control trials to assess the effects of DMPA on mood. There have, however, been several observational studies that demonstrate that the clinically significant difference in mood is rare with MPA.[114]

Headaches are the most common reason cited for discontinuation of MPA in the first year. There is no conclusive evidence to suggest that the use of DMPA increases the severity of tension of migraine headaches and the presence of headaches is not a contraindication to initiation or continuation of MPA use. However, if women find that their headache incidence increases while on this method, counseling regarding the benefits of perhaps changing to another method should be initiated.

Metabolical Effects of DMPA

DMPA does not increase liver production of proteins as the estrogen component of combined hormonal contraception does. Therefore, there is no evidence to suggest that clotting factors are increased with DMPA use. A WHO study also reported that blood pressure measurements are unchanged with DMPA users over 2 years.[115] Injectable progestins do not appear to be associated with increased risk of VTE, MI, or stroke. This makes DMPA a very desirable method of contraception in women with comorbidities, in an aging cohort, or in potentially obese women because these women may all have other risk factors for venous or arterial thromboembolic events.

Bone Mineral Density

The FDA recently placed a black box warning on MPA labeling which stated that MPA has been shown to be associated with a decrease in bone mineral density in women with long-term use. Many studies have demonstrated that bone mineral density is decreased in women on MPA.[116-118] No studies, however, demonstrated that MPA users are at increased risk of a fracture.[117,119] Furthermore, the use of a DEXA (dual energy x-ray absorptiometry) scan, the test used to assess bone mineral density, may not be a valid test in the premenopausal population. Studies also suggest that the loss in bone density is likely to be entirely reversible with discontinuation of MPA. This biological effect of a progestin-dominant hormonal milieu on bone mineral density can be likened to that of a breastfeeding woman, who also will experience a transient loss in bone mineral density while breastfeeding, which appears to be reversible after breastfeeding is discontinued. For women who do well on MPA as their long-term contraceptive method, we encourage continued use for the duration of time that contraception is required. We do not recommend discontinuing the use of MPA based on concerns of bone mineral density alone. Women and adolescents who use MPA for contraception or control of menorrhagia should be encouraged to have a calcium-rich diet, or perhaps to supplement with calcium if necessary during this time. DEXA scan should not be used routinely in women who are using DMPA for contraception.

Recommendations for Initiating MPA Use

Women should be counseled about the importance of repeat dosing every 3 months of MPA, as well as about the primary side effect experience: abnormal bleeding for the first one to two injections followed by a high likelihood of amenorrhea. If these bleeding patterns are acceptable to the patient, then MPA is a reasonable method. Women who are cycling can be dosed within the first 5 days of the onset of their menses and do not require backup contraception after this point. Women can also receive MPA within the first 5 days postpartum. MPA can be used immediately after abortion or miscarriage. Women who are using lactational amenorrhea as their contraceptive method can institute

MPA at any time so long as hCG value is negative. For a woman seeking a repeat dose of DMPA beyond the 13-week period, if she is still amenorrheic and her hCG value is negative, it is appropriate to re-dose at this time. Concerns about new pregnancy should always be balanced with the understanding that pregnancies that have occurred with DMPA during DMPA use have not demonstrated any evidence of increase risk of teratogenicity in this population or on adverse pregnancy outcomes.

Combined Estrogen-Progestin Injectable Contraception

A combined method of injectable contraception is no longer on the U.S. market. However, in other parts of the world this method is available and it is a highly discreet method of contraception. Because of the combined estrogen and progestin in the injectable formulation, the bleeding irregularities that are associated with progestin-only injection (DMPA) are not experienced with this combined method. Progestin and estrogen combined injectables contain a low-dose of a long-acting progestin and a low-dose of an estradiol. The most widely used method contains 17α-hydroxyprogesterone caproate (250 mg) and estradiol valerate (5 mg). This is a commonly used method in Asia and in Latin America. Previously in the United States, a combined injection of medroxyprogesterone acetate, 25 mg, and estradiol cypionate (E_2C), 5 mg, was available under the name Lunelle, but this is not currently the case.

MPA-E_2C is a well-tolerated method of contraception with a reasonable side effect profile.[120] The amount of pregnancy data is sparse owing to the short amount of time that it was on the market. However, in clinical trials, no pregnancies occurred in 782 women over 8008 cycles.[83] Side effects and clinical effects of the injectable combined hormonal contraceptive are similar to those of oral contraceptive pills.

Spermicides and Barrier Methods

Spermicides are a nonprescription barrier method of reversible contraception. All spermicidal agents contain a surfactant, usually 1000 mg nonoxynol-9 (N-9), that immobilizes or kills sperm on contact. They also provide a mechanical barrier and can be used with barrier methods of contraception such as condoms or diaphragms; therefore they need to be placed into the vagina before each coital act. Spermicides used alone, are 75% to 85% effective in preventing pregnancy.[121] The effectiveness of these agents increases with increasing age of the woman and is similar to that of the diaphragm in all age and income groups.

There was a great deal of hope invested in the possibility that N-9 might act as an anti-infective agent as well as a spermicide, and prevent against Human Immunodeficiency Virus (HIV) and other sexually-transmitted infections (STIs). While initial *in vitro* studies were promising, clinical trials have demonstrated that N-9 containing spermicides do not provide protection against HIV or STI. According to a recent study by the WHO, N-9 appears to increase HIV transmission in high-risk women, perhaps by causing lesions and ulcerations in the genital mucosa. These effects were also found to be dose-related, so at lower doses, the gel did not have a protective or promotive effect on HIV transmission or disruptions in the vaginal ecology.[122] N-9 does not appear to effect transmission of other STIs, such as gonorrhea or chlamydia.

Microbicides

Microbicides are products in development designed to protect against HIV and other STIs. Many of the candidate microbicides may also be effective contraceptives. The development of microbicides is urgent as the incidence of transmission of HIV and STIs is greatest in women of reproductive age.[123] Currently, the only proven way to protect against the sexual transmission of HIV is with the male condom, which is not always available and may not be a viable option for all women because it requires male cooperation. Many microbicidal compounds are currently under development, and they will provide additional protection when used with condoms, but may be used as primary protection for those who are unable or unwilling to use condoms consistently.

The pharmacological action of microbicides is based on several different mechanisms: (1) kills or immobilizes pathogens, (2) prevents the infective agent from entering target cells, (3) prevents a pathogen from replicating once it has entered cells, (4) boosts the vagina's or rectum's own defense system, and (5) acts like an invisible condom that forms a barrier between the pathogen and vaginal tissues. The focus is on developing such products that can be applied in advance before sexual intercourse, and spread evenly over the vagina, cervix, or rectum.

Many potential microbicides are in various phases of development, but most are in early phases of clinical trial or in preclinical stages. A recent study in several African nations demonstrated the effectiveness of a Tenovifir gel (9-[(R)-2-phosphonomethoxypropyl]) adenine monohydrate (PMPA). Overall, these researchers reported an estimated 39% reduction in HIV transmission.[124] Currently, studies are underway to test different methods of administration including applying the microbicide gel at different intervals to study whether or not that improves the effectiveness of the gel. Furthermore there is hope that the antiretroviral medications may also be effective against other STIs including: herpes simplex, bacteria, yeast, fungi, and other viruses.

Some of the groups of compounds that are being developed as microbicides are noted in Table 36.4.

Barrier Methods

Diaphragm

Diaphragms are soft latex or silicone barriers that cover the cervix. They provide contraception by blocking the entrance to the uterus and preventing the sperm from fertilizing the egg. They may also be used in conjunction with spermicides or microbicides that immobilize the sperm, and therefore, lead to better contraception. Diaphragms provide no protection against HIV or sexually transmitted infections, however may be combined with mircrobicides in the future to provide dual protection against pregnancy and STIs.

Currently, available diaphragms must be fitted by a healthcare provider. The largest size that does not cause

Table 36.4 Microbicide Compounds Under Development

Microbicide Group	Mode of Action	Examples
Surface active agents (detergents)	Penetrate cervical mucus, disrupt viral envelopes, cover the surface of viral STDs	Nonoxynol 9, octoxynol 9, benzalkonium chloride, menfegol, and *n*-docosanol
Sulfated compounds	Bind to virus or host cell receptors and block viral uptake	Dextrin sulfate, Dextran 2 sulfate, carageenan, polystyrene sulfonate, heparin sulfate: cholic acid, cellulose sulfate, PC-515, and naphthalene sulfonate (PRO 2000)
Anti-HIV compounds	Antiretroviral agents that prevent virus replication	Tenofovir (adenine antiretroviral drug), serine proteinase inhibitor, novel aryl phosphate derivatives of azidothymidine (AZT), nevirapine gel or cream, and cyanovirn N
Natural defense compounds	Increase host natural defenses against STIs by maintaining the normal acidic pH of the vagina in the presence of semen, contain lactobacillis which produces hydrogen peroxide killing HIV and STDs	Lactobacillus suppositories, buffer gel, and acid gel (ACIDFORM)

discomfort or undue pressure on the vaginal epithelium should be used. After the fitting, the woman should remove the diaphragm and reinsert it herself. She should then be examined to make sure the diaphragm is covering the cervix. The diaphragm should be used with a spermicide and be left in place for at least 8 hours after the last coital act. If repeated intercourse takes place or coitus occurs more than 8 hours after insertion of the diaphragm, additional spermicide should be used. Although spermicide use with the diaphragm is advised, it has not been conclusively demonstrated that pregnancy rates are lower when a spermicide is used with a diaphragm than when the diaphragm is used alone. The number of urinary tract infections in women who use diaphragms is significantly higher than in nonusers, probably because of the mechanical obstruction of the outflow of urine by the diaphragm. A one-size-fits-most nonlatex diaphragm is currently being studied, and, if approved for use, has the potential to simplify this method of contraception significantly.

Advantages

- Not easily felt by either partner (reasonably discrete)
- Immediately effective
- Immediately reversible
- Female-controlled
- Nonhormonal

Disadvantages

- Requires personalized fitting by healthcare professional
- Difficulty in insertion may be experienced
- May need to be refitting after full term pregnancy, pelvic or abdominal surgery
- Must be used with every coital act
- Moderately effective

Cervical Cap

The *cervical cap* is a cup-shaped plastic or rubber device that fits around the cervix. It may be either a cavity rim cap (Prentif and Oves), a cap that fits the vaginal walls around the cervix. (Femcap, Dumas, Vimule) or a large cap-like device (Lea's shield). Femcap is the only cervical cap Food and Drug Administration (FDA)-approved for use in the United States. These barrier methods can be left in place longer than the diaphragm. The various types of caps are manufactured in different sizes and should be fitted to the cervix by a healthcare provider.

The cap should be left on the cervix for no more than 48 hours, and a spermicide should always be placed inside the cap before use.[125] Failure rates with the cervical cap for nulliparous women are similar to those observed with the diaphragm, but for parous women failure rates are twice as high.

Constraints of Fitting of a Cervical Cap

- Fitting is to be done by a healthcare professional
- Vaginal delivery may lead to a scarred or irregularly shaped cervix
- Antiflexed uterus is necessary for cavity rim caps
- All sizes are not available
- Refitting may be needed after child birth

Male Condom

The male condom is the only method of contraception that has been shown to prevent transmission of sexually transmitted infections (STIs) including HIV, so its use by individuals at risk for infection should be encouraged. The male condom should not be applied tightly against the tip of the penis, but rather the condom tip should extend beyond the end of the penis by about half an inch to collect the ejaculate. Care must be taken on withdrawal not to spill the ejaculate. When used by strongly motivated couples, the male condom is very effective. The male condom has a 'typical use' failure rate of 14%. Recent studies have shown that teens were most likely to use condoms for birth control and 68% to 80% used a condom when they became sexually active.[5,126] The male condoms provide a physical barrier that prevents sperm and egg interaction. They are intended for one time use only.

Female Condom

A female condom was approved for marketing in the United States in 1994. It consists of a soft, loose-fitting sheath and two flexible polyurethane rings. One ring lies inside the vagina at the closed end of the sheath and serves as an insertion mechanism and internal anchor.

The outer ring forms the external edge of the device and remains outside the vagina after insertion, thus providing protection to the labia and the base of the penis during intercourse. The condom is pre-lubricated and is intended for one-time use only. Fitting by a health professional is not required. In comparison to the male condom, the female condom has the advantage of being able to be inserted before beginning sexual activity and to be left in place for a longer time after ejaculation occurs. Because the female condom also covers the external genitalia, it should offer greater protection against the transfer of certain sexually transmitted organisms, particularly genital herpes. Because polyurethane is stronger than the latex used in male condoms, the female condom is less likely to rupture.

In a multicenter clinical trial, the cumulative pregnancy rate in U.S. centers at 6 months was 12.4%. The 6-month pregnancy rate with perfect use was 2.6%. However the typical 1-year failure rate is 21%.[2] At the end of 6 months in the U.S. study, about one-third of the women had discontinued use of this method. Clinical trials with the female condom have not directly compared its use with other barrier techniques. Trussell and colleagues,[127] using the data of other studies, concluded that the efficacy rate of the female condom with perfect use would be similar to that of the diaphragm and cervical cap, but the failure rate of the female condom with typical use would be higher than that of the diaphragm. Because of the lack of prospective clinical trials with the male condom, no statistical comparison of the effectiveness of the two types of condoms can be made. Moreover, the effectiveness of the female condom for reducing sexual infection transmission has not been evaluated. However, because polyurethane does not allow virus transmission, it should reduce a woman's risk of acquiring human immunodeficiency virus infection.

Contraception in Women of Special Subpopulations

Given the overall safety of hormonal contraception, especially in young healthy women, and when compared to the risk that pregnancy conveys, there are few instances when the risks of providing contraception outweigh the benefits. Overall mortality in the general population is not increased by OC use.[128] However, there are occasions when special consideration to contraceptive method must be given due to a woman's comorbidities or health circumstances.

Noncontraceptive Benefits of Hormonal Contraception

The noncontraceptive health benefits of hormonal contraception are derived from a variety of epidemiological studies that have been conducted since combined hormonal contraceptive pills were initially marketed in the 1960s. A study of combined hormonal contraceptive pills containing less than 35 μg of ethinyl estradiol (those that are routinely in clinical use today) estimate that use of these oral contraceptive pills allows for a 70% reduction in ovarian cancer (Fig. 36.4), 50% reduction in endometrial cancer, 45% reduction in uterine blood loss, 50% reduction in dysmenorrhea, 65% reduction in premenstrual symptoms, 50% reduction in acne, 20% reduction in benign ovarian tumors, and 80% reduction in ectopic pregnancy.[129] Twelve case-control studies and three cohort studies have examined the relation between OCs and endometrial cancer, and all but two of these studies have indicated that the use of these agents has a protective effect against endometrial cancer, the third most common cancer among U.S. women.[130,131] Women who use OCs for at least 1 year have an age-adjusted relative risk of 0.5 for diagnosis of endometrial cancer between the ages of 40 and 55 years compared with nonusers. This protective effect is related to duration of use, increasing from a 20% risk reduction with 1 year of use to a 40% reduction within 2 years of use, to about a 60% reduction with 4 years of use. A prospective study of almost 30 years showed that OC users had one-third the risk of endometrial cancer as nonusers (RR, 0.34; CI, 0.17 to 0.66) and one-third the risk of dying of uterine cancer (RR, 0.3), compared with age-matched non-OC users.[132,133]

Eighteen studies on ovarian cancer and oral contraceptives have found a reduction in risk (Fig. 36.4).[74] The relative risk of ovarian cancer among ever-users of OCs was 0.64, a 36% reduction. The magnitude of the decrease in risk is directly related to the duration of OC use, increasing from about a 40% reduction with 4 years of use to a 53% reduction with 8 years of use and a 60% reduction with 12 years of use. Beyond 1 year, there is about an 11% reduction in ovarian cancer risk for each of the first 5 years of use. The protective effect begins within 10 years of first use and continues for at least 20 years after the use of OCs ends. In the 1968 to 1995 analyses of the RCGP data,[133] OC users were half as likely to develop ovarian cancer as nonusers (RR, 0.49; CI, 0.3 to 0.8) and had a 40% decreased risk of death due to ovarian cancer (RR, 0.5). In a large case-control study, OC users had a 40% decrease in risk of ovarian cancer that increased to 70% reduction with 10 or more years of use.[134]

With the advent of the ability to test for hereditary predispositions to breast and ovarian cancer, the potential value for chemoprophylaxis with hormonal contraceptive regimens has become more important. Results from three studies indicate that women with *BRCA1* and *BRCA2* mutations who use combined hormonal contraceptive pills have a decreased risk for developing ovarian cancer.[79] It is important to counsel women who are at increased risk for ovarian cancer due to hereditary reasons that chemoprophylaxis can offer a safe and effective way to reduce their risk of developing ovarian cancer. Furthermore, the use of combined hormonal contraception does not increase a woman's risk of developing breast cancer.[73,77] In a large study by the Centers for Disease Control, current or past use of combined hormonal contraceptive pills did not increase the risk for developing breast cancer in women who were 35 to 64 years of age.[135] Women who are at increased risk of breast cancer due to genetic or family history were not at increased risk if they used combined hormonal contraceptives. Most studies show a similar pattern of risk with hormonal contraceptive use regardless of a woman's reproductive history or her family history of breast cancer when it comes to her personal risk for breast cancer development.

Recent studies have shown that OCP use is not associated with an increase in breast cancer incidence, even in those who carry the *BRCA1* and *BRCA2* mutations.[79]

The odds ratio for developing breast cancer with COC use was 0.74 (CI 0.55 to 0.99) for women with neither mutation; 0.18 (CI 0.08 to 0.42) for women with *BRCA1* mutation; and 0.92 (CI 0.3 to 2.84) with the *BRCA2* mutation. Therefore, it is evident that the use of the low-dose hormonal contraceptive pills that are currently in clinical practice today do not increase the risk for breast cancer in women who are genetically predisposed to developing this disease and afford a risk reduction for ovarian cancer in this same population.[78,79]

Given the overall safety of hormonal contraception, especially in young healthy women, and when compared to the risk that pregnancy conveys, there are few instances when the risks of providing contraception outweigh the benefits. Overall mortality rate in the general population is not increased by OC use.[128] However, there are occasions when special consideration to contraceptive method must be given due to a woman's co-morbidities or health circumstances.

Hormonal Contraceptive Implants

The current etonogestrel implant now available cannot be assessed yet for differential efficacy in obese and nonobese populations. The number of pregnancies thus far has been too low for such conclusions to be drawn. However, the previously used six-rod levonorgestrel implant was associated with a fivefold increase in risk of pregnancy in women who weighed more than 70 kg or 154 pounds. These women are also at increased risk for endometrial cancer; progestin-only methods, specifically the progestin-only intrauterine contraceptive device, may be an appropriate choice in obese women because efficacy does not appear to be decreased in this population and it provides protection against endometrial hyperplasia.

Women with Cardiovascular Disease

A meta-analysis of 10 studies found the odds ratio (OR) for myocardial infarction (MI) and ischemic stroke in OC users versus nonusers was 2.01 (95% CI 1.63 to 2.48). Second-generation OCs were associated with a significant increased risk of both MI and ischemic stroke events (OR 1.85, 95% CI 1.03 to 3.32; and 2.54, 95% CI 1.96 to 3.28, respectively) and third-generation OCs, for ischemic stroke outcome only (OR 2.03, 95% CI 1.15, 3.57).[135] Another meta-analysis found that current OC users have an overall adjusted OR for MI of 2.48 (95% CI 1.91 to 3.22) compared to never-users. The risk of MI for past OC users is not significantly different from that for never-users, overall OR 1.15 (95% CI 0.98 to 1.35; $P = 0.096$).[136]

There is an emerging population of women who are adult survivors of congenital heart disease. This population represents a challenge as pregnancy may either be contraindicated or certainly impose increased risk. Careful assessment of the independent risk of thrombosis in these women, based on the specific heart defect and the degree of correction, must be made when evaluating such patients for appropriate contraception. In general, using progestin-only methods or intrauterine devices is probably the safest mode in these patients. Barrier methods will likely not give sufficient efficacy, and although estrogen-containing methods may be appropriate in some subsets of this population, this choice may require careful attention and thought. Progestin-only methods, however, are safe in women who are at increased risk of MI or thrombosis and therefore can be used in this population.

Hereditary Thrombophilia

In a retrospective family cohort study, women with hereditary deficiencies of protein S, protein C, or antithrombin, who were taking an oral contraceptive had an annual incidence of venous thromboembolism of 3.5%, increasing to 12% in women with more than one type of hereditary thrombophilia.[137] A systematic review found a total of 10 studies together that provided "good" evidence of a greater risk of VTE (risk ratios of 1.3 to 25.1) and cerebral vein or cerebral sinus thrombosis among COC users with factor V Leiden mutation when compared with nonusers who have the mutation.[71] The evidence for prothrombin and other thrombogenic mutations was not as strong as for factor V Leiden mutation. It is unclear whether the type of COC or duration of use modifies the risk of VTE among women with thrombogenic mutations.[43] However, one study found that women with factor V Leiden polymorphisms may remain asymptomatic after prolonged use of OCs. Some authors support the use of SHBG levels and activated protein C resistance as markers of thromboembolic risk with OCPs.[128] The WHO discourages use of OCs in women with either an underlying thrombophilia or a history of a previous VTE.[45] These women are good candidates for progestin-only contraceptives or intrauterine contraceptives.

The complete reference list can be found on the companion Expert Consult Web site at www.expertconsult.com.

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