Alice C. Levine *Editor*

Adrenal Disorders

Physiology, Pathophysiology and Treatment

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Series Editor Foreword

With its multilayered anatomy and complex physiology, the adrenal gland is one of the most important life-sustaining organs in the human body. Its development and hormone biochemistry are intricate, and its disorders are fascinating. Unlike diseases of glucose metabolism or the thyroid gland, those of adrenal glands are relatively uncommon. As a result, many physicians lack familiarity with the manifestations of adrenal gland dysfunction. It is therefore the responsibility of endocrinologists not only to understand, diagnose, and manage adrenal gland diseases but also to educate physicians in other specialties about presentation of these conditions. The goal is to maintain appropriate level of clinical suspicion when a patient presents with a possible adrenal gland disorder.

The current volume is edited by Dr. Alice C. Levine (Professor of Medicine and the Co-Director of the Adrenal Center at the Icahn School of Medicine at Mount Sinai in New York City) with contributions by an internationally renowned group of authors. In my opinion, it represents an invaluable tool that will help to meet the above challenges. The book is logically divided into three parts: Part I addresses normal adrenal physiology; Part II deals with genetics and pathophysiology; and Part III describes the diagnosis and management of adrenal disorders. This monograph will be immensely useful not only for practicing endocrinologists but also for endocrine fellows, medical residents, and medical students as they learn the intricacies of adrenal gland genetics, development, structure, function, and dysfunction.

New York, NY, USA   Leonid Poretsky, MD

Preface

It is with great pleasure that I introduce this unique book on adrenal disorders. My mentor, Dr. J. Lester Gabrilove, coauthored the very first textbook on Adrenal Disorders over 50 years ago. That book was published just after the isolation and identification of a number of adrenal hormonal fractions as well as corticotropin. Since that time, the molecular era of steroidogenesis ensued with the cloning and functional characterization of steroid receptors, steroidogenic enzymes, adrenal transcription factors, and the determination of the molecular basis for adrenal diseases. Over the past 5 years there has been an explosion of new insights into the factors controlling adrenal development and steroidogenesis, the genetic pathophysiology of adrenal tumors, and the diagnosis and treatment of adrenal disorders.

The book is divided into three major sections. The first section elucidates the factors that control normal adrenal zonation/development, adrenal steroidogenesis, and the pharmacology of glucocorticoids. The second section focuses on genetics and pathophysiology, specifically regarding autoimmune Addison disease, congenital adrenal hyperplasia, primary aldosteronism, adrenocortical tumors/hyperplasia, and pheochromocytomas/paragangliomas. Finally, the last section is clinically oriented, detailing the diagnosis and treatment of adrenal insufficiency, adrenal Cushing syndrome, primary aldosteronism, pheochromocytomas/paragangliomas, and adrenal cortical carcinoma. The book is translational in nature and designed to provide a framework for both clinicians and basic scientists to better understand the cross-talk and opportunities in going from bench to bedside and back to the bench.

In order to accomplish this ambitious endeavor, I have recruited esteemed friends and colleagues from around the globe to share their expertise. I thank them for their time and effort that resulted in this comprehensive and important work.

The Preface to Dr. Gabrilove's book ends with "This seems to be a good time to pause, take stock and incorporate the broad new knowledge into our thinking. There is, of course, a great deal still left undone and inadequately explored. The currently available background and the new technological advances in this and related fields should provide the impetus for another forward surge." Fifty-six years later, we have

indeed surged ahead and improved the lives of patients with adrenal disorders to an extent that was unimaginable to previous generations. This book takes stock of that progress but ultimately, like the previous volume, is designed to inform and inspire future scientists and physicians to continue the charge.

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New York, NY   Alice C. Levine, MD

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Part I
Physiology

Chapter 1
Adrenal Zonation and Development

Emanuele Pignatti, Sining Leng, Diana L. Carlone, and David T. Breault

Introduction

The adrenal cortex is a major site of steroid hormone production. In adult mammals it is comprised of three concentric layers or zones of steroid-producing cells surrounding the adrenal medulla [1, 2]. The outer layer of the cortex, the zona glomerulosa (zG), represents ~15% of the cortical mass and produces the mineralocorticoid aldosterone, which is essential for sodium retention, intravascular volume, and blood pressure regulation. Excess aldosterone production, as seen in primary

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Fig. 1.1 Concentric layers of the mouse adrenal gland. (**a**) Schematic of the various regions of the adrenal. (**b**) A representative H&E longitudinal mouse adrenal section. Zones are identified by white dashed lines. *c* capsule, *zG* zona glomerulosa, *zF* zona fasciculata, *zX* X-zone, *m* medulla
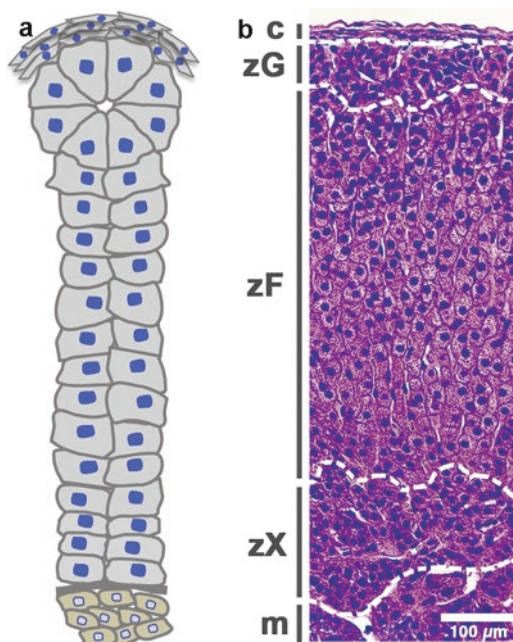
aldosteronism, is a major cause of hypertension and cardiovascular damage [3, 4]. The middle layer of the cortex, the zona fasciculata (zF), is ~8 times larger than the zG and produces the glucocorticoid corticosterone (in rodents) and cortisol (in humans), which impacts immunity, metabolism, development, and behavior. A third layer, the zona reticularis (zR), is present in humans, some nonhuman primates (e.g., rhesus macaques, marmosets), ferrets, and the spiny mouse. It lies between the zF and the medulla and produces androgens, such as dehydroepiandrosterone (DHEA) and its sulfated derivative DHEA-S [5]. While the mouse adrenal lacks a true zR, it does contain a transient X-zone (zX), which appears to be a remnant of the fetal adrenal cortex [6] and is thought to be involved in progesterone metabolism [7] (Fig. 1.1).

Embryonic Adrenal Development

Adrenal embryonic development has been extensively studied [8]. In the mouse, development begins on embryonic day 9 (E9.0), or around 28 days post coitum (28 dpc) in the human, when cells in the coelomic epithelium first express the master transcriptional regulator steroidogenic factor 1 (SF1, also known as NR5A1 and AD4BP), which results in the emergence of the adrenogonadal lineage. SF1+ cells then delaminate into the adjacent mesenchyme giving rise to the adrenogonadal primordium (AGP). AGP cells, marked by expression of the *Sf1-fetal adrenal enhancer (FAdE)*, then give rise to the fetal adrenal anlagen around E10.5 (~33 dpc).
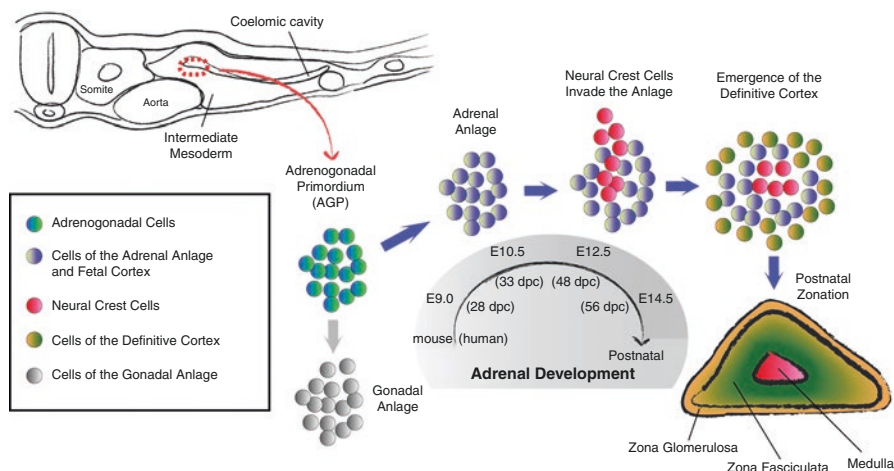
Fig. 1.2 Embryonic adrenal development in the mouse. Schematic illustration of the cellular changes during mouse and human adrenal development. The adrenogonadal primordium (AGP) originates from a thickening of the coelomic epithelium designated by the red dashed circle around E9.0 (28 dpc). At E10.5 (33 dpc), the adrenal anlage separates from the AGP and is then invaded by neural crest cells, precursors of the medullary chromaffin cells around E12.5 (48 dpc). From E14.5 (56 dpc) onward, the fetal cortical cells are slowly replaced by the definitive cortex, which gives rise to functional zones around the time of birth. Once formed, the zones are maintained throughout life

Next, cells from the neural crest invade the fetal adrenal ~E12.5 (~48 dpc), which go on to form the adrenal medulla. Subsequently, ~E14.5 (~56 dpc) the fetal cortex slowly begins to regress, while the definitive (adult) cortex emerges beneath the newly formed capsule, though distinct zones are not yet formed (Fig. 1.2). Establishing a connection between the definitive cortex and the fetal cortex, elegant lineage-tracing studies have demonstrated that the definitive cortex is indeed a direct descendent of the fetal cortex [9, 10].

Postnatal Adrenal Development

In contrast to early embryonic development, the mechanisms underlying postnatal adrenal development, which lead to the formation and maintenance of the adrenal's distinct zones, remain poorly understood. Detailed knowledge of how these mechanisms mediate zonation has important implications for understanding normal homeostatic functions as well as the pathological conditions that arise within the adrenal cortex. For example, it is known that control of steroidogenic output is dependent, in part, on proper maintenance of zonation over time [1]. Consistent with this, impaired zonation has been implicated in a range of conditions, including primary aldosteronism, cortisol-producing adenomas, primary pigmented nodular adrenocortical disease

(PPNAD), congenital adrenal hyper- and hypoplasia, and adrenocortical carcinoma [11]. While the precise mechanisms underlying each of these conditions remain to be fully characterized, recent advances in our understanding of the cellular and molecular mechanisms underlying normal tissue homeostasis have made it possible to begin to explore key structure/function relationships within this tissue.

Adrenal Morphology

The adrenal cortex is an epithelial tissue circumscribed by a mesenchymal capsule (Fig. 1.1). The cells of the zG are organized in distinct morphological clusters, known as glomeruli, a highly conserved structure [12], which is surrounded by a basement membrane and a fenestrated capillary network [13]. zG cells are densely packed and contain scant cytoplasm, abundant rough endoplasmic reticulum, and a small number of lipid droplets and mitochondria [12, 14, 15]. In contrast, zF cells are arrayed in cord-like structures and exhibit distinctly different morphological features. zF cells are larger and more loosely packed than zG cells and contain extensive smooth endoplasmic reticulum, large gap junctions, numerous lipid droplets, and mitochondria characterized by tubulovesicular cristae [12, 14]. Also, like in the zG, zF cells are surrounded by a basement membrane and a rich capillary network. While the cells in the zR are morphologically similar to zF cells, they contain fewer lipid droplets with additional lysosomes and lipofuscin pigment granules [16]. In mice, X-zone cells are smaller than zF cells, contain an eosinophilic cytoplasm, and demonstrate a range of mitochondrial shapes with tubular cristae [6, 17].

Signaling Pathways and Zonation

The presence of morphologically distinct, yet physically contiguous, adrenocortical zones suggests tight regulation of each zone's identity, relative size, and overall function. Recent advances in our understanding of how angiotensin II (AngII), potassium ions (K⁺), and adrenocorticotropic hormone (ACTH) regulate adrenal homeostasis may ultimately provide key insights into the origins of adrenal zonation and the dynamic regulation of these zones that occurs in response to physiological cues [18–27]. It is likely that multiple signaling pathways also contribute to adrenal zonation. Considerable progress has been made regarding the role of the canonical Wnt/β-catenin signaling pathway and the role of the ACTH/cyclic adenosine monophosphate (cAMP) pathway in setting the morphological and functional boundaries between the zones [11, 18, 21, 25, 28–34].

The canonical Wnt signaling pathway is active in the outer region of the cortex, overlapping with the morphological zG, and drives a transcriptional program that facilitates the production of the mineralocorticoid aldosterone [11, 29, 32]. Consistent with this, in vitro and in vivo experiments demonstrate that constitutive activation of the canonical Wnt pathway leads to an upregulation of aldosterone

biosynthesis and an expansion of the morphological zG, while inhibition of the pathway leads to inactivation of aldosterone biosynthesis and contraction of the zG [28–32, 34, 35]. In contrast, the ACTH/cAMP signaling pathway is dominant in the zF and mediates the downstream transcriptional effects of ACTH on the synthesis and secretion of glucocorticoids [36–38]. Additionally, recent evidence suggests a reciprocal inhibitory effect of these two pathways, whereby Wnt signaling maintains zG zonal identity and size and also serves to inhibit expression of the zF program [30, 34]. Critical mediators of these effects include two key Wnt pathway ligands: Rspo3 (secreted from the capsule) and Wnt4 (expressed in the zG). Consistent with this, ectopic activation of Wnt signaling inactivates the zF steroidogenic program [11, 29, 30]. On the other hand, stabilization of the ACTH/cAMP signaling pathway results in activation of the zF steroidogenic program and inhibition of the Wnt signaling pathway leading to contraction of the zG [30, 33].

The overall significance of these signaling pathways in the regulation of adrenal homeostasis and zonation is made clear by the effects of somatic gain-of-function mutations giving rise to (1) aldosterone-producing adenomas (APAs) (associated with aberrant activation of the Wnt pathway) and (2) PPNAD (arising from mutations in *PRKAR1A* mutations, which leads to constitutive activation of ACTH/cAMP-dependent signaling) [39, 40].

Centripetal Migration and Cortical Renewal

Once established, the zG and the zF are continuously renewed throughout life and undergo dynamic hormonal feedback regulation. Despite the functional importance of these separate layers, surprisingly little is known about the cellular mechanisms that underlie their formation and ongoing maintenance. Recently, two members of the sonic hedgehog family, GLI1 and SHH, were identified as markers for adrenal progenitor cells that reside in the capsule and subcapsular regions (adjacent to the zG), respectively [41]. Consistent with the classical model of centripetal migration [42], proposed more than 70 years ago, these progenitor cells give rise to terminally differentiated zG cells, which then migrate centripetally and are thought to undergo cell fate conversion into zF cells before undergoing apoptosis at the corticomedullary junction [43].

Generation of *Cyp11b2*-Cre Mice

To define the molecular and cellular mechanisms underlying adrenal lineage development, we recently targeted the *Cyp11b2* (*aldosterone synthase*) locus in mice, to generate a knock-in/knock-out *Cyp11b2*-Cre allele (officially known as Cyp11b2tm1.1(cre) Brit). Combined with other strains, these mice facilitate lineage-tracing, cell fate analysis and tissue-specific knock-out studies, specifically within zG cells [20]. CYP11B2 is required for the final steps of aldosterone synthesis, and its gene expression is restricted to terminally differentiated cells in the zG [43], making it a highly

specific marker for zG cells. Although given the heterogeneous nature of *Cyp11b2* expression with the zG under normal conditions, it is not as sensitive as other validated zG markers (e.g., β-catenin, Dab2, Dlk1) [11, 44–47]. Importantly, mice heterozygous for the *Cyp11b2*-Cre allele maintain normal levels of aldosterone and plasma renin activity (PRA), essential components of the renin-angiotensin aldosterone system (RAAS), indicating normal feedback regulation is maintained. In contrast, mice homozygous for the *Cyp11b2*-Cre allele are aldosterone deficient and demonstrate increase levels of PRA.

Direct Cell Fate Conversion

To investigate whether zG cells undergo direct cell fate conversion to zF cells, lineage-tracing studies were performed by combining *Cyp11b2*-Cre mice with the Rosa26 lineage reporter strain, which expresses membrane-targeted Tomato at baseline and expresses membrane-targeted green fluorescent protein (GFP) following Cre-mediated recombination (Fig. 1.3a) [20]. These studies revealed activation of the endogenous *Cyp11b2* locus around the time of birth, and GFP-marked cells were entirely restricted to the zG. During the first few weeks of postnatal
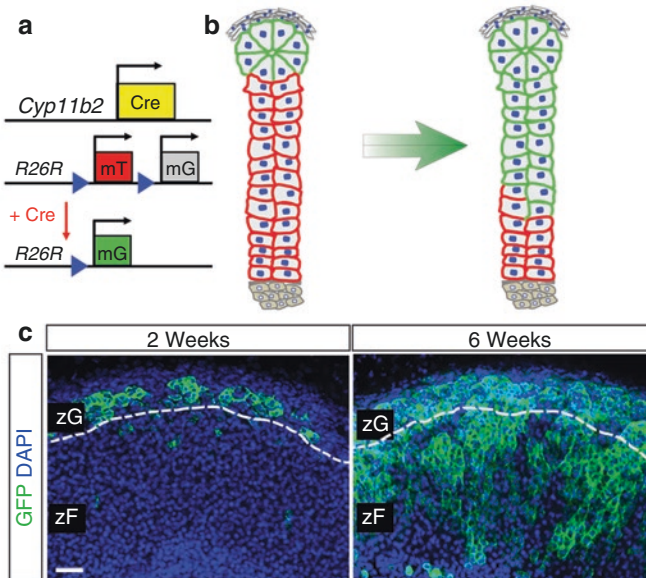
Fig. 1.3 zG cells give rise to zF cells through direct conversion. (**a**) Schematic illustration of the Cyp11b2-Cre and the Rosa26-mTmG allele (R26R) alleles before and after Cre-mediated LoxP recombination, which leads to deletion of mTomato and expression of mGFP. (**b**) Schematic illustration showing centripetal migration of GFP+ cells from the zG to the zF. (**c**) Representative immunofluorescent images showing centripetal migration of GFP+ cells from the zG (left, 2 weeks of age) to the zF (right, 6 weeks of age) in female mice. Scale bar, 50 μm

development, the zG was progressively marked by GFP expression (Fig. 1.3b), which subsequently gave rise to zF cells in a radial fashion, ultimately remodeling the entire zF by ~12 weeks of age (Fig. 1.3c) [20]. zG to zF cell fate conversion also functions during adrenal regeneration following dexamethasone suppression [20]. Together, these observations establish that differentiated zG cells give rise to zF cells through a process of direct cell fate conversion during postnatal adrenocortical zonation and regeneration, consistent with the model of centripetal migration.

Role of SF1 in Cell Fate Conversion and zG Homeostasis

Understanding the mechani sms that regulate cell fate conversion has important implications for both normal and pathological states. The ability of one differentiated cell to be converted into another differentiated cell type, without passing through an undifferentiated state, has been described following the overexpression of specific transcription factors. For example, fibroblasts can be converted into myoblasts following expression of MyoD [48], and embryonic and mesenchymal stem cells can be converted into steroid-producing cells following expression of SF1 [49, 50]. The observations that SF1 plays a critical role during steroidogenic development and is sufficient to activate a steroidogenic program raised the possibility that it may play a role in cell fate conversion. Consistent with this, we observed that deletion of SF1 within zG cells prevented their conversion to zF cells (Fig. 1.4a) [20]. While the overall size of the zG remained essentially unchanged, detailed histological analysis

Down-regulated	Function
Cyp11b2	Aldosterone Synthase
Per1	Overexpression increases aldosterone secretion and expression; is stimulated by Angiotensin II
Bhlhe40/DEC1	Circadian Clock Gene; Essential role in phase-shift of clock gene expression in adrenal, Interacts with Per1
Nr4a2/Nurr1	Orphan nuclear receptor, expressed in zG, involved in zonation, target of Wnt signaling
Nr4a1/Nur77	Orphan nuclear receptor, involved in zonation
Apcdd1	APC down-regulated 1, DRAPC1, target of Wnt signaling
Up-regulated	
Frzb/Sfrp3	Soluble Wnt Inhibitor, Secreted Frizzled related Protein 3, Expressed in rat zF
Sfrp2	Soluble Wnt Inhibitor, Secreted Frizzled related Protein 2

Fig. 1.4 Deletion of SF1 impairs zG-to-zF conversion and alters gene expression. (**a**) Representative immunofluorescent images of wild-type and SF1 KO adrenals demonstrating contribution of GFP+ cells to the zF. Note the absence of GFP+ cells in the zF in SF1 KO adrenals. Both images are taken from 10-week-old female mice. Scale bar, 50 μm. (**b**) Heat map representation of differentially expressed genes from wild-type and SF1 KO whole adrenals. Dendrograms represent hierarchical clustering of genes and samples. (**c**) Select list of genes that are down- or up-regulated in SF1 KO whole adrenals compared to wild-type whole adrenals

revealed that lineage-marked zG cells had a dramatically altered cell shape, raising the possibility that these cells had undergone dedifferentiation. In addition, functional analysis revealed a state of compensated hypoaldosteronism, indicated by normal aldosterone levels and a nearly threefold increase in the levels of PRA.

To identify the mechanisms by which SF1 regulates cell fate conversion and zG homeostasis, we performed transcriptome analysis comparing total RNA from SF1 KO and wild-type adrenals using Affymetrix microarray analysis. Of 35,556 probes analyzed, 240 expressed a greater than two-fold difference in expression level and 105 of those contained unique genes (Fig. 1.4b). Among the genes showing the greatest fold changes were members of the Wnt/β-catenin signaling pathway and members of the clock gene family (Fig. 1.4c). Both of these pathways have been implicated in adrenal homeostasis, though what role they play in zG homeostasis and zonation remains largely unknown. Finally, these studies also revealed that zF cells were functionally normal, as evidenced by measurement of basal corticosterone secretion, and indicate that an "alternate (zG-independent) pathway" can contribute to zF formation. Exactly how this alternative pathway directs zF formation as well as whether it functions during normal adrenal homeostasis remains to be determined. One possibility is that when normal tissue homeostasis is severely disrupted, such as in the case of zG-specific SF1 deletion, mesenchymal cells in the capsule harboring stem-/progenitor-like potential may become activated to directly replenish the zF. Changes in gene expression identified in the microarray analysis (Fig. 1.4b) may provide new insight into these mechanisms.

Conclusions and Future Directions

In summary, the mechanisms underlying adrenocortical homeostasis and zonation during postnatal development remain largely unknown, though critical insights have recently been made. It is clear, for example, that direct conversion of zG cells into zF cells represents the major cellular mechanism by which the cortex is maintained under normal homeostatic conditions. However, it remains less clear as to the extent zG cells, alone, sustain long-term cortical renewal or to what degree zG cells rely on replenishment from the capsule, an important signaling center. Genetic lineage-tracing experiments performed by several laboratories have unequivocally demonstrated that the mesenchymal capsule can serve as a source for cellular replenishment for all steroidogenic zones as well as non-steroidogenic stromal cells [9, 41, 51]. However, an important issue raised by these studies is that constant centripetal migration of cells appears to require a much higher cellular turnover rate than provided by capsular cell activity. Hence, it is possible that differentiated cortical cells, especially the more proliferative zG population, may, in fact, play a key role in supporting the self-renewal of this tissue. Understanding which cells underlie adrenocortical self-renewal has important implications for (1) the development of future regenerative medicine strategies and for (2) understanding the pathogenesis of

adrenal neoplasms. Going forward, the ability to perform "pulse-chase" lineage-tracing studies utilizing inducible mouse models will help to define the self-renewing potential of mature zG cells and to better understand the mechanisms underlying adrenal homeostasis.

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Chapter 2
Regulation of Adrenal Steroidogenesis

Marjut Pihlajoki, Markku Heikinheimo, and David B. Wilson

Introduction

The adrenal cortex is a major source of steroid hormones. Anatomically and functionally distinct adrenocortical zones synthesize specific classes of steroids in response to various stimuli. Adrenal steroids impact a myriad of physiological processes in the fetus and adult, including intrauterine homeostasis, organ maturation, salt/water balance, carbohydrate metabolism, and the response to stress. This chapter highlights the regulation of steroidogenesis in the adrenal cortex. Diseases associated with aberrant production of adrenal steroids are discussed.

Overview of Adrenal Steroidogenesis

The principal steroid hormones produced by the human adrenal cortex are the mineralocorticoid aldosterone, the glucocorticoid cortisol, and the 19-carbon (C_{19}) androgen precursor dehydroepiandrosterone (DHEA). Adrenal steroids are synthesized from cholesterol through the sequential actions of a series of cytochrome P450

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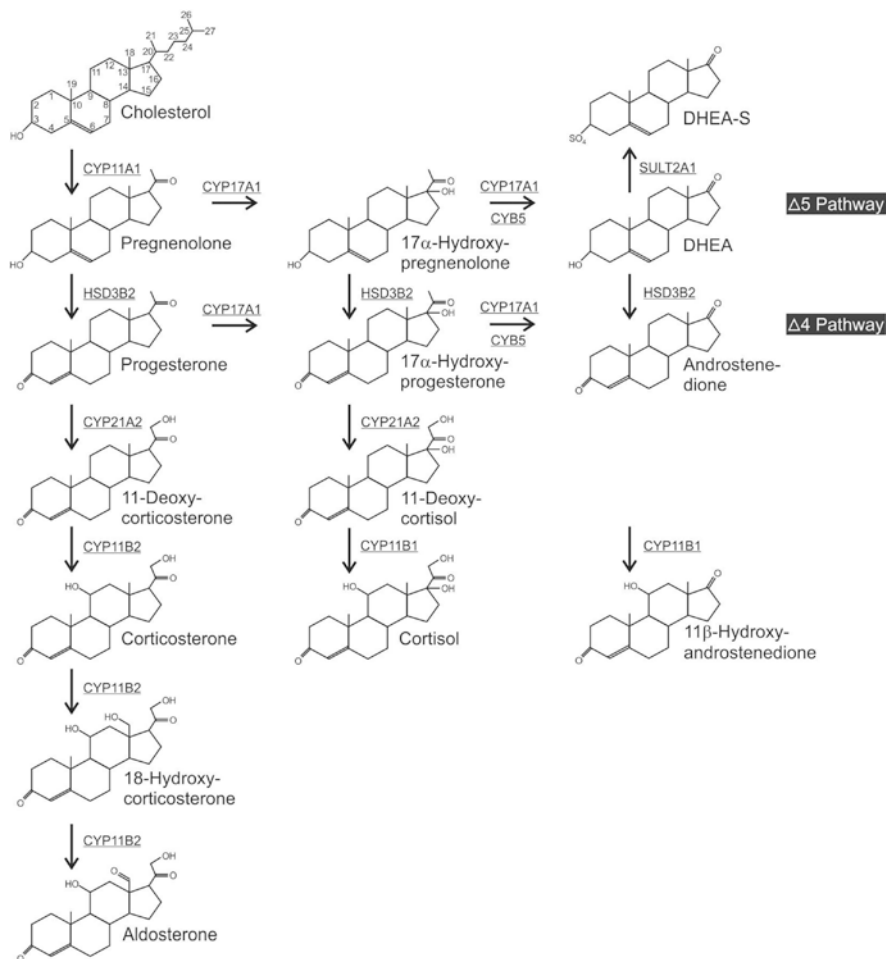
Fig. 2.1 Steroid biosynthetic pathways in the human adrenal cortex. Shown are enzymes (underlined) and intermediates in the biosynthesis of adrenal steroid hormones. 17α-Hydroxypregnenolone is the preferred substrate for the 17,20-lyase reaction of CYP17A1. Consequently, the Δ5 pathway to DHEA is favored over the Δ4 pathway to androstenedione. The adrenal gland produces small quantities of other steroids not shown here. An expanded view of adrenal androgen production is presented later

(CYP)-mixed function oxidases and hydroxysteroid dehydrogenases (HSDs) (Fig. 2.1) [1]. Steroid hormones are not stored in adrenocortical cells. Instead, adrenal steroid secretion relies on de novo synthesis, a process that requires a ready supply of cholesterol [2].

To initiate steroidogenesis, cholesterol undergoes facilitated transport from a replenishable pool in the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM), where CYP11A1 (side-chain cleavage enzyme)
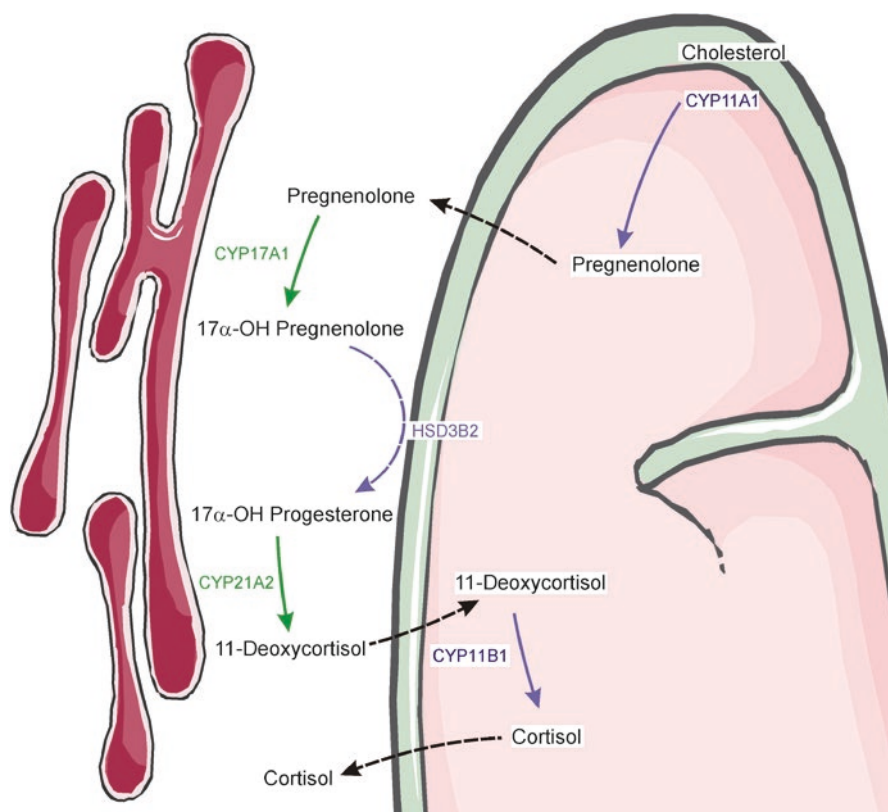
Fig. 2.2 Steroidogenic intermediates shuttle between mitochondria and the ER. The biosynthetic pathway for cortisol is shown; similar shuttling takes place during the synthesis of other adrenal steroid hormones. Enzymatic reactions that occur in mitochondria are shown in *purple*, whereas those that occur in the ER are in *green*. *Dashed lines* indicate passive diffusion across mitochondrial membranes. Prepared using image vectors from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/)

catalyzes the conversion of cholesterol to pregnenolone [1]. Transcription of the *CYP11A1* gene is regulated in a hormonally-responsive manner and determines the net steroidogenic capacity of a cell [3]. Pregnenolone diffuses out of mitochondria and serves as the precursor for the ensuing steps of steroidogenesis, most of which take place in the endoplasmic reticulum (ER) (Fig. 2.2). The final steps of cortisol and aldosterone biosynthesis, catalyzed by the enzymes CYP11B1 and CYP11B2, respectively, occur in mitochondria. Thus, intermediates in the corticoid biosynthetic pathway shuttle between mitochondria and the ER. The electron donors for CYP enzymes in these two cellular compartments are summarized in Table 2.1.

Table 2.1 Cytochrome P450 enzymes involved in adrenal steroidogenesis

CYP classification	Location	Enzyme	Electron donor
Type I	Mitochondria	CYP11A1	NADPH via a flavoprotein (ferredoxin reductase) and an iron-sulfur protein (ferredoxin)
		CYP11B1	
		CYP11B2	
Type II	ER	CYP17A1	NADPH via a flavoprotein [P450-oxidoreductase (POR)]
		CYP21A2	

Each of these enzymes uses molecular oxygen and electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to metabolize substrates

Zones of the Adrenal Cortex

In both the fetus and adult, the adrenal cortex is divided into concentric zones that produce different classes of steroid hormones [4, 5].

Human Fetal Adrenal Cortex

At the eighth week of human gestation, the fetal adrenal cortex comprises two morphologically distinct layers: an outer definitive zone (Dz) and an inner fetal zone (Fz) [6]. The Dz is thin and contains small basophilic cells, whereas the Fz is thick and contains large eosinophilic cells (Fig. 2.3). The Dz does not synthesize significant amounts of steroid hormones, but the Fz produces large quantities of DHEA and its sulfated counterpart DHEA-S. Cells of the Fz express *CYP17A1*, a dual function enzyme that catalyzes both a 17α-hydroxylation reaction and a 17,20-lyase reaction required for C_{19} steroid production [1]. The lyase reaction is selectively enhanced through allosteric interactions with cytochrome b_5 (CYB_5), a protein that is abundant in the Fz [1]. A third cortical zone, the transitional zone (Tz), develops shortly after the appearance of the Fz and Dz. The Tz secretes cortisol, a hormone that promotes maturation of the lungs and other organs [8].

C_{19} steroids secreted by the Fz are converted into estrogens through the actions of enzymes in the liver and/or placenta. The fetal pituitary, adrenal, liver, and placenta constitute a functional entity known the feto-placental unit [9] (Fig. 2.4). The concentration of estrogens in maternal plasma increases abruptly mid-gestation, reflecting production by this unit [10]. Estrogens support pregnancy by promoting maternal breast development, blood volume expansion, and uterine growth/contractility, although intact fetal adrenocortical function is not a prerequisite for term gestation or birth [11].

Adrenocorticotropic hormone (ACTH), a peptide secreted by the anterior pituitary gland, is a major regulator of fetal adrenal growth and function. ACTH promotes the production of both C_{19} steroids and cortisol in the fetal adrenal. Disruption of hypothalamic/pituitary function (e.g., in the anencephalic fetus) impairs Fz growth and decreases estrogen levels in the maternal circulation [8].

Another important regulator of steroidogenesis in the fetus is placenta-derived corticotropin-releasing hormone (CRH), a peptide that both directly and indirectly

Fig. 2.3 Structure of the human fetal adrenal gland. The zones of the fetal cortex are the Dz, Tz, and Fz. The Tz and Fz produce cortisol and C_{19} androgen precursors, respectively. An early burst of cortisol production by the Tz during the 9th week of gestation, coinciding with a transient increase in expression of *HSD3B2*, is thought to safeguard female sexual development by limiting the production of androgen precursors by the Fz [7]. After birth the Dz differentiates into the functionally distinct zones of the adult cortex. *Cap* capsule, *DHEA-S* dehydroepiandrosterone sulfate, *Dz* definitive zone, *Fz* fetal zone, *med* medulla, *Tz* transitional zone
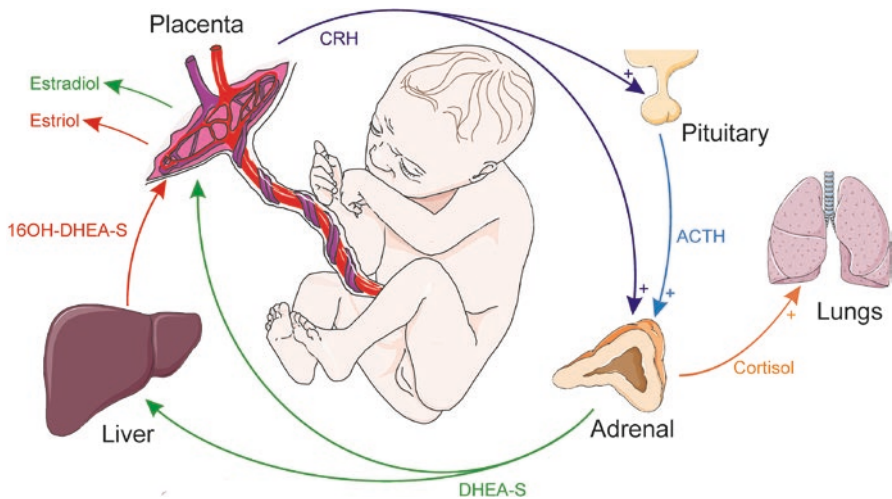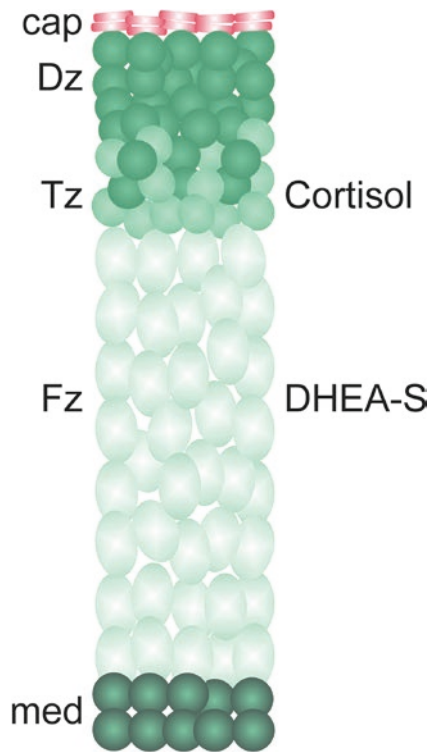
Fig. 2.4 Steroid production by the feto-placental unit. Placental CRH and pituitary-derived ACTH promote cortisol and DHEA-S secretion by the fetal adrenal gland. DHEA-S is converted into estrogens (estradiol and estriol) by enzymes in the liver and placenta. The resultant estrogens support pregnancy, while cortisol promotes the maturation of the lungs and other organs in the fetus. *ACTH* adrenocorticotropic hormone, *CRH* corticotropin-releasing hormone, *DHEA-S* dehydroepiandrosterone sulfate, *16OH-DHEA-S* 16-hydroxydehydroepiandrosterone sulfate. Prepared using image vectors from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/)

stimulates fetal adrenal cells to produce cortisol and androgens [12, 13] (Fig. 2.4). Unlike hypothalamic CRH secretion, which is subject to feedback inhibition by glucocorticoids (see later), placental CRH release is enhanced by cortisol. At birth the placenta separates from the body, and this CRH feed-forward loop is interrupted. The newborn adrenal gland, which is almost as large as the kidney, shrinks dramatically over the first 2 weeks of life owing to apoptotic involution of the Fz. Regression of the Fz is accompanied by a reduction in C_{19} steroid production [1]. Postnatally, the Dz differentiates into the anatomically and functionally distinct zones of the adult cortex.

Human Adult Adrenal Cortex

The adult adrenal cortex of humans contains three layers: the zona glomerulosa (zG), zona fasciculata (zF), and zona reticularis (zR) (Fig. 2.5) [4, 14]. The cortex is enveloped by a capsule that provides structural support and houses stem/progenitor cells of the cortex. A complex network of blood vessels within the adrenal gland ensures
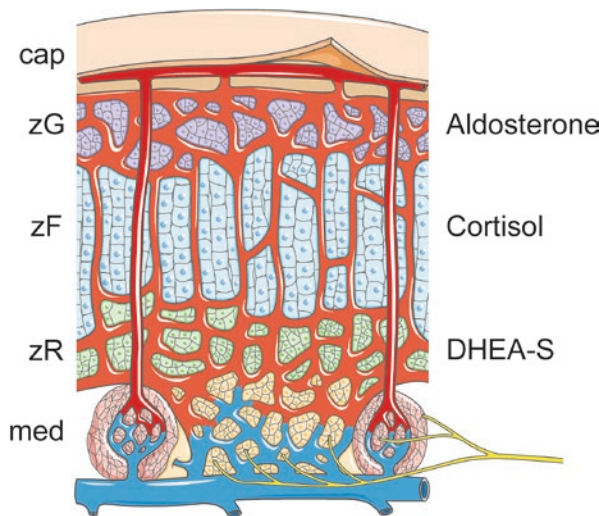
Fig. 2.5 Structure of the adult adrenal gland. The gland is covered by a capsule. The cortical zones are the zG, zF, and zR, which produce aldosterone, cortisol, and DHEA-S, respectively. Adrenal glands are highly vascularized, ensuring the efficient delivery of stimulants and export of steroid hormones into the systemic circulation. A subcapsular arteriolar plexus receives oxygenated blood and distributes it to the underlying tissue via two types of vessels: (1) sinusoids that supply the adrenal cortex and medulla and (2) medullary arteries that directly supply to the medulla. The adrenal medulla, which is innervated by preganglionic sympathetic fibers, functions as part of the sympathetic nervous system, releasing catecholamines into the circulation. *Cap* capsule, *DHEA-S* dehydroepiandrosterone sulfate, *med* medulla, *zF* zona fasciculata, *zG* zona glomerulosa, *zR* zona reticularis. Prepared using image vectors from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/)

the efficient delivery of stimulants and the export of corticoids into the circulation [15, 16]. Blood flows centripetally in the gland, so inner cortical zones and the adrenal medulla are exposed to high concentrations of locally-produced steroids.

zG

The outermost zone, the zG, secretes aldosterone. The major physiological stimuli of aldosterone secretion are angiotensin II (Ang II) and extracellular K^+, but ACTH also enhances production of this steroid hormone [17]. Aldosterone binds to the mineralocorticoid receptor (MR) in cells of the distal nephron, leading to retention of Na^+ and excretion of K^+ and H^+ by the kidneys [18]. By modulating Na^+ balance, aldosterone impacts extracellular fluid volume and blood pressure. Aldosterone also plays important roles in certain cardiovascular, renal, and inflammatory diseases [19–21]. For example, aldosterone exerts direct actions on cardiomyocytes, contributing to cardiac fibrosis and congestive heart failure [22]. Distinctive molecular markers of the zG include CYP11B2 (the mitochondrial enzyme that catalyzes the final steps of aldosterone biosynthesis) and AT_1R (the Ang II receptor) [23]. The zG lacks expression of *CYP17A1* and therefore is not able to synthesize cortisol or androgen precursors.

zF

The largest cortical zone, the zF, produces the stress hormone, cortisol, as part of the hypothalamic-pituitary-adrenal (HPA) axis (Fig. 2.6). ACTH secreted by the pituitary is the principal stimulus for cortisol production [24]. Cells in the zF respond to
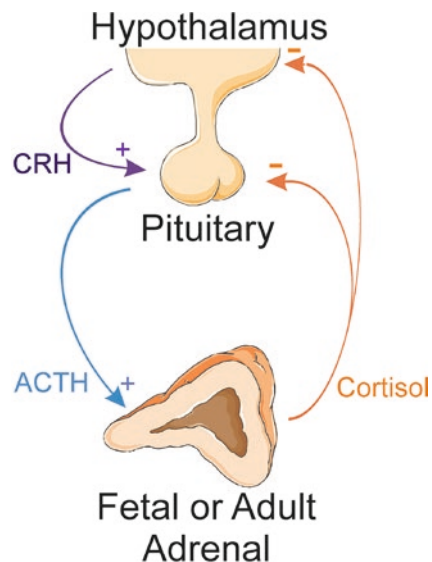
Fig. 2.6 HPA axis. In response to stress or other physiological cues, hypothalamic neurons secrete CRH, which stimulates the release of ACTH from the pituitary gland. ACTH promotes the secretion of cortisol by the adrenal cortex. Cortisol, in turn, inhibits the axis at the level of the pituitary and hypothalamus. *ACTH* adrenocorticotropic hormone, *CRH* corticotropin-releasing hormone, *HPA* hypothalamic-pituitary-adrenal. Prepared using image vectors from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/)

ACTH via its G-protein-coupled receptor, the melanocortin 2 receptor (MC2R), working in conjunction with melanocortin 2 receptor accessory proteins (MRAPs). Adrenal-derived cortisol binds to the glucocorticoid receptor (GR), which is expressed in a wide array of cell types including hepatocytes, muscle cells, lymphocytes, neurons, and neuroendocrine cells. Cortisol functions to (1) increase blood glucose concentrations through gluconeogenesis; (2) facilitate the catabolism of proteins, lipids, and carbohydrates; (3) suppress the immune system; and (4) induce enzymes for catecholamine biosynthesis in the medulla [25, 26]. A distinctive molecular marker of the zF is CYP11B1, a mitochondrial enzyme required for the biosynthesis of cortisol [23].

zR

The innermost layer of the cortex, the zR, secretes the C_{19} steroids DHEA, DHEA-S, androstenedione, and 11β-hydroxyandrostenedione, which are often termed "adrenal androgens" [1]. DHEA-S is the most abundant adrenal steroid in the circulation of adults [27]. In contrast to more potent androgens, such as testosterone and dihydrotestosterone (DHT), adrenal androgens exhibit little or no binding/activation of the androgen receptor (AR) [28]. Consequently, adrenal C_{19} steroids function mainly as precursor molecules that can be converted into more potent androgens in peripheral tissues (e.g., hair follicles, sebaceous glands, prostate, and genital skin).

Cells of the zR, like those of the zG and zF, respond to ACTH. In contrast to other zones of the cortex, the zR does not become functionally active until 6–8 years of age (adrenarche), coinciding with increased expression of *CYB5*, the allosteric regulator that enhances the 17,20-lyase activity of CYP17A1 [1]. CYB5 serves as a distinctive molecular marker of the zR; another characteristic marker of this zone is SULT2A1, the enzyme responsible for sulfation of DHEA [29].

Comparative Adrenal Anatomy of Small Animal Models

Rodents are used as experimental models for the study of adrenal physiology, but there are noteworthy differences between the adrenocortical zonation patterns of rodents and humans [30]. The zR is absent from the adrenal gland of the mouse. The adrenal cortex of the young mouse contains an ephemeral juxtamedullary layer, the X-zone, that regresses during puberty in males and the first pregnancy in females [31]. Lineage tracing studies have shown that the X-zone is a derivative of the fetal cortex [31], but the physiological function of the X-zone remains unclear [32]. The rat adrenal cortex has an additional layer, the undifferentiated zone (zU), located between the zG and zF. The zU contains a transitional population of cells [33].

Species also differ in the complement of steroidogenic enzymes and cofactors expressed in the adrenal cortex, and these differences have biological ramifications. For example, cells in the zF and zR of humans express *CYP17A1*, so cortisol is the

principal glucocorticoid secreted by the adrenal gland of this species [34]. Cells in the zF of adult mice and rats lack CYP17A1, so corticosterone is the main glucocorticoid produced, and adrenal androgens are not made. A newly recognized rodent-like model is the spiny mouse (genus *Acomys*) [35]. In contrast to the laboratory mouse (genus *Mus*), the adrenal cortex of the spiny mouse has a zR. Cells in the zF and zR of the spiny mouse produce cortisol and DHEA, respectively. Thus, the adrenal physiology of the spiny mouse recapitulates that of humans [9].

Role of Zonal Remodeling in the Regulation of Steroidogenesis

The adrenal cortex is a dynamic tissue that undergoes continual renewal. Senescent cells are replenished through division and differentiation of stem/progenitor cells in the periphery of the gland (i.e., the adrenal capsule and subcapsule) [36–38]. The newly formed cells migrate centripetally to repopulate cortical zones. This cellular turnover facilitates adrenocortical remodeling in response to physiological demand for steroids. Cortical zones can reversibly expand, contract, or alter their biochemical profiles in response to physiological or pharmacological triggers (Table 2.2). Zonal remodeling is one of the major mechanisms by which steroid production is modulated.

Table 2.2 Impact of various stimuli on adrenocortical remodeling and function

Zone (species)	Physiological or pharmacological stimulus	Effect	References
zG (rat)	↓ Na$^+$ or ↑ K$^+$ in diet	Expands the zone, increasing aldosterone production	[17, 23, 39]
	↑ Na$^+$ or ↓ K$^+$ in diet	Contracts the zone, decreasing aldosterone production	
	Captopril or other ACE inhibitors	Contracts the zone, decreasing aldosterone production	
	Endothelin or serotonin	Stimulates aldosterone production	
	Dopamine or atrial natriuretic peptide	Inhibits aldosterone production	
	Arginine vasopressin	Expands the zone	
zF (rat)	ACTH	Expands the zone, increasing glucocorticoid production	[23]
	Dexamethasone	Contracts the zone, decreasing glucocorticoid production	
zR (primates)	Adrenarche	Increases *CYB5* expression, enhancing DHEA production	[1, 40–42]
	Social status in marmosets	Adult females develop a functional zR based on status in the group	
	Cortisol and androstenedione	Stimulates DHEA production through competitive inhibition of HSD3B2 activity	

Secreted Peptides Implicated in Adrenocortical Growth and Steroidogenesis

The archetypal peptide hormones impacting adrenocortical function are ACTH and Ang II, but many other hormones and growth factors, working alone or in combination, have been shown to regulate adrenocortical cell function [36, 38] (Table 2.3).

Table 2.3 Secreted proteins that regulate adrenocortical cell growth and function

	Factor	Function	References
Endocrine hormones	ACTH	Stimulates cortisol and androgen biosynthesis; some of its tropic actions are mediated indirectly via growth factors	[8]
	Ang II	Stimulates aldosterone production	[23]
	Placental CRH	Directly stimulates fetal adrenal cells to produce cortisol and androgens	[13]
	Human chorionic gonadotropin (hCG)	Drives the growth of the fetal adrenal gland during the first trimester of pregnancy	[8]
	Luteinizing hormone (LH)	The LH receptor has been shown to be functionally active in the adrenal of adults during pregnancy and other high gonadotropin states	[43, 44]
	Activins	Inhibit adrenocortical cell growth/survival and modulate steroidogenesis	[45, 46]
	Inhibins	Inhibit activin signaling in adrenocortical cells	[38, 46]
Growth factors	Insulin-related growth factors (IGF1/2)	Promote adrenocortical cell mitosis and survival; enhance the effect of ACTH on steroidogenesis in fetal and adult adrenocortical cells	[47, 48]
	Epidermal growth factor (EGF)	A potent mitogen for cultured fetal and definitive zone cells from mid-gestation human fetal adrenal glands	[49]
	Fibroblast growth factor-2 (FGF2)	Acts as an adrenocortical cell mitogen; binds to zG cells	[50]
Developmental signaling molecules	Sonic hedgehog (SHH)	Ligand secreted by subcapsular cells; promotes steroidogenic differentiation of stem/progenitor cells in the capsule	[51]
	Delta-like homologue-1 (DLK1)	Transmembrane protein that is cleaved and secreted by the rat zU; regulates the differentiation of steroidogenic cell progenitors in the capsule	[33]
	Wingless-related integration site-4 (WNT4)	Wnt/β-catenin signaling is critical for zG differentiation and maintenance; downregulation of this signaling is required for zG-to-zF conversion	[52, 53]
	R-spondin-3 (RSPO3)	Ligand that potentiates Wnt/β-catenin signaling and is required for zG differentiation	[54]

Hormones and paracrine factors traditionally associated with reproductive function, such as luteinizing hormone, activins, and inhibins, can affect adrenocortical steroidogenesis.

Uptake and Intracellular Trafficking of Cholesterol: Prelude to Steroidogenesis

Initiation of steroidogenesis entails the following steps: (1) mobilization of cholesterol, the precursor of all steroid hormones, from endogenous or exogenous sources, (2) transport of cholesterol to the OMM, and (3) transfer of cholesterol from the OMM to the IMM.

Mobilization of Free Cholesterol from Intracellular and Extracellular Sources

Cholesterol can be derived from a combination of sources: (1) de novo biosynthesis, (2) import of lipoprotein-associated cholesteryl esters (CEs) via endocytosis of the low-density lipoprotein receptor (LDLR), (3) uptake of esterified cholesterol through the high-density lipoprotein (HDL) scavenger receptor 1 (SR-B1), and (4) hydrolysis of CEs stored with lipid droplets. These redundant sources ensure that adequate cholesterol is available for steroidogenesis.

De Novo Cholesterol Biosynthesis

Cholesterol is synthesized from mevalonate and isoprenoid precursors [55]. The rate-limiting step of cholesterol biosynthesis is catalyzed by hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase. Expression of HMG-CoA reductase is regulated by isoforms of sterol-regulatory element-binding protein (SREBP), a transcription factor that coordinate the synthesis, uptake, and metabolism of cholesterol and fatty acids [56]. Newly synthesized SREBP is membrane-bound and resides in the ER, where it interacts with SREBP cleavage-activating protein (SCAP), a key cellular cholesterol sensor [57] (Fig. 2.7). When cells are deficient in cholesterol, SCAP and other proteins escort SREBP from the ER to Golgi. Once in the Golgi, SREBP is proteolytically processed to generate a "mature" transcription factor that travels to the nucleus and activates genes required for de novo cholesterol synthesis (e.g., HMG-CoA reductase) and for internalization of LDL [58]. When cells are replete with cholesterol, SREBP is retained in the ER, thereby limiting its proteolytic activation.

De novo synthesis is not the principal source of cholesterol for steroidogenesis, as evidenced by the fact that patients treated with statins, inhibitors of HMG-CoA reductase, exhibit normal cortisol secretion [59, 60]. Nevertheless, de novo synthesis remains an important source of cholesterol under certain physiological and pathophysiological states [61].
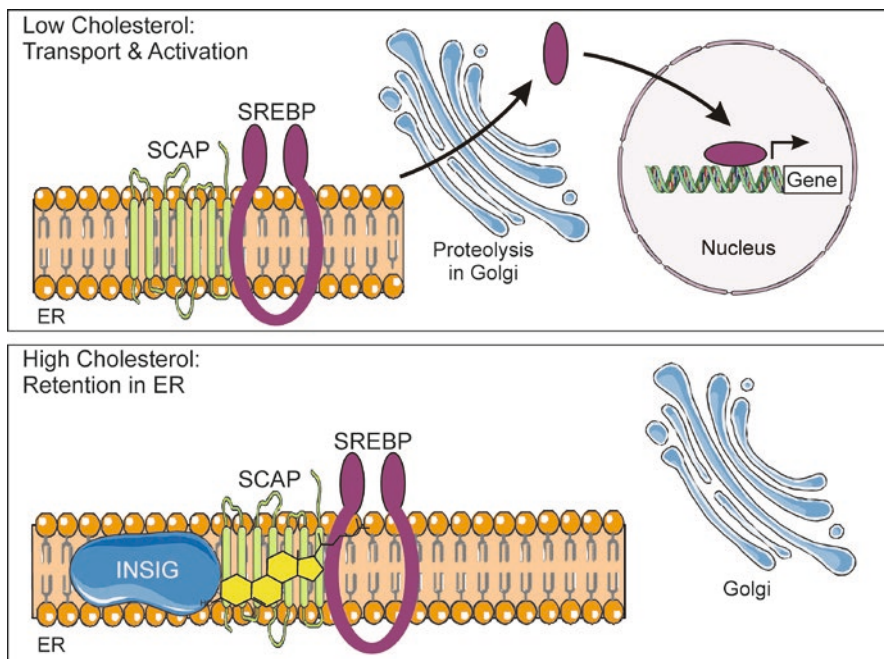
Fig. 2.7 Activation of SREBP, a key transcriptional regulator of steroidogenesis, is regulated by the cholesterol content of the ER membrane. Cholesterol homeostasis is maintained by SREBP, a membrane-associated transcription factor, working in conjunction with SCAP, a cholesterol sensor. When cholesterol levels are low, SCAP and other proteins escort SREBP from the ER to Golgi. Once in the Golgi, SREBP is proteolytically processed to generate a "mature" transcription factor that travels to the nucleus and increases the expression of genes required for cholesterol synthesis and uptake. High levels of cholesterol trigger a conformational change in SCAP, causing the SCAP-SREBP complex to associate with INSIG, an ER anchor protein. Consequently, SREBP is retained in the ER and not proteolytically activated. *ER* endoplasmic reticulum, *INSIG* insulin-induced gene, *SCAP* SREBP cleavage-activating protein, *SREBP* sterol-regulatory element-binding protein. Prepared using image vectors from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/)

Import of Esterified Cholesterol via the LDL Receptor

Approximately 80% of the cholesterol used in the synthesis of adrenal steroids derives from uptake of plasma lipoproteins by LDLR (Fig. 2.8) [62]. Apolipoprotein E (apoE) and apolipoprotein B (apoB)-containing lipoprotein particles bind to LDLR and are internalized via clathrin-coated pits [63]. In late endosomes, lipoprotein-derived CEs are hydrolyzed by lysosomal acid lipase (LIPA) to produce free cholesterol. Loss-of-function mutations in *LIPA* cause Wolman disease and its milder variant, cholesteryl ester storage disease [64]. The liberation of LDL-derived cholesterol from late endosomes requires the protein products of two other genes *NPC1* and *NPC2* [65]. NPC2 binds and facilitates the hydrolysis of CEs by
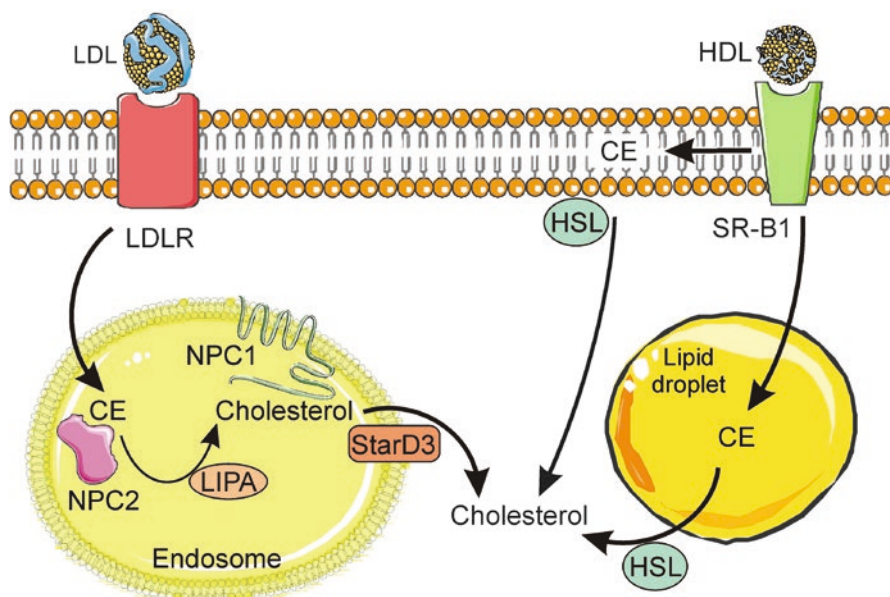
Fig. 2.8 Import of esterified cholesterol via lipoprotein receptors. Adrenocortical cells take up circulating LDL via receptor mediated endocytosis. In late endosomes, LDL-derived CE is bound by NPC2 and hydrolyzed by LIPA to yield free cholesterol, which is then transferred to NPC1, inserted into the endosomal membrane, and exported. A cholesterol-binding protein, StarD3, co-localizes with the NPC system and may participate in the egress of cholesterol from endosomes. SR-B1 mediates the uptake of CE from HDL. Some of the CE delivered via SR-B1 is incorporated into the plasma membrane, and some is directly incorporated into lipid droplets. HDL-derived CE is hydrolyzed to free cholesterol by HSL. *CE* cholesteryl ester, *HDL* high-density lipoprotein, *HSL* hormone-sensitive lipase, *LDL* low-density lipoprotein, *LDLR* LDL receptor, *LIPA* lysosomal acid lipase, *NPC1/NPC2* Niemann-Pick type C disease proteins 1 and 2, *StarD3* START domain protein 3, *SR-B1* scavenger receptor class B, type 1. Prepared using image vectors from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/)

LIPA. The resultant-free cholesterol is transferred to NPC1, inserted into the endosomal membrane, and then exported. Mutations in *NPC1* or *NPC2* cause Niemann-Pick type C disease, another lysosomal storage disorder [66].

A cholesterol-binding protein, StarD3 (MLN64), co-localizes with NPC proteins and may participate in the egress of LDL-derived cholesterol from endocytic vesicles. StarD3 is a member of the START domain (StarD) family of proteins [67]. The START [StAR (*ST*eroidogenic *A*cute *R*egulatory protein)-related lipid *T*ransfer] domain is a conserved sequence that binds sterols and other lipids [68]. The human START domain family comprises 16 proteins, 5 of which (StarD1/D3/D4/D5/D6) bind cholesterol. StarD3 contains a leader sequence that targets the molecule to late endosomes. Enforced expression of a mutant form of StarD3 lacking the START domain causes accumulation of free cholesterol in lysosomes [69]. The roles of other START domain proteins, including the founding member of the family, StarD1 (better known as StAR), are discussed later.

Uptake of Esterified Cholesterol via SR-B1

Scavenger receptor class B, type 1 (SR-B1) mediates the uptake of HDL CEs through a mechanism that differs from the endocytic pathway used by the LDL receptor (Fig. 2.8) [70]. SR-B1 localizes to lipid rafts, membrane domains rich in cholesterol and sphingolipids. SR-B1 forms a nonaqueous channel [71]. Binding of HDL to SR-B1 results in the "selective uptake" of esterified cholesterol into the plasma membrane, followed by the release of the resultant lipid-depleted HDL particles back into the circulation. Some CEs delivered via SR-B1 appear to be incorporated directly into lipid droplets [72, 73]. CEs imported into the plasma membrane or lipid droplets by SR-B1 may be hydrolyzed to free cholesterol by hormone-sensitive lipase (HSL). Uptake through SR-B1 is the major source of cholesterol for steroidogenesis in rodents [74]. SR-B1 impacts steroidogenesis in human cell cells, too. Incubation of human HAC15 adrenocortical cells with cholesterol-free synthetic HDL leads to an efflux of cholesterol and a decrease in cortisol production [75].

Hydrolysis of Cholesteryl Esters Stored in Lipid Droplets

Hydrolysis of CEs stored within lipid droplets is the preferred source for cholesterol in the setting of acute hormonal stimulation. HSL, an enzyme activated in response to binding of ACTH or Ang II to their cognate receptors, catalyzes the hydrolysis of lipid droplet-associated CEs [73, 76]. ACTH activates HSL via PKA-mediated phosphorylation, whereas Ang II activates HSL via mitogen-activated protein kinases (MAPKs) (see later). In response to phosphorylation by these kinases, HSL is translocated from the cytoplasm to either the plasma membrane or the surface of lipid droplets, facilitating hydrolysis of CEs at these sites. In addition to catalyzing the hydrolysis of CEs, HSL interacts with various cholesterol-binding proteins to help direct the cholesterol to the OMM for steroidogenesis [77].

Esterification of Excess Free Cholesterol by SOAT1

Excess-free cholesterol, which is toxic to cells, may be esterified by the ER enzyme sterol O-acyltransferase 1 (SOAT1) [also called acyl-CoA:cholesterol O-acyltransferase 1 (ACAT1)]. The resultant CEs may be incorporated into lipid droplets for storage. Drugs that inhibit SOAT1 activity, such as mitotane and ATR-101, induce the accumulation of cholesterol and free fatty acids in the ER of adrenocortical cells (Fig. 2.9) [78–80]. This increase in cholesterol limits SREBP activation, resulting in downregulation of target genes that mediate cholesterol synthesis and uptake. The accumulation of cholesterol and free fatty acids induces ER stress, which triggers transcription of unfolded protein response (UPR) genes [81]. Persistent ER stress leads to upregulation of pro-apoptotic *BAX* and repression anti-apoptotic *BCL2*, thus inducing cell death [79]. Targeted deletion of *SOAT1* in an
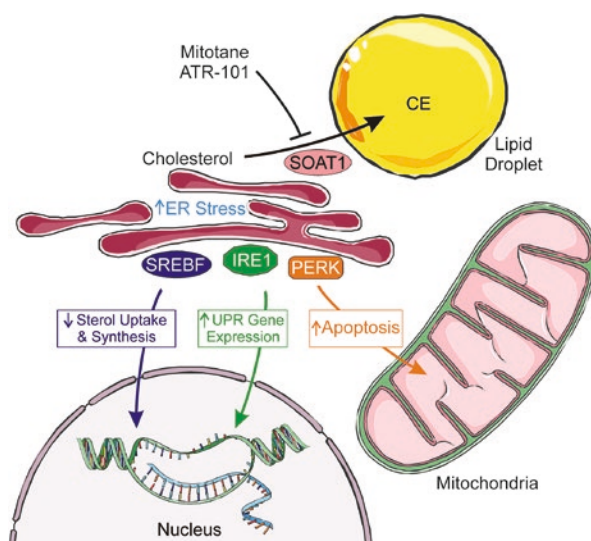
Fig. 2.9 Pharmacological inhibition of SOAT1, the enzyme that esterifies excess cholesterol, impairs adrenocortical steroidogenesis and cell survival. Inhibition of SOAT1 by either mitotane or ATR-101 leads to an increase in free cholesterol and fatty acids that (1) cause downregulation of SREBF-dependent genes that mediate cholesterol synthesis and uptake and (2) trigger ER stress, leading to IRE1-dependent splicing of XBP1 mRNA and subsequent transcription of UPR genes. As ER stress persists, increased expression of *PERK* induces increased *CHOP* expression, which triggers expression of pro-apoptotic *BAX* and repression of anti-apoptotic *BCL2*, thus inducing cell apoptosis. *CE* cholesteryl ester, *CHOP* CCAAT-enhancer-binding protein homologous protein, *ER* endoplasmic reticulum, *PERK* PKR-like ER kinase, *SREBF* sterol-regulatory element-binding protein, *SOAT1* sterol O-acyltransferase 1, *UPR* unfolded protein response, *XBP1* X-box-binding protein. Prepared using image vectors from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/)

adrenocortical cell line recapitulates the effects of pharmacological inhibitors of SOAT1 [78]. Inhibition of cholesterol esterification is thought to be the mechanism of action of mitotane, the only drug approved for the treatment of patients with adrenocortical carcinoma (ACC) (Fig. 2.9). ATR-101 is undergoing preclinical and clinical testing as a new drug for the treatment of ACC [78].

Transport of Free Cholesterol to the OMM

Mitochondria contain a limited amount of cholesterol, the majority of which resides in the OMM [82, 83]. Therefore, cholesterol consumed during steroidogenesis must be continually replenished. Mitochondrial stores cannot be restocked with cholesterol via simple diffusion across aqueous cytoplasmic spaces, because this compound is relatively insoluble in water. Instead, "free" cholesterol is trafficked to

mitochondria via two general mechanisms: (1) vesicular transport, an energy-dependent process entailing budding and fusion of membrane vesicles, and (2) non-vesicular transport via soluble cholesterol-binding proteins [61, 71]. Close physical interactions between mitochondria and other subcellular compartments (e.g., ER, lipid droplets) promote cholesterol transfer.

Role of the Cytoskeleton in Cholesterol Delivery to the OMM

Intracellular vesicular trafficking of cholesterol requires rearrangement of an integrated network of cytoskeletal elements, composed of intermediate filaments, microtubules, and other structures [84–86]. Some cytoskeletal rearrangements appear to be driven by the protein myristoylated alanine-rich C-kinase substrate (MARCKS), which is phosphorylated in adrenocortical cells in response to hormonal stimulation [87] and can associate with actin filaments and the membrane surfaces [88]. Another key cytoskeletal protein is the intermediate filament vimentin, which binds to lipid droplets and to HSL and appears to regulate cholesterol delivery to mitochondria. Pharmacological or genetic disruption of cytoskeletal structure impacts cholesterol trafficking. For example, vimentin null mice exhibit decreased ACTH-stimulated corticosterone levels [89].

Role of SNARE Proteins in Cholesterol Delivery to Mitochondria

SNARE proteins [an acronym derived from "SNAP (*S*oluble *N*-ethylmaleimide-sensitive factor *A*ttachment Protein) *RE*ceptor"] are GTP-binding proteins that mediate vesicle fusion [90]. The most extensively studied SNARE proteins are those that mediate the fusion of synaptic vesicles with the presynaptic membrane in neurons, but recent studies suggest that these proteins also regulate cholesterol transfer [91]. Certain SNARE proteins, including α-SNAP and SNAP25, are associated with lipid droplets [92]. Gene-silencing experiments and in vitro reconstitution assays have shown that specific SNARE proteins [α-SNAP, SNAP25, syntaxin (STX)-5, and STX-17] are required for efficient cholesterol movement to mitochondria for steroidogenesis [91]. A subset of SNAREs, including STX-5, exhibit cholesterol-binding properties, and this may aid in the trafficking of cholesterol from lipid droplets to the OMM [93, 94].

MAM-Facilitated Transfer of Cholesterol to the OMM

Trafficking of cholesterol to mitochondria is also facilitated by the mitochondria-associated ER membrane (MAM), a distinct subdomain of the ER that is reversibly tethered to the OMM by lipids and protein filaments [71, 95, 96]. MAMs serve as hubs that coordinate metabolic interactions between these two organelles. MAMs have been implicated in a wide array of cellular processes, including lipid transport
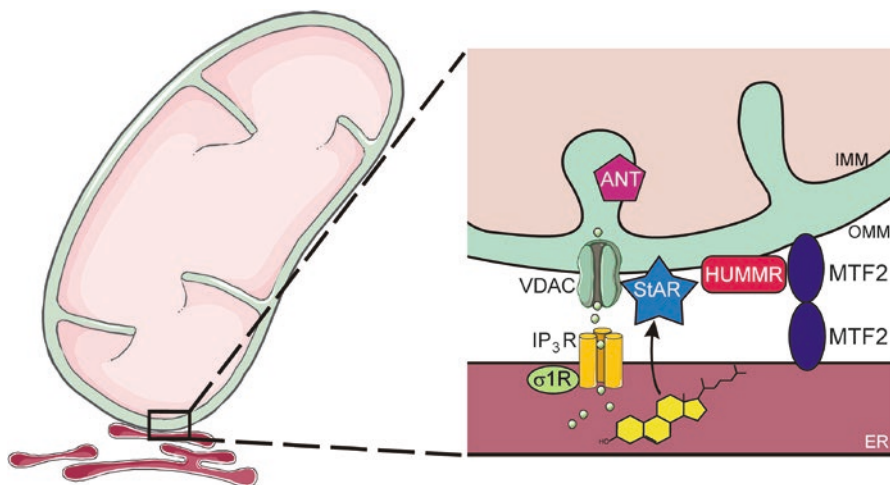
Fig. 2.10 Mitochondria-associated ER membrane (MAM). The ER communicates with mitochondria via the MAM, a specialized subdomain of the ER with the characteristics of a lipid raft. MAMs serve to coordinate metabolic interactions (e.g., lipid transport, Ca^{2+} signaling) between these two organelles. The following MAM components are illustrated: MTF2 (a tether), VDAC (a channel), σ-1 (a receptor), StAR (a transport protein), HUMMR (an OMM adaptor protein), ANT (an IMM nucleotide translocase), and IP$_3$R, (a receptor and Ca^{2+} channel). *ANT* adenine nucleotide translocase, *ER* endoplasmic reticulum, *IMM* inner mitochondrial membrane, *HUMMR* hypoxia-upregulated mitochondrial movement regulator, *IP$_3$R* inositol (1,4,5) trisphosphate receptor MTF2, mitofusin 2, *OMM* outer mitochondrial membrane, *σ1R* sigma-1 receptor, and *VDAC* voltage-dependent anion channel. Prepared using image vectors from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/)

(e.g., mitochondrial import of phosphatidylserine), Ca^{2+} signaling, energy metabolism, mitochondrial dynamics, and apoptosis [97, 98].

MAMs are composed of cholesterol-rich lipid rafts and resident membrane proteins (Fig. 2.10). MAM proteins include: (1) mitofusin 2 (MTF2), which functions as a tether; (2) FATE1, which modulates ER-mitochondrial distance [99]; (3) the voltage-dependent anion channel (VDAC), an OMM-associated protein that allows for the passage of ions, metabolites, and signaling molecules (e.g., ATP) across the membrane (see later); and (4) sigma-1 receptor, which promotes cholesterol compartmentalization and trafficking [100, 101]. It has been proposed that cholesterol transfer from OMM to IMM occurs at specialized contact sites bridging the two membranes composed of VDAC and IMM adenine nucleotide translocase (ANT).

Genetic evidence supporting a role for the MAM in steroidogenesis has emerged from the isolation and characterization of mutant cell lines with altered intracellular cholesterol homeostasis. One such cell line harbors a mutation in the H/ACA small nucleolar RNA (snoRNA) U17, which regulates hypoxia-upregulated mitochondrial movement regulator (HUMMR), an OMM adaptor protein that promotes the formation of ER/mitochondrial contact sites [102].

Role of StarD4/D5/D6 in Cholesterol Trafficking

As mentioned earlier, there are five START proteins in the human genome that can bind cholesterol. Two of these have leader peptides that direct them to specific organelles: StarD1 (StAR itself), which is targeted to mitochondria, and StarD3, which is targeted to endosomes (see later). The remaining members of this subgroup—StarD4, StarD5, and StarD6—lack leader peptides and appear to be cytosolic proteins involved in non-vesicular cholesterol transport in various cell types, including steroidogenic cells. StarD4 is thought to be the principal agent delivering cholesterol to the OMM from elsewhere in the cell [67]. StarD5 binds cholesterol and bile acids, whereas StarD6 binds cholesterol and steroid hormones.

Transfer of Cholesterol from the OMM to the IMM

Once cholesterol reaches the OMM, a group of proteins led by steroidogenic acute regulatory protein (StAR) facilitate the transport of cholesterol to the IMM, the site of pregnenolone synthesis by CYP11A1. The delivery of cholesterol from the OMM to the IMM is the rate-limiting step in steroid production in adrenocortical cells. Hormones, such as ACTH and Ang II, rapidly stimulate this process. Transport occurs preferentially at sites of close contact between the OMM and IMM, and such sites are more plentiful in tubulovesicular than lamelliform mitochondria. The preponderance of tubulovesicular mitochondria in the zF compared to the zG reflects higher levels of steroidogenesis in the former [23].

StAR

The acute response to hormone stimulation is characterized by a rapid increase in the rate of steroid hormone biosynthesis, and classic studies indicated that the induction of steroidogenesis requires new protein synthesis (i.e., is cycloheximide-sensitive) [103, 104]. The acute steroidogenic response is controlled by StAR (StarD1), a rapidly synthesized and short-lived phosphoprotein [105, 106]. The principal function of StAR is to move cholesterol from the OMM to the IMM.

   The expression and activation of StAR are tightly regulated by various signaling kinases, including PKA, protein kinase C (PKC), and extracellular signal-regulated kinases 1 and 2 (ERK1/2) [107, 108]. In response to hormonal stimulation, StAR is phosphorylated [109, 110], which enhances its activity [111]. Hormonal stimulation also modulates the activity of various transcription factors that regulate *StAR* expression [112, 113] (Fig. 2.11). For example, PKA-mediated activation of HSL leads to increased production of not only free cholesterol but also oxysterols, which activate liver X receptors (LXRs) that upregulate *StAR* transcription in a feed-forward manner for steroidogenesis [114, 115].
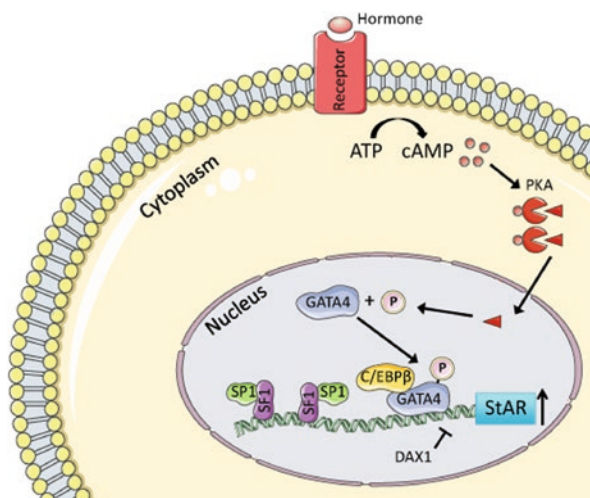
Fig. 2.11 Transcriptional regulation of *StAR* expression in steroidogenic cells. Shown is the regulation of *StAR* expression in testicular Leydig cells; similar mechanisms operate in fetal and adult adrenocortical cells. Hormonal stimulation leads to increases in the level of cAMP. Binding of cAMP to the regulatory subunit of PKA (PRKAR1A) allows dissociation of the catalytic subunit (triangle) and its translocation to the nucleus, where it phosphorylates target transcription factors, including GATA4. SF1 is essential for basal StAR expression and binds another widely expressed transcription factor, Sp1. CCAAT-enhancer-binding protein (C/EBPβ) acts synergistically with GATA4. DAX1 functions as a repressor. Prepared using image vectors from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/)

The *StAR* gene encodes a 37 kDa "precursor" protein that harbors a mitochondrial targeting sequence [108, 116]. When StAR engages the OMM, portions of the molecule become membrane-associated, and StAR undergoes a conformational change that allows it to bind and release cholesterol [117]. As StAR is imported into the mitochondria, it undergoes proteolytic processing from a 37 kDa to a "mature" 30 kDa form [3]. This 30 kDa form localizes to the mitochondrial matrix and retains full StAR activity. Each molecule of StAR is thought to recirculate across the mitochondrial membranes many times, delivering up to 400 molecules of cholesterol to the IMM [118]. To prevent mitochondrial impairment from excess StAR accumulation, the 30 kDa form of the molecule is eventually degraded by proteases in the mitochondrial matrix [119]. Acute mitochondrial accumulation of StAR provokes a retrograde mitochondrial-nuclear signaling and transcriptional upregulation of the proteases involved in the degradation of StAR [119].

The importance of StAR for steroidogenesis is underscored by the disease lipoid congenital adrenal hyperplasia (lipoid CAH), an autosomal recessive disorder caused by loss-of-function mutations in *StAR* [120, 121]. Lipoid CAH is characterized by enlarged, lipid-laden adrenal glands that make minimal steroids. The condition is lethal in the neonatal period unless promptly diagnosed and treated with corticosteroids. Targeted ablation of the *Star* gene in mice recapitulates the phenotype of lipoid CAH in humans [122–124].

StAR's Entourage

StAR interacts with a variety of membrane-associated proteins. It has been proposed that these proteins are organized into a dynamic complex termed the transduceosome, which functions to translocate cholesterol efficiently to the IMM [125]. In addition to StAR, the transduceosome contains VDAC, the translocator protein (TSPO), a TSPO-associated protein PAP7, and other proteins [126]. In steroidogenic tissues that do not express StAR, such as the placenta, the remaining components of the transduceosome, working in conjunction with StarD3, may provide basal cholesterol transport to the IMM for production of pregnenolone.

Voltage-Dependent Anion Channel

VDAC is an OMM channel-forming protein that regulates the passage of ions and small molecules through the OMM. This function determines membrane potential, thereby regulating cell metabolism [127]. There are three isoforms of VDAC, termed VDAC-1, VDAC-2, and VDAC-3 [95]. In addition to controlling metabolism, VDAC isoforms influence programmed cell death by facilitating the release of apoptotic proteins located in the intermembranous space and serving as the proposed target of pro- and anti-apoptotic members of the BCL2 family [127].

VDAC, acting in conjunction with the σ-1 receptor in the MAM, helps facilitate cholesterol transfer for the initiation of pregnenolone synthesis [101]. VDAC interacts with the 37 kDa StAR precursor at the OMM and controls proteolytic processing, allowing the translocation of StAR into mitochondria as a mature 30 kDa protein [96, 128].

Translocator Protein: Necessity is the Mother of Contention

Translocator protein (TSPO), also known as the peripheral benzodiazepine receptor (PBR), is a highly conserved OMM protein [129]. *TSPO* is expressed in most mammalian tissues and found at highest levels in steroid-producing cells. Early evidence suggested that TSPO supports steroidogenesis by modulating cholesterol transport into mitochondria. The TSPO/PBR ligand PK11195 was shown to stimulate steroidogenesis in both adrenocortical and Leydig cell lines [130]. Mutagenesis and modeling studies indicated that TSPO could bind cholesterol with high affinity [131, 132]. TSPO and StAR were shown to be closely associated in fluorescence energy transfer experiments [133].

Recent experiments, however, have challenged the necessity of TSPO for steroidogenesis, inciting contentious debate [95, 129, 134–136]. Gene targeting studies in cell lines and mice have yielded conflicting results, ranging from no effect on

steroidogenesis to impairment of ACTH-stimulated glucocorticoid production [135, 137–140]. The precise function of TSPO remains an area of active investigation [141].

PAP7/ACBD3

PBR-associated protein 7 (PAP7) was identified in a yeast-two hybrid screen employing TSPO/PBR as bait [142]. In steroidogenic cells, PAP7 binds TSPO and the PKA regulatory subunit 1α (PKAR1A) [142, 143]. The PKA holoenzyme comprises two regulatory subunits and two catalytic subunits. Binding of cAMP to the regulatory subunits of PKA triggers the release of catalytic subunits, thereby activating the enzyme. PAP7 has been proposed to function as a kinase-anchoring protein that recruits and confines the PKA holoenzyme at key intracellular sites. A rise in cAMP leads to local release of the catalytic subunits, which phosphorylate StAR present at the OMM [144]. In addition to binding PKAR1A, PAP7 contains an acyl-coenzyme A (CoA)-binding domain, prompting its renaming to acyl-CoA-binding domain containing protein 3 (ACBD3).

Regulation of Cortisol Secretion

Glucocorticoids are essential for basal homeostasis and the response to stress, so production of cortisol is tightly regulated by HPA axis and locally acting factors.

ACTH Synthesis and Release

ACTH, a 39-amino acid peptide secreted by the anterior pituitary, is the principal stimulus for cortisol production [24]. When the HPA axis is activated by stress or other stimuli, neurons in the hypothalamic paraventricular nucleus secrete CRH and arginine vasopressin (AVP) into the hypophyseal portal circulation [145, 146]. CRH is the main stimulant of ACTH release. AVP acts synergistically with CRH to enhance ACTH secretion, although AVP is ineffective in the absence of CRH [147].

ACTH is derived from pro-opiomelanocortin (POMC), a 214-amino acid precursor synthesized in the anterior pituitary and limited other sites [148]. POMC can be proteolytically processed to generate ACTH and several other peptides, including α-melanocyte-stimulating hormone (α-MSH) and β-endorphin [149]. The N-terminal 18 amino acids of ACTH confer its biological activity, so shorter synthetic peptides [ACTH (1–24) and ACTH (1–18)] are used clinically in lieu of

full-length ACTH. The sequence of α-MSH, which is contained within the ACTH peptide, stimulates the production of melanin by melanocytes, resulting in skin hyperpigmentation when secreted in excess, as in the ACTH-dependent Cushing syndrome.

ACTH promotes steroidogenesis at three levels: (1) acute ACTH exposure rapidly (within minutes) stimulates the transport of cholesterol from the OMM to the IMM, (2) longer term exposure to ACTH (hours) promotes transcription of genes encoding steroidogenic enzymes, notably *CYP11A1*, and (3) prolonged exposure to ACTH (weeks to months) promotes zonal expansion and adrenal growth [126, 150].

Feedback Inhibition of the HPA Axis

Cortisol is the main negative regulator of the HPA axis (Fig. 2.6). Feedback inhibition by cortisol is mediated via binding to GRs in the pituitary and hypothalamus. Cortisol also acts on suprahypothalamic centers (e.g., hippocampus) to further limit the secretion of CRH [151]. Feedback inhibition in suprahypothalamic centers is mediated by cortisol binding to GRs and MRs [152].

Circadian Rhythm of Glucocorticoid Secretion

ACTH is secreted in regular pulses of varying amplitude. Peak ACTH secretion occurs in the early morning, coinciding with a rise in cortisol secretion [153]. The diurnal production of cortisol, which anticipates times of increased activity and energy demand, reflects an interplay between environmental cues (e.g., fluctuations in light intensity) and an internal circadian timekeeping system [154]. Disruption of this interplay contributes to the phenomenon of jetlag [155].

The circadian timekeeping system is composed of a central clock [the hypothalamic suprachiasmatic nucleus (SCN)] and subsidiary peripheral clocks in nearly every cell type, including adrenocortical cells [154]. The SCN is entrained to the light-dark cycle [156]. Highlighting the importance of the central clock, the diurnal glucocorticoid rhythm is altered by lesions of the SCN [157] or constant light exposure [158]. Signals from the SCN are relayed via the sympathetic nervous system to peripheral clocks in the adrenal cortex (Fig. 2.12). The rhythmic secretion of glucocorticoids from the adrenal gland is thought to synchronize peripheral circadian rhythms of tissues downstream of the SCN [155, 159].

The central and peripheral circadian clocks are composed of interlocking positive and negative transcriptional-translational feedback loops that oscillate with a periodicity of approximately 24 hours [154]. Genetically-engineered mice lacking of key components of the molecular clock exhibit either chronic elevation or
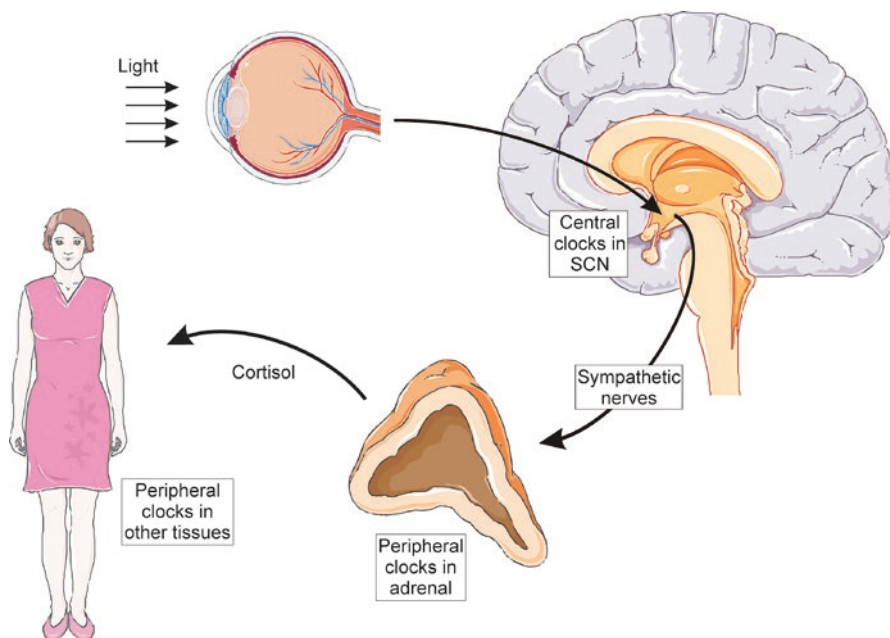
Fig. 2.12 Clock regulation of glucocorticoid secretion. Light received at the retina entrains central circadian clocks in the suprachiasmatic nucleus (SCN). Signals from the SCN are relayed via the sympathetic nervous system to peripheral clocks in the adrenal cortex. The rhythmic secretion of cortisol from the adrenal gland helps synchronize peripheral circadian rhythms of tissues downstream of the SCN. Prepared using image vectors from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/)

suppression of glucocorticoid levels; rhythmic expression of certain steroidogenic genes, including *Star*, is altered in the adrenocortical cells of these animals [160–165]. Peripheral clocks in the adrenal cortex are thought to modulate sensitivity to ACTH, although the mechanistic basis for this effect is unclear.

MC2R and MRAPs

The trophic effects of ACTH are mediated through its plasma transmembrane G-protein-coupled receptor MC2R (Fig. 2.13). Melanocortin 2 receptor accessory proteins (MRAPs) are small membrane proteins that are essential for ACTH signaling. MRAP deficiency is one of the causes of hereditary unresponsiveness to ACTH [166]. Two types of MRAPs have been identified: MRAP1 and MRAP2. In humans, there are two distinct isoforms of MRAP1, termed MRAPα and MRAPβ, which share the same N-terminus and transmembrane domain but differ in their C-terminal
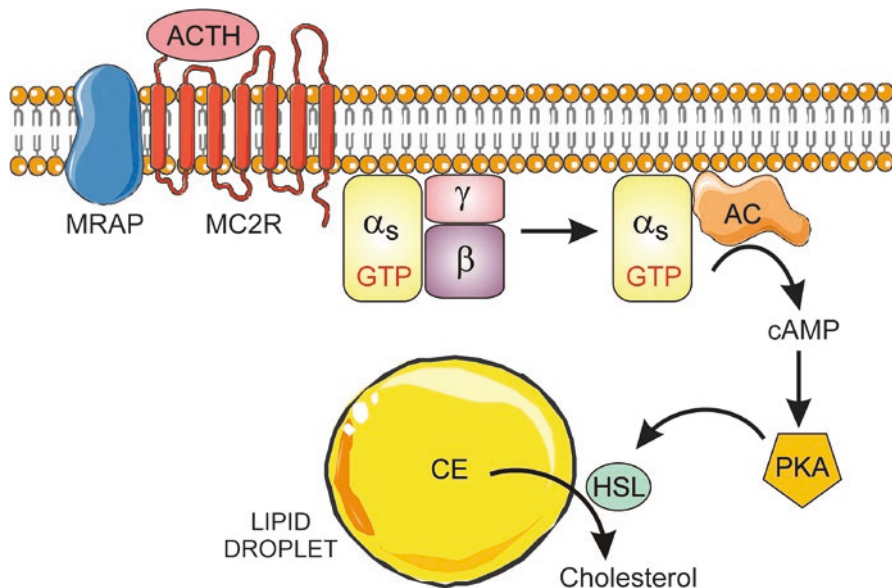
Fig. 2.13 ACTH-stimulated mobilization of cholesterol from lipid droplets. ACTH binds to its receptor MC2R. The ligand-bound receptor, in conjunction with the accessory protein MRAP, triggers the activation of AC via the heterotrimeric G-protein G_s. The resultant increase in cAMP activates PKA, leading to phosphorylation of HSL. The latter enzyme hydrolyzes CE in lipid droplets, producing free cholesterol for steroidogenesis. *AC* adenylate cyclase, *CE* cholesteryl ester, *HSL* hormone-sensitive lipase, *MC2R* melanocortin 2 receptor, *MRAP* melanocortin 2 receptor accessory protein, *PKA* protein kinase A. Prepared using image vectors from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/)

domains. MRAP1 isoforms are involved in intracellular trafficking and signaling of MC2R. MRAPα has been implicated in targeting of MC2R to the plasma membrane, whereas MRAPβ appears to enhance cAMP production by ACTH-bound MC2R [153]. MRAP2 also is involved in trafficking of MC2R to the plasma membrane.

Activation of cAMP Signaling

Binding of ACTH to MC2R causes the α-subunit of the stimulatory heterotrimeric G-protein G_s to associate with adenylate cyclase (AC), resulting in cAMP production [167]. This rise in cAMP enhances lipid synthesis, protein synthesis, and protein phosphorylation, all of which promote cholesterol trafficking to mitochondria and the production of steroid hormones [167]. Most of these effects are mediated by PKA. cAMP binds to the regulatory subunits of PKA, allowing the catalytic subunits of PKA to phosphorylate downstream effectors. For example, PKA-induced

phosphorylation and activation of HSL enhances free cholesterol formation, while phosphorylation of StAR promotes transport of cholesterol from the OMM to the IMM [111]. PKA-dependent phosphorylation enhances CE uptake by enhancing the transcription and stability of SR-B1 [70]. PKA also phosphorylates transcription factors, leading to enhanced expression of steroidogenic genes such as CYP11A1 [167]. The transcription factors phosphorylated include steroidogenic factor 1 (SF1), cAMP response element-binding protein (CREB), and activator protein 1 (AP1) [150]. PKA-mediated phosphorylation of L-type Ca^{2+} channels activates a slow but sustained influx of Ca^{2+} that triggers additional signaling pathways.

Loss-of-function mutations in the protein kinase A regulatory subunit gene (*PRKAR1A*) cause excessive cAMP production. Such mutations underlie Carney complex, a syndrome associated with the pituitary-independent Cushing syndrome and adrenocortical neoplasia. Conditional deletion of *Prkar1a* in the adrenal cortex of mice leads to not only excess glucocorticoid production but also impaired apoptosis [168], mediated in part by crosstalk between the PKA and mammalian target of rapamycin (mTOR) pathways [169].

Somatic gain-of-function mutations in the *PRKACA* gene, which encodes the catalytic subunit of PKA, cause the ACTH-independent Cushing syndrome due to cortisol-producing adenomas [170, 171]. Germline duplications of this gene can cause bilateral adrenal hyperplasia [170].

Although the initial and most significant actions of ACTH are mediated through cAMP and the subsequent activation of PKA, there are also PKA-independent effects of cAMP, such as those mediated by Epac (the Exchange protein directly activated by cAMP) [172, 173]. One Epac isoform, Epac2, is highly expressed in the adrenal cortex and functions to activate Rap GTPases [174]. Epac proteins regulate various cellular processes via mechanisms that include modulation of gene expression and cytoskeletal rearrangements [153, 173, 174]. Treatment with an Epac-selective analogue of cAMP is sufficient to stimulate the expression of steroidogenic enzymes and cortisol secretion [175].

Phosphodiesterases

The level of intracellular cAMP is determined not only by its rate of synthesis by AC but also its rate of degradation by phosphodiesterases (PDEs) [176]. In mammals there are 11 families of PDEs, each with distinctive properties [177]. In mice, treatment with a PDE-selective inhibitor increases phosphorylation of HSL and basal corticosterone secretion [178]. Loss-of-function mutations in *PDE8B* or *PDE11A* have been linked to bilateral adrenal hyperplasia and Cushing syndrome in humans [176, 178].

Hydrolysis of cAMP by PDEs produces AMP, which in turn stimulates the AMP-activated protein kinase (AMPK) [179]. Activated AMPK represses the expression of transcription factors known to stimulate steroidogenesis (e.g., NUR77 and cJUN) and activates the expression of repressors of steroidogenesis (e.g., DAX1 and cFOS) [180] (Fig. 2.14).
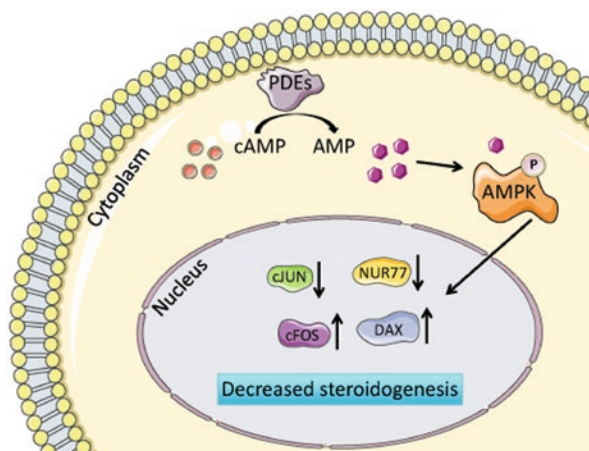
Fig. 2.14 Termination of cAMP signaling. Hydrolysis of cAMP by PDEs produces AMP, which in turn stimulates AMPK. Activated AMPK represses the expression of transcription factors known to stimulate steroidogenesis (e.g., NUR77 and cJUN) and activates the expression of repressors of steroidogenesis (e.g., DAX1 and cFOS). Prepared using image vectors from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/)

Interplay of cAMP Signaling and Arachidonic Acid Metabolism

ACTH stimulation induces the release of arachidonic acid (AA) from phospholipid stores, and this fatty acid and its metabolites have been shown to enhance steroidogenesis [181, 182]. Intracellular levels of free AA are tightly regulated by not only phospholipases, such as phospholipase A_2 (PLA_2), but also by acyl-CoA synthetases and thioesterases. Most acyl-CoA synthetases exhibit a broad specificity with respect to fatty acid substrate, but acyl-CoA synthetase 4 (ACSL4) prefers AA as a substrate [183, 184]. Originally characterized as an activity in platelets [185], ACSL4 is highly expressed in steroidogenic tissues including the adrenal gland and gonads [183, 186]. The expression of *Acsl4* mRNA is upregulated through a cAMP-dependent pathway, and pharmacological inhibition of ACSL4 reduces steroidogenesis in adrenocortical cell lines [187]. The hormonal regulation of steroid synthesis requires the concerted action of ACS4 and a second enzyme, mitochondrial acyl-CoA thioesterase 2 (ACOT2), which cleaves arachidonoyl-CoA into its component parts. These two enzymes constitute an AA generation/export system, which releases AA in mitochondria in response to ACTH stimulation [188]. AA is then metabolized into lipoxygenated or epoxygenated products that induce the expression of *StAR* [182, 187, 188].

Other Signaling Pathways Activated by ACTH

ACTH triggers a transient increase in total protein tyrosine phosphatase (PTP) activity in zF cells and a concomitant decrease in the phosphotyrosine level of several proteins [189]. Treatment with PTP inhibitors reduces hormone-induced

stimulation of steroidogenesis [153], and PTPs have been implicated in the regulation of StAR induction and cholesterol transport to the IMM [189]. One particular PTP, PTPN11, has been shown to regulate the expression of *ACSL4* [190].

MAPKs have also been implicated in the action of ACTH [24]. This kinase family comprises the extracellular signal-related kinases ERK1/2 (p42/p44[mapk]), the p38 MAPKs, and the p54 stress-activated JNK protein kinases [191]. MAPK targets in steroidogenic cells include HSL [76] and CYP17A1 (see later).

JAK/STAT (Janus kinase/signal transducer and activator of transcription) signaling is also activated by ACTH. Pharmacological or siRNA-mediated inhibition of JAK2 impairs ACTH-induced steroidogenesis [192]. Nuclear JAK2 regulates the amount of active CREB through tyrosine phosphorylation and prevention of proteasomal degradation, which in turn leads to transcriptional upregulation of *StAR* [192].

ACTH-Independent Mechanisms Regulating Cortisol Secretion

Although ACTH is the principal stimulus for glucocorticoid secretion, there are several ACTH-independent mechanisms that regulate cortisol release from the adrenal cortex. A variety of neuropeptides, neurotransmitters, and cytokines bind receptors on the surface of zF cells and modulate glucocorticoid secretion [193]. Additionally, factors secreted by endothelial cells and adipocytes influence the secretion of adrenal steroids [194, 195]. The close anatomical relationship among adrenocortical cells, medullary chromaffin cells, and nerve endings facilitates paracrine modulation of adrenal secretion of glucocorticoids [193].

Regulation of Aldosterone Secretion

The zG is optimized for the synthesis of aldosterone. Cells in this zone express *CYP11B2* (aldosterone synthase) but not *CYP17A1*, the enzyme that directs steroid substrates toward cortisol and androgen synthesis. The principal controllers of aldosterone production are Ang II and extracellular K^+, although ACTH also plays a role [17]. Ang II and extracellular K^+ act mainly by generating a cytosolic Ca^{2+} signal [196]. The signaling pathways and effectors employed include phospholipase C (PLC), inositol 1,4,5-trisphosphate (IP_3), Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), diacylglycerol (DAG), PKC, MAPKs, tyrosine kinases, and PKA. Stimulation of these signaling pathways leads to the direct activation of several transcription factors. This, in turn, increases the expression of steroidogenic genes, including *CYP11B2*.

ACTH Signaling in the zG

In addition to regulating cortisol secretion in zF cells, ACTH can induce aldosterone production in the zG. As in the zF, ACTH acts on zG cells by binding to its receptor, MCR2, and activating AC via G_s [17]. This leads to increased cAMP/PKA

signaling, which facilitates the movement of cholesterol from the OMM to the IMM and activates transcription of key steroidogenic genes. Binding of ACTH to its receptor also impacts the electrical properties of zG cells [24].

Ang II Signaling

The zG controls extracellular fluid volume and salt balance as part of the renin-angiotensin-aldosterone system [17, 23]. Renin is a protease secreted by the juxta-glomerular apparatus of the kidney in response to extracellular fluid depletion, low sodium concentrations, or hypotension. Renin cleaves angiotensinogen, a glycoprotein constitutively secreted into the serum by the liver, yielding the decapeptide angiotensin I (Ang I). Angiotensin-converting enzyme (ACE), a protein expressed on the surface of pulmonary and renal endothelial cells, subsequently converts Ang I into to the vasoactive octapeptide Ang II.

The binding of Ang II to AT_1R induces coupling to $G_{\alpha q}$, resulting in activation of PLC [153, 196] (Fig. 2.15). The latter hydrolyzes phosphatidylinositol-4,5-bisphosphate
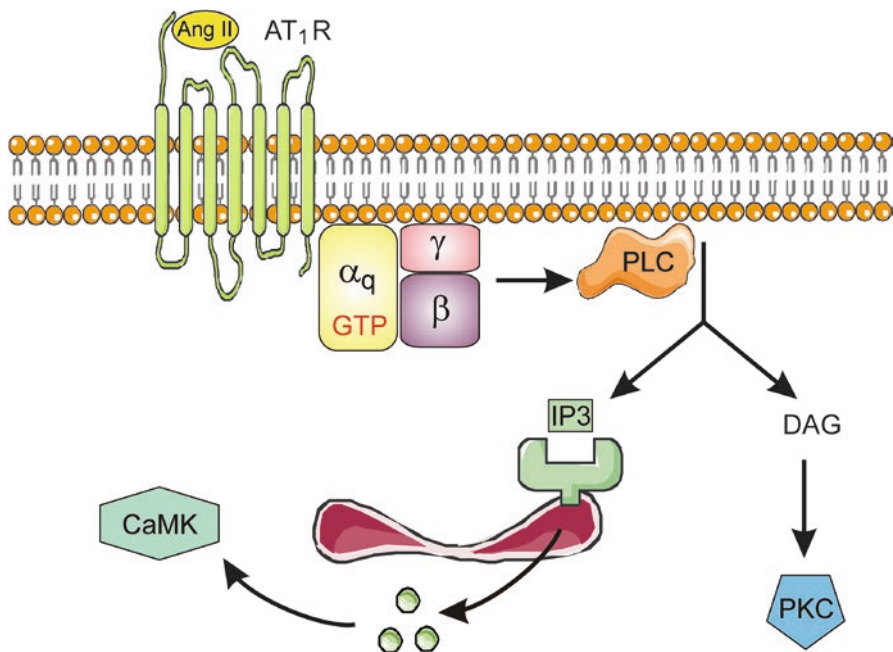
Fig. 2.15 Signaling pathways regulated by Ang II. Ang II binds to AT1R on zG cells and activates PLC via the heterotrimeric G-protein Gq, leading to the production of the second messengers IP$_3$ and DAG. IP$_3$ binds to its receptor and releases Ca^{2+} from intracellular stores into the cytoplasm. This triggers activation of CaMKs. DAG activates PKC. *Ang II* angiotensin II; *AT$_1$R* angiotensin II receptor, type 1; *CaMK* Ca^{2+}/calmodulin-dependent protein kinase; *DAG* diacylglycerol; IP$_3$, inositol (1,4,5) trisphosphate; *PKC* protein kinase C; *PLC* phospholipase C. Prepared using image vectors from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/)

[PI(4,5)P$_2$] to produce the second messengers IP$_3$ and DAG. IP$_3$ binds to its receptor and mobilizes Ca^{2+} from intracellular depots into the cytoplasm. This leads to activation of CaMKs. DAG binds to PKC, resulting in the phosphorylation and activation of additional second messenger cascades that modulate the activity of transcription factors involved in aldosterone production [24, 197].

The binding of Ang II to its receptor also inhibits K$^+$ channels (Fig. 2.16). This inhibition causes cell depolarization, leading to the opening of voltage-gated Ca^{2+} channels. The ensuing increase in cytoplasmic Ca^{2+} activates CaMKs, which drive cell proliferation and the expression of *CYP11B2*, the enzyme that catalyzes the last steps of aldosterone synthesis [197]. In a fashion analogous to PKA in zF cells, the Ca^{2+} signal in zG cells leads to activation of HSL and StAR (via ERK1/2 or CaMK) [76, 198]. Additionally, the cytoplasmic Ca^{2+} signal is transferred to the mitochondrial matrix, where it enhances reduction of NAD$^+$ and NADP$^+$ [196].
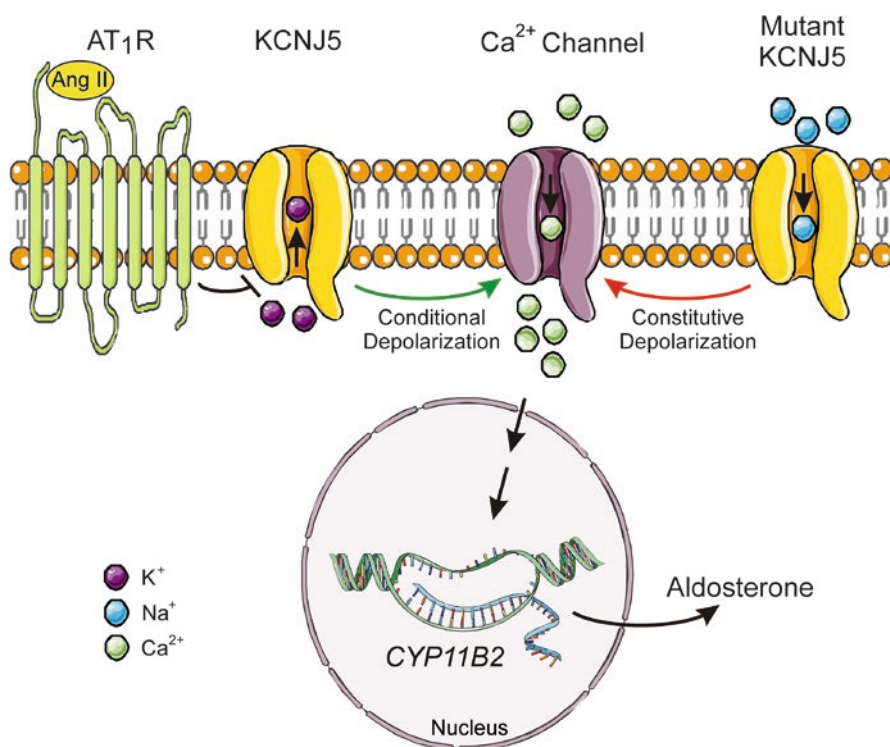
Fig. 2.16 Regulation of aldosterone production by voltage-gated channels in normal and pathological states. Ang II binds to its receptor (AT$_1$R) on zG cells and decreases efflux of K$^+$, thereby conditionally depolarizing the cell and increasing the entry of Ca^{2+} into the cytoplasm. This increase in cytoplasmic Ca^{2+} stimulates cell proliferation and transcription of CYP11B2 via CaMK and related signaling pathways, resulting in increased aldosterone production. *KCNJ5* gene mutations associated with familial hyperaldosteronism type 3 result in unselective potassium Kir 3.4 channels. This loss of selectivity causes constitutive cell depolarization, uncontrolled cell proliferation, and excessive aldosterone secretion. Prepared using image vectors from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/)

The resultant increases in NADH and NADPH enhance ATP production and steroidogenesis, respectively.

The clinical consequences of excessive Ca^{2+} signaling are evident in familial hyperaldosteronism type 3 (FH3), a disease caused by mutations in *KCNJ5* [199]. These mutations perturb the selectivity filter of the inward rectifying potassium channel (Kir3.4) and allow Na^+ entry into the cell, resulting in constitutive membrane depolarization, Ca^{2+} influx, and enhanced aldosterone production (Fig. 2.16) [200].

Stimulation of Aldosterone Secretion by Extracellular K^+

Small changes in extracellular K^+ elicit changes in the plasma membrane potential of zG cells [201]. Elevated concentrations of extracellular K^+ depolarize the plasma membrane and activate voltage-dependent Ca^{2+} channels. The resulting increase in cytosolic Ca^{2+} triggers aldosterone production by the mechanisms detailed above. Aldosterone secretion increases linearly as the K^+ concentration exceeds 3.5 meq/L [202].

Other Signaling Pathways Activated by Ang II or K^+

Ang II also activates phospholipase D, which hydrolyzes phosphatidylcholine into phosphatidic acid, which can be metabolized to DAG by lipid phosphate phosphatases [153]. DAG activates PKC isoforms, which in turn activate ERK1/2. Ang II can activate PLA_2 to generate AA. Binding of Ang II to AT_1R can induce activation of the JAK/STAT pathway and receptor tyrosine kinases such as the EGF receptor [17, 153, 196].

Regulation of C_{19} Steroid Secretion

Spectrum of C_{19} Steroids Produced by the Adrenal

The zR secretes a variety of C_{19} steroids including DHEA, DHEA-S, androstenedione, and 11β-hydroxyandrostenedione [28, 203, 204]. The vast majority of circulating DHEA and DHEA-S is derived from the adrenal glands, and about half of circulating androstenedione is adrenal in origin, the balance arising from gonads [28]. To efficiently produce these C_{19} steroids, cells in the zR exhibit a specific biochemical profile: high CYP17A1 17,20 lyase activity (owing in part to

high levels of the allosteric regulator CYB$_5$), low HSD3B2 activity, and high SULT2A1 activity.

Most of the C$_{19}$ steroids produced by the adrenal gland have little or no androgenic activity, but these compounds may be metabolized into more potent androgens and estrogens in peripheral tissues. For example DHEA and DHEA-S may be converted to androstenedione and then testosterone in tissues that express HSD3B1 and HSD17B1/5, such as adipose tissue or skin. The resultant testosterone can be metabolized further by 3-oxo-5α-steroid 4-dehydrogenase (SRD5A) to yield the highly potent androgen DHT or by aromatase to yield estradiol. The adrenal gland contributes ~1% to the total circulating testosterone in males and ~50% in females [205]. The peripheral conversion of adrenal C$_{19}$ steroids to more potent androgens is of importance in the pathogenesis and treatment of castration-resistant prostate cancer [206].

Analysis of blood collected from adrenal veins has shown that the adrenal gland has the capacity to produce small quantities of more potent androgens such as testosterone and 11β-hydroxytestosterone [203]. The adrenal cortex lacks HSD17B3, the enzyme that converts androstenedione to testosterone in testicular Leydig cells. Instead, testosterone and 11β-hydroxytestosterone are synthesized in adrenal cells using another isoform of 17β-HSD termed AKR1C3 (HSD17B5). An expanded androgen biosynthetic pathway is diagrammed in Fig. 2.17. Metabolites in this expanded pathway may be useful for monitoring adrenal androgen production in certain disease states, such as 21-hydroxylase deficiency [207].
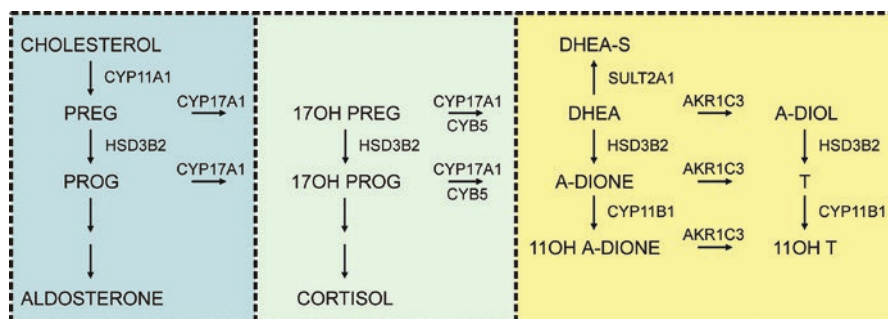
Fig. 2.17 Expanded view of the adrenal C$_{19}$ steroid biosynthetic pathway. Adrenal vein sampling studies have shown that small quantities of potent androgens (T, 11OH T) are generated from C$_{19}$ precursors. The enzymes responsible are highlighted in the yellow box. *11OH A-dione* 11β–hydroxyandrostenedione, *11OHT* 11β–hydroxytestosterone, *A-diol* androstenediol, *A-dione* androstenedione, *AKR1C3* 17β-hydroxysteroid dehydrogenase type 5, *CYB5* cytochrome b5, *CYP11A1* cytochrome P450 cholesterol side-chain cleavage, *CYP11B1* 11β-hydroxylase type 1, *DHEA* dehydroepiandrosterone, *DHEA-S* DHEA sulfate, *HSD3B2* 3β-hydroxysteroid dehydrogenase type 2, *SULT2A1* steroid sulfotransferase type 2A1, *T* testosterone
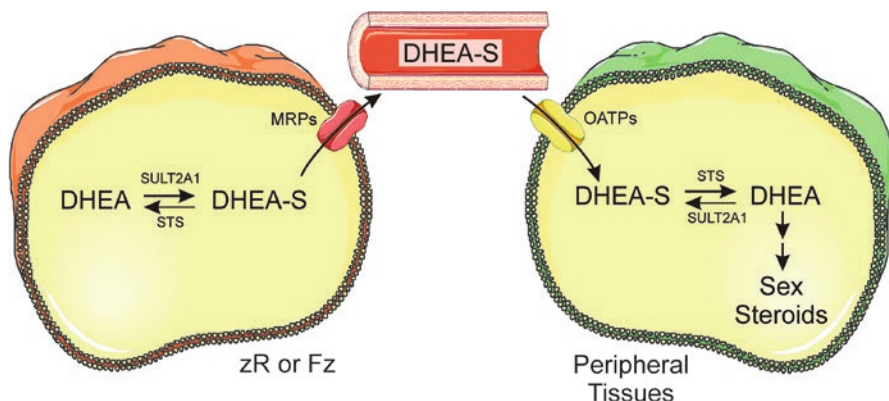
Fig. 2.18 Reversible sulfation allows DHEA, a hydrophobic steroid, to be transported to peripheral tissues where it can be metabolized into more potent androgenic and estrogenic steroids. After biosynthesis in the zR or Fz, DHEA is sulfated by SULT2A1 to facilitate transit in the circulation. Cellular efflux of DHEA-S occurs through MRPs, while OATPs allow import of DHEA-S into the target cell. Steroid sulfatase converts DHEA-S into DHEA. *MRPs* multidrug-resistant proteins, *OATPs* organic acid transport proteins, *STS* steroid sulfatase. Prepared using image vectors from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/)

Reversible Sulfation of DHEA

Most of the DHEA produced by the adrenal is sulfated to increase water solubility and allow circulatory transport [208]. The sulfotransferase SULT2A1 converts DHEA to DHEA-S, an inactive steroid that serves as a reservoir for the peripheral formation of bioactive hormones. SULT2A1 requires the sulfate donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) for catalytic activity. In addition to facilitating circulatory transport of precursors, sulfation acts as a buffer to prevent adrenal androgen excess. Defects in DHEA-S sulfation caused by impaired synthesis of PAPS result in excessive amounts of DHEA, which is further converted to androstenedione and testosterone [209]. In affected females this manifests as premature pubarche and hyperandrogenic anovulation.

Cells can import DHEA-S via organic anion-transporting polypeptides for intracellular desulfation by steroid sulfatase (STS) and subsequent generation of androgenic and estrogenic compounds (Fig. 2.18) [208].

Role of ACTH in the Regulation of C_{19} Steroid Production

The regulation of adrenal C_{19} steroid production is not fully understood, but ACTH is thought to be the main stimulus for production of these compounds [203]. Dexamethasone suppression of ACTH levels decreases circulating adrenal C_{19} steroids. Like ACTH, adrenal C_{19} steroids exhibit a diurnal pattern of expression,

although the rhythmic fluctuation in DHEA-S is muted owing to its long half-life [210]. Further supporting a role for ACTH in the regulation of adrenal C_{19} steroid production, children with loss-of-function mutations in *MC2R* fail to experience adrenarche and the attendant increase in adrenal C_{19} steroids [211]. Clearly, however, signals other than ACTH influence adrenal C_{19} steroid production, as evidenced by the fact that in normal children DHEA-S levels increase at the time of adrenarche, whereas ACTH and cortisol do not. This age-related dissociation between circulating levels of cortisol and DHEA-S has spurred researchers to seek the still elusive adrenal androgen-stimulating hormone.

Modulation of CYP17A1 Activity

The 17,20-lyase activity of CYP17A1 is affected by phosphorylation of specific serine residues [204]. Dephosphorylation of CYP17A1 with protein phosphatase 2A decreases lyase activity and androgen synthesis [212]. Gene-silencing studies have shown that MAPK14 (p38a) is the kinase responsible for enhancing 17,20 lyase activity by phosphorylation [213]. Another kinase, ROCK1, appears to regulate MAPK signaling and target p38a for CYP17A1 phosphorylation [214].

Transcriptome profiling of adrenocortical zones has shown that bone morphogenetic protein 4 (BMP4) is differentially expressed between the zG and zR [215]. Cell culture studies suggest that BMP4 is an autocrine/paracrine negative regulator of C_{19} steroid synthesis and works by suppressing *CYP17A1* expression [215].

Other Factors Implicated in the Regulation of Androgen Production

When subjected to serum starvation, the human ACC cell line H295R adopts a hyperandrogenic phenotype, marked by increased production of DHEA, reduced HSD3B2 activity, increased CYP17A1 phosphorylation, and higher 17,20-lyase activity [216]. Transcriptional profiling of serum-starved H295R cells has implicated two additional factors, retinoic acid receptor-β (RARB) and angiopoietin-like protein 1 (ANGPTL1), in the regulation of androgen production [204]. The transcription factor RARB stimulates the *StAR*, *CYP17A1*, and *HSD3B2* promoters, while the secreted protein ANGPTL1 modulates CYP17A1 expression by inducing ERK1/2 phosphorylation.

Key Transcription Factors Involved in Steroidogenesis

Among the plethora of transcription factors implicated in the regulation of adrenal steroidogenesis, several play noteworthy roles.

SF1

SF1 (also called Ad4BP or NR5A1), the prototype of steroidogenic transcription factors, regulates a wide array of genes including enzymes involved in adrenal steroidogenesis [217, 218]. Enforced expression of SF1 in embryonic or mesenchymal stem cells is sufficient to activate steroidogenic gene expression [219, 220], and transgenic expression of *Sf1* in fetal adrenal progenitor cells leads to ectopic adrenal formation (Zubair et al. 2009).

Traditionally, SF1 has been classified as an orphan nuclear receptor, but studies have shown that certain lipids bind this transcription factor and regulate its activity [221]. For example, SF1 activity can be modulated by phosphorylation of the 3-position of the inositol head group of $PI(4,5)P_2$ while this phospholipid is bound to SF1 [222]. SF1 activity also is controlled by gene dosage, transcriptional regulation, posttranslational modification, and association with positive and negative cofactors [223–225].

Sf1$^{-/-}$ mice exhibit agenesis of both the adrenal glands and gonads [226]. Individuals with loss-of-function mutations in the DNA-binding domain of SF1 exhibit primary adrenal failure and gonadal dysgenesis. However, most loss-of-function mutations in human *SF1* are not associated with adrenal insufficiency but rather isolated XY gonadal dysgenesis. In addition to regulating steroidogenesis, SF1 has been implicated in the control of glycolysis, cell proliferation, cytoskeletal rearrangements, and apoptosis [217, 218].

DAX1

DAX1 (also called *NR0B1*) is an X-linked gene that encodes a repressor of steroidogenic gene expression [227]. DAX1 is an acronym for *D*osage-sensitive sex reversal, *A*drenal hypoplasia critical region on chromosome *X*. In response to ACTH, SF1-positive subcapsular progenitors downregulate *Dax1* and differentiate into corticoid-producing cells. DAX1 deficiency in humans and mice leads to excessive differentiation of subcapsular progenitors and eventual depletion of the stem/progenitor cell compartment [228, 229]. DAX1 deficiency typically affects boys and presents as primary adrenal insufficiency in early infancy or childhood, hypogonadotropic hypogonadism at puberty and impaired spermatogenesis [230]. Cytomegaly, a hallmark of adrenal dysfunction associated with *DAX1* deficiency [227, 228, 230, 231], is thought to be a compensatory response to a reduced number of cortical cells or to progenitor cell exhaustion [229].

CREB

CREB (cAMP response element-binding protein) is a transcription factor that binds to cAMP response elements (CREs) in the promoters or enhancers of genes [232]. CREB proteins are activated by phosphorylation on Ser142 by various kinases, including PKA and CaMKs. When activated, CREB recruits transcriptional coactivators,

including CREB-binding protein, thereby modulating gene expression [232]. CREB has been implicated in the regulation of multiple steroidogenic enzymes. CREB also plays a key role in the circadian clock; mutant mice lacking the Ser142 phosphorylation site in CREB have difficulty entraining to light-dark cycles [233].

GATA6

GATA6 is expressed in both the fetal and adult cortex [234–237]. GATA6 acts in synergy with SF1 and other transcription factors to enhance the expression of genes involved in adrenal steroidogenesis [237, 238]. In humans GATA6 is hypothesized to regulate the production of adrenal androgens and possibly glucocorticoids [234, 237, 239–241]. Heterozygous loss-of-function mutations in human *GATA6* have been linked to pancreatic agenesis, cardiac malformations, and biliary tract abnormalities, but not primary adrenocortical defects [242–244]. Targeted deletion of *Gata6* in SF1+ cells of the mouse results in a thin adrenal cortex, cytomegaly, and blunted corticoid production [245]. *Gata4/Gata6* double-knockout mice generated with *Sf1*-cre exhibit severe adrenal hypoplasia. Female double-knockout mice die from adrenocortical insufficiency, whereas their male counterparts survive due to heterotopic corticoid production by cells in the testes [246–248].

Other Factors Involved in Steroidogenesis

Additional insights into the factors that regulate steroidogenesis have emerged from studies of patients with familial glucocorticoid deficiency (FGD) and other forms of congenital adrenal hypoplasia/insufficiency [166, 249, 250]. Such patients may be classified into two categories: those without extra-adrenal features (non-syndromic adrenal hypoplasia) and those with extra-adrenal features (syndromic adrenal hypoplasia). Causative genes for these two categories are shown in Table 2.4.

Table 2.4 Causes of primary adrenal hypoplasia/insufficiency that shed light on the regulation of steroidogenesis

Category	Disease	Gene	Reference
Non-syndromic (no extra-adrenal manifestations)	FGD	*MC2R*	[251]
	FGD	*MRAP*	[252]
	X-linked congenital adrenal hypoplasia	*DAX1 (NR0B1)*	[230]
	FGD	*NNT*	[253]
Syndromic (extra-adrenal manifestations)	FGD	*TXNRD2*	[254]
	AAA syndrome	*AAAS*	[255]
	IMAGe syndrome	*CDKN1C*	[256]
	Variant FGD	*MCM4*	[257]
	MIRAGE syndrome	*SAMD9*	[258]

Factors Affecting Redox Homeostasis

The adrenal cortex is particularly susceptible to oxidative stress, so inherited mutations that alter redox homeostasis often manifest clinically as adrenocortical insufficiency.

NNT

Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, are generated during steroidogenesis and oxidative phosphorylation. Detoxification of ROS in mitochondria depends on NADPH for regeneration of reduced glutathione (GSH) from oxidized glutathione (GSSG, glutathione disulfide). Nicotinamide nucleotide transhydrogenase (NNT) is a redox-driven H^+ pump of the IMM (Fig. 2.19). This enzyme uses energy from the mitochondrial H^+ gradient to reduce $NADP^+$ to NADPH [253]. Thus, NNT deficiency can negatively impact steroidogenesis in two ways: (1) by limiting production of NADPH, the electron donor for mitochondrial steroidogenic enzymes (CYP11A1, CYP11B1, CYP11B2), and (2) by allowing mitochondrial damage from excessive ROS. Loss-of-function mutations in *NNT* account for 5–10% of FGD patients [259]. Certain substrains of C57Bl/6 J mice, a widely used inbred line, harbor spontaneous *Nnt* mutations [260]. These mice exhibit higher levels of adrenocortical cell apoptosis and impaired glucocorticoid production. Consequently, experiments assessing adrenal steroidogenesis in C57Bl/6 J mice must be interpreted cautiously [261].

TXNRD2

The selenoprotein TXNRD2 is a mitochondrial thioredoxin reductase that contributes to redox homeostasis (Fig. 2.19). *TXNRD2* is highly expressed in the adrenal cortex, where it functions to limit oxidative stress by inactivating ROS. A homozygous loss-of-function mutation in the *TXNRD2* gene has been reported in consanguineous family [254]. TXNRD2 deficiency appears to be associated with extra-adrenal manifestations. *Txnrd2* ablation causes fatal cardiac and hematopoietic defects in mice [262, 263], and two novel heterozygous mutations in *TXNRD2* were identified in 3 of 227 patients with a diagnosis of dilated cardiomyopathy [264].

ALADIN

AAA syndrome, characterized by *A*drenal insufficiency, *A*lacrima, *A*chalasia, and a progressive neurological disorder, is caused by recessive mutations in *AAAS*. This gene encodes ALADIN, a nuclear pore complex gene [265]. Pathogenic mutations sequester ALADIN in the cytoplasm. Silencing of the *AAAS* gene in H295R ACC cells impairs redox homeostasis and inhibits steroidogenesis [255]. It is hypothesized that defective import of specific nuclear proteins allows oxidative damage in the adrenal glands and other tissues.
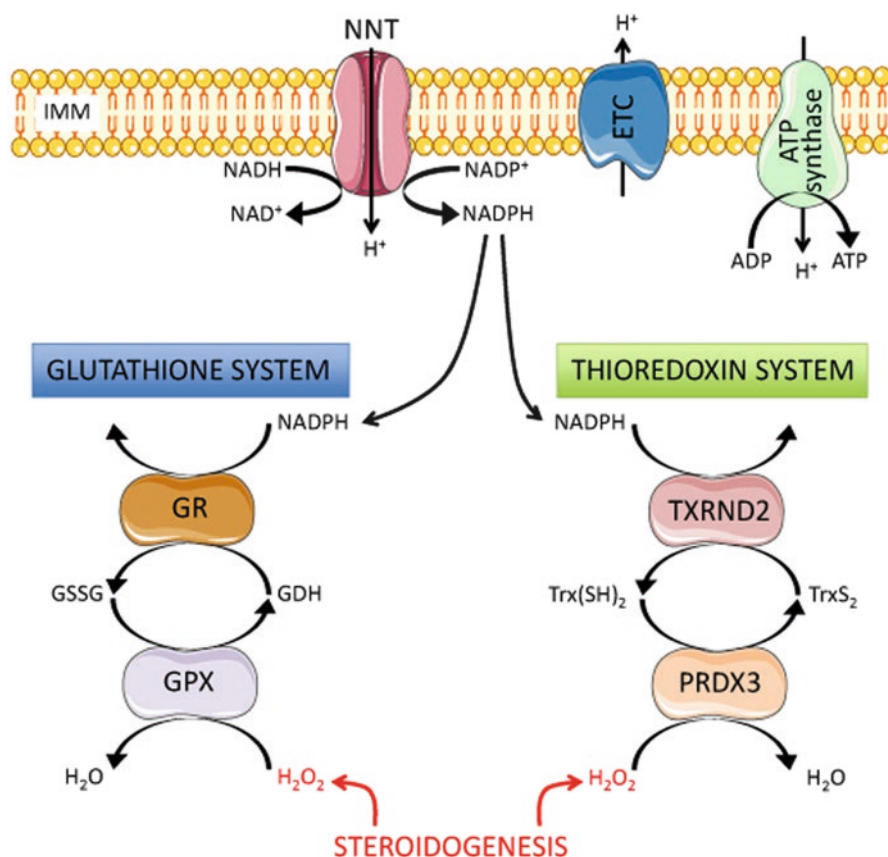
Fig. 2.19 Roles of NNT and TXRNR2 in steroidogenesis. NNT generates NADPH, the electron donor for the mitochondrial steroidogenic enzymes CYP11A1, CYP11B1, and CYP11B2. NNT and TXRND2 function to detoxify ROS that are the by-products of steroidogenesis and oxidative phosphorylation. *ETC* electron transport chain, *GSSG* glutathione disulfide, *GSH* glutathione, *GPX* glutathione peroxidase, *GR* glutathione reductase, *IMM* inner mitochondrial membrane, *TXNRD2* thioredoxin reductase 2, *PRDX3* peroxiredoxin 3. Prepared using image vectors from Servier Medical Art (www.servier.com), licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/license/by/3.0/)

Factors Affecting Adrenocortical Cell Growth

CDKN1C (p57Kip2)

Heterozygous gain-of-function mutations in *CDKN1C*, which is paternally imprinted and encodes the cell cycle regulator p57Kip2, cause IMAGe syndrome [256]. The hallmarks of IMAGe syndrome are *I*ntrauterine growth retardation, *M*etaphyseal dysplasia, *A*drenal hypoplasia congenita, and *G*enital abnormalities. *CDKN1C* is expressed in the adrenal cortex and is upregulated by ACTH treatment,

suggesting that its normal function in the adrenal cortex may be to limit cell proliferation. Supporting this premise, loss-of-function mutations in *CDKN1C* cause Beckwith-Wiedemann syndrome, an overgrowth syndrome associated with adrenal hyperplasia [266].

MCM4

Recessive mutations in the mini chromosome maintenance-deficient 4 (*MCM4*) homologue gene have been identified in a variant of FGD [257, 267]. Clinical manifestations of this FGD variant include short stature, chromosomal breakage, natural killer cell deficiency, and progressive adrenocortical insufficiency. The mechanistic basis for this loss in adrenal steroidogenic capacity is unclear. MCM4 is part of a DNA repair complex essential for DNA replication and genome stability in various cell types.

SAMD9

Sporadic heterozygous mutations in *SAMD9*, which encodes a facilitator of endosome fusion, cause MIRAGE syndrome. Clinical manifestations include *M*yelodysplasia, *I*nfection, *R*estriction of growth, *A*drenal hypoplasia, *G*enital phenotypes, and *E*nteropathy [258]. Expression of pathogenic SAMD9 variants in wild-type fibroblasts causes profound growth inhibition. Patient-derived fibroblasts exhibit restricted growth, increased size of early endosomes, and intracellular accumulation of giant vesicles carrying a late endosome marker. These abnormalities suggest that pathogenic *SAMD9* mutations enhance endosome fusion. Patient-derived fibroblasts have decreased plasma membrane expression of the EGF receptor, likely due to defective recycling of the receptor. The adrenal glands of affected individuals are small and disorganized, with foamy-appearing adrenocortical cells.

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Chapter 3
Pharmacology of Glucocorticoids

Baha M. Arafah

Cortisol Synthesis/Secretion

Cortisol is the primary product of the zona fasciculata of the adrenal cortex and is the dominant glucocorticoid in humans. Its secretion is tightly controlled by ACTH as part of the hypothalamic-pituitary adrenal (HPA) regulation. Cortisol secretion follows a diurnal rhythm such that in people with normal wake-sleep cycles, it peaks in the early morning hours and declines gradually thereafter reaching a nadir at approximately midnight. However, throughout the day and irrespective of the time of the day, ACTH and subsequently cortisol secretion can rapidly increase in response to acute stimuli and/or stresses. Cortisol secretion is a dynamic process that depends on de novo synthesis of the hormone in response to a spike in ACTH production as the adrenals do not store pre-synthesized product [1–3].

Although the details of cortisol synthetic process are reviewed elsewhere, some aspects will be briefly discussed here. It is worth pointing out that steroidogenesis is initiated by LDL-cholesterol mobilization from a pool in the outer mitochondrial membrane to the inner mitochondrial membrane; a process mediated by the steroidogenic acute regulatory (StAR) protein. Thus, with ACTH stimulation, an increase in intracellular C-AMP occurs which in turn induces the StAR protein. This is the rate-limiting step in steroidogenesis where the side-chain cleavage enzyme (also known as CYP11A1, 450 scc) converts cholesterol into pregnenolone. Within the zona fasciculate and under the regulation of ACTH, pregnenolone undergoes several changes that are mediated by or controlled by specific key enzymes such as CYP17A1, HSD 3B2, CYP21A2, and CYP11B1 that culminate in the synthesis of cortisol [1–3].

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Cortisol Dynamics in the Circulation

The majority (90–92%) of cortisol in the circulation is protein bound, while the remaining 8–10% is free or unbound [4–8]. It is the free or unbound fraction that dictates physiologic functions of the hormone. Approximately 75% of the total cortisol in the circulation is bound to corticosteroid-binding globulin (CBG) or transcortin, while 10–15% is albumin bound. The binding of cortisol to CBG is not linear and reaches a saturation point in subjects with normal CBG secretion when the total serum cortisol is near 22–25 µg/dL [4, 6–8]. That is, at that level of total serum cortisol concentration, all the CBG binding sites are saturated, and therefore, an increase in cortisol secretion beyond that will be albumin bound or unbound (i.e., free).

CBG is an alpha-2 globulin that is increased in subjects with hepatitis and also in states of estrogen excess such as pregnancy or estrogen replacement therapy including the use of oral contraceptives [4–9]. Standard assays for serum cortisol measurements determine the total concentration, i.e., the protein-bound and the free fractions. Thus, it is not surprising to note that in conditions where CBG levels are increased, serum total cortisol levels would appear higher than expected although the free fraction remains normal as one would predict [4–8]. Thus, it would be prudent to interpret serum total cortisol measurements in such cases with caution. It follows that all dynamic testing of the HPA function (e.g., cosyntropin stimulation or dexamethasone suppression) would yield misleading and unreliable results in such patients as they rely on total cortisol measurements [10]. The reverse is also true in that when both binding proteins are low, measured serum total cortisol will appear lower than predicted and can raise concern for impaired adrenal function even though the free fraction remains appropriately normal [11, 12]. Measurement of serum free cortisol level can address the problem of high or low binding proteins [11–13]. However, at this point measurements of serum free cortisol are technically difficult and are not widely available in a timely manner. An alternative to measuring serum free cortisol would be the determination of cortisol concentration in the saliva [14, 15]. It is well known that the cortisol in the saliva is in equilibrium with that of the free cortisol fraction in the circulation. It is also known that any alteration in serum free cortisol is reflected within minutes with parallel change in the concentration of the hormone in the saliva [16].

Chemical Structure of Cortisol and Commonly Used Synthetic Glucocorticoids

The basic chemical structure of cortisol is shown in Fig. 3.1 as a 21-carbon molecule derivative of cholesterol with four rings (A through D) and a side chain attached to the D ring [1–3]. The essential components of the cortisol molecule responsible for glucocorticoid activity include hydroxyl groups at the 11, 17, and 21 positions along with a keto group at position 3 and a delta 4–5 double bond as highlighted in Fig. 3.1. Synthetic glucocorticoids have the same basic chemical structure as the cortisol molecule but with additional modifications that alter
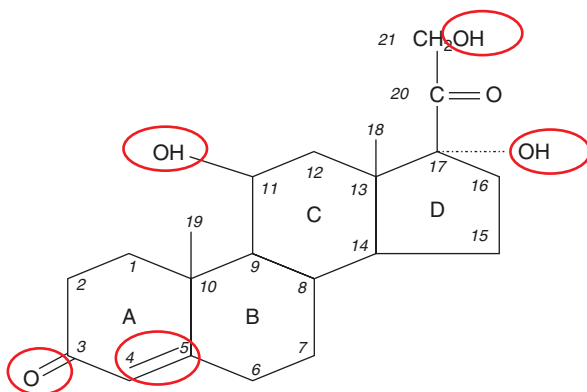
Fig. 3.1 Chemical structure of hydrocortisone (cortisol) showing the four rings, A through D, along with numbering system for the carbon molecules. Also shown are the important features necessary for glucocorticoid activity (*circled in red*). These include hydroxyl group at positions 11, 17, and 21 as well as a keto group at position 3 and a double bond at 4–5 positions. It should be noted that the chemical structure of cortisone is identical to that of hydrocortisone with the exception that there is a keto group (=O) instead of a hydroxyl group (OH) at the 11 position

1) Add double bond at 1—2 position= prednisolone
2) Add double bond at 1—2 position + CH3 at position 6=Methylprednisolone
3) Add F at 9 α position: Fludrocortisone
4) Add double bond at 1—2 and F at 9 α position= Δ^1 Fludrocortisone
5) Add double bond at 1-2 + F at 9 α and CH3 at 16 α position= Dexamethasone
6) Add double bond at 1-2 + F at 9 α and CH3 at 16 β position= Betamethasone
7) Add double bond at 1-2 + F at 9 α and OH at 16 position= Triamcinolone

Fig. 3.2 Modifications in the basic chemical structure of cortisol can yield different compounds with variable anti-inflammatory and Na-retaining effects shown in *red color* and *black circles*. While some compounds have predominantly Na-retaining effects (e.g., fludrocortisone), others have potent anti-inflammatory effects without significant mineralocorticoid activity (e.g., dexamethasone). The figure shows specific addition to the basic structure resulting in the formation of commonly used synthetic glucocorticoids

affinity to the glucocorticoid and mineralocorticoid receptors as well as protein binding, metabolism, and pharmacokinetics [17–21]. Figure 3.2 shows several additions to the cortisol molecule that yield commonly available synthetic gluco- and mineralocorticoids. In most instances, the primary outcome achieved with

modifying the basic chemical structure of hydrocortisone molecule is to enhance the anti-inflammatory while decreasing or not necessarily altering the Na-retaining effects of the compound.

It is important to point out that all synthetic glucocorticoids have alterations (double bond at 1–2 positions) or additions to positions 6, 9, and 16 (Fig. 3.2). A double bond at 1–2 positions of the hydrocortisone molecule results in the formation of prednisolone, and further addition of a methyl group at the 6 position results in the formation of methylprednisolone that possesses enhanced anti-inflammatory effects in comparison to the Na-retaining properties. The addition of the same (double bond at 1–2 positions) to cortisone results in the formation of prednisone. It is also important to point out that cortisone is not biologically active as it has a double bond at position 11 and would require the enzyme 11-β-hydroxysteroid dehydrogenase (11-β-HSD) to convert it to the active form, namely, hydrocortisone or cortisol [19, 25–27]. Likewise, prednisone has to be converted to the active moiety, prednisolone by the same enzyme [19, 25–27]. The addition of fluoride at position 9 to the hydrocortisone molecule results in the formation of fludrocortisone which has the highest mineralocorticoid activity among all synthetic adrenal steroids and is also a potent glucocorticoid as well [17]. In contrast, the addition of fluoride at position 9 and a double bond at 1–2 and a methyl group at position 16 results in the formation of dexamethasone with the most potent anti-inflammatory and the least (almost nonexistent) mineralocorticoid activity. Betamethasone (16-β-methyl group) has a similar structure to dexamethasone (16-α-methyl group) and is widely used as nasal and aerosol sprays for allergic and inflammatory lung disease. Alterations in the basic structure of the cortisol molecule near C-18 tend to decrease the mineralocorticoid effects of these compounds. The relative anti-inflammatory and Na-retaining effects of commonly used glucocorticoids [2, 3, 20, 21] are shown in Table 3.1.

Table 3.1 Commonly used oral and parenteral preparations of glucocorticoids

	Dose equivalent in mg[b]	Anti-inflammatory potency	Na-retaining/mineralocorticoid potency
Hydrocortisone	20	1	1.0
Cortisone acetate[a]	25	0.8	0.8
Prednisone[a]	5	4	0.8
Prednisolone	4	4	0.8
Methylprednisolone	4	5	0.5
Triamcinolone	4	5	Negligible
Dexamethasone	0.375–0.75	30	Negligible

Modified from Schimmer BP, Parker KL. Adrenocorticotropic hormone; adrenocortical steroids and their synthetic analouges. In, The pharmacological Basis of Therapeutics, 11th ed, Brunton, LL, Lazo JS, Parker KL (eds), McRraw Hill, NY

[a]Both cortisone and prednisone are not biologically active until they are hydroxylated at the 11 position and converted to cortisol and prednisolone, respectively

[b]These are approximate dose equivalents with significant individual differences related to genetics, age, gender and variations in drug metabolism

Metabolism of Glucocorticoids

The major site of cortisol metabolism is the liver where it is reduced, oxidized, or hydroxylated and the products of these reactions are conjugated with sulfate or glucuronic acid and excreted in the urine [1, 3]. The main pathway in the metabolism of glucocorticoids is reduction of the 3-keto (by 3 α-HSD) and delta-4 double bond (by 5 α and 5 β reductase). The 11-β-HSD type 1 is a key enzyme in cortisol metabolism as it possesses dehydrogenase and reductase activities [25–27]. The enzyme has the ability to inactivate cortisol by oxidation in several tissues such as liver, bone, adipose tissue, and the central nervous system while the reverse (i.e., reduction or conversion of cortisone into cortisol) occurs primarily in the liver. Additional pathways of cortisol metabolism include oxidative removal of the side chain (to yield a 19-carbon molecule) or hydroxylation at position 6 to yield 6-β-hydroxycortisol. The latter pathway predominates when cortisol secretion is excessive [28, 29].

Several factors can alter hepatic metabolism of cortisol such as a change in thyroid hormone secretion where hyperthyroidism accelerates, while hypothyroidism decreases cortisol metabolism [30, 31]. A recent study showed that cortisol metabolism and clearance are decreased during critical illness that accounts, in part, for the continued rise in serum levels [32]. Renal failure does not influence enzymatic metabolism of cortisol although the clearance of the glucuronide-associated metabolites is diminished. No significant changes in the clearance of glucocorticoids are observed in patients on peritoneal dialysis. However, the clearance of synthetic glucocorticoids is increased in patients with renal failure on hemodialysis [33]. A recent study showed that moderate impairment in renal function (eGFR, 30–60 ml/min/1.73 m^2) results in an increase in baseline AM serum cortisol level as well as a higher serum level following dexamethasone administration [34]. The latter finding should be taken into account when one interprets the results of dexamethasone suppression test.

The other site of cortisol metabolism is the kidney where cortisol is converted into cortisone by 11-β-HSD type-2 enzyme which prevents cortisol from binding to the mineralocorticoid receptor [26, 27]. Synthetic glucocorticoids are metabolized through similar pathways: reduction, oxidation, hydroxylation, and conjugation [20, 23, 24]. Their metabolism is also subjected to similar alteration to those noted with cortisol. Importantly, however intake of other drugs will have a more dramatic impact on the metabolism of most synthetic glucocorticoids. For example, mitotane [35], phenytoin [36, 37], carbamazepine [9] phenobarbital [38], and rifampin [28, 39] increase 6-β-hydroxylation and as such accelerate the metabolism of halogenated glucocorticoids including dexamethasone, betamethasone, and fludrocortisone. The clearance of glucocorticoids is also decreased in women on oral contraceptives [40, 41] and others on estrogen replacement therapy as a result of increased transcortin. Similar outcomes are observed in patients receiving strong inhibitors of CYP 3A4 such as clarithromycin [42].

Pharmacokinetics of Synthetic Glucocorticoids

In contrast to cortisol, most synthetic glucocorticoids except prednisolone [21–24] have a very low affinity for CBG. Prednisolone has approximately half of the affinity for CBG as compared to cortisol, while the rest of synthetic glucocorticoids have minimal (<1%) affinity to the binding protein. Other than cortisol or prednisolone, synthetic glucocorticoids in the circulation are either weakly bound to albumin (60–70%) or are in the free form (30–40%). The plasma half-lives of synthetic glucocorticoids are longer than that of cortisol, and there are significant interindividual variations that relate to genetic variation and the influence of simultaneously used drugs as discussed earlier. Clearance of synthetic glucocorticoids such as prednisone and prednisolone decreases with age and the time of drug administration being slower in the morning than later in the day. A small percentage of administered glucocorticoids can be detected in breast milk of nursing women.

The half-life of endogenously synthesized cortisol in the circulation is variable but ranges between 60 and 110 min when the concentration is within the maximal binding capacity of CBG which is 22–25 μg/dL [4]. When cortisol concentrations reach levels above that, the proportions of free and albumin-bound cortisol are increased; and thus, the free fraction is higher than usual 8–10%. The half-life of exogenously administered hydrocortisone is also variable depending primarily on the dose, presence of liver and kidney diseases, as well as other comorbidities such as critical illness [32]. The half-lives of synthetic glucocorticoids (other than cortisol and perhaps prednisolone) are higher than that of cortisol reaching nearly 3–4 h for dexamethasone. It is important to emphasize the fact that there are significant individual variability in the activity of enzymes involved in glucocorticoid metabolism and therefore the prospective half-life of each compounds in humans [23]. Furthermore, the concomitant intake of other drugs that alter the activity of enzymes involved in glucocorticoid metabolism can also modulate the plasma half-life of each glucocorticoid [9, 24, 35–39]. In addition, concurrent illnesses such as thyroid diseases [30, 31] or liver failure [43, 44] can modulate the effects of glucocorticoids and alter their plasma half-lives.

As discussed elsewhere, cortisol metabolism and clearance are decreased during critical illness which accounts, in part for the continued rise in serum levels [32]. It is not known whether this will also be true about other glucocorticoids. Also as noted elsewhere, the clearance of cortisol as well as that of other glucocorticoids is increased in patients with hyperthyroidism [30, 31] and in patients with renal failure during hemodialysis [33]. In light of the low binding of synthetic glucocorticoids to transcortin and albumin, patients with nephrotic syndrome do not experience major changes in synthetic glucocorticoid clearance as compared to those with normal binding proteins [22, 33]. Fluorinated glucocorticoids such as dexamethasone and betamethasone cross the placenta and therefore would impact fetal tissue and suppress the fetal adrenal cortex. In contrast, the placenta inactivates prednisone and prednisolone. Therefore, betamethasone would be the preferred glucocorticoid to use when the intended action is directed at the fetus such as enhanced fetal lung maturity in those where premature delivery is anticipated [45]. However, when

glucocorticoids are used to treat a pregnant woman with a glucocorticoid-responsive illness, the preferred choice would be prednisone/prednisolone and not dexamethasone. The clearance of exogenously administered synthetic glucocorticoids is slowed in patients with advanced liver failure as a result of decreased metabolism.

As stated earlier, the pharmacokinetics of glucocorticoids can be modified by certain drugs that affect glucocorticoid metabolism. Other drugs that can influence glucocorticoid pharmacokinetics include those that reduce the GI absorption such as aluminum/magnesium hydroxide but not the proton pump inhibitors or the H-2 blockers [44, 46]. It is important to appreciate the fact that there are genetic differences in patients' ability to metabolize drugs over and above the presence of associated illnesses or concurrent medications use. It is clear however that subjects who metabolize glucocorticoids slowly tend, as one would predict, to have a higher chance for adverse events [47].

Routes of Administration of Commonly Used Glucocorticoids

The rate of absorption of synthetic glucocorticoid through the GI tract is quite similar among available preparation. Glucocorticoids can be administered intravenously, intramuscularly, intra-articularly, and orally, as a nasal aerosol, topically on the skin, conjunctiva, or other mucous membranes such as the buccal and rectal mucosa [20, 48–51]. Glucocorticoids are generally insoluble in water, and thus they are formulated primarily as salts such as sodium phosphates or succinates for intravenous use. While hydrocortisone is absorbed quickly after an intramuscular injection, other glucocorticoids are absorbed at a much slower and at times unpredictable rate such as cortisone acetate and triamcinolone. Absorption after intra-articular injections occurs slowly over days to weeks and leads to continuous systemic exposure that is often associated with adverse effects including suppression of the HPA axis. The absorption after oral administration is rapid occurring within 30–45 min and can be decreased by concomitant use of calcium/magnesium-containing drugs. Most topical and inhaled glucocorticoids such as fluticasone are absorbed to a certain degree and are often associated with adverse events. Many patients on chronic inhaled glucocorticoids have partial suppression of the HPA axis. Absorption of topically administered glucocorticoids is variable and depends to a large extent on the drug used, the skin abnormality and its integrity where inflamed and open skin tends to result in much higher absorption as compared to an intact skin [49, 50]. A thin region of the skin (face as compared to the arms) and the presence of occlusive dressing can also increase absorption.

Glucocorticoid Actions

Although the molecular mechanisms of glucocorticoid actions are discussed in detail elsewhere, a brief description is included in this chapter. Glucocorticoids exert their effects on many cells and tissues throughout the body through binding to

the glucocorticoid receptor (GR) that is a member of the nuclear hormone receptor superfamily. GR resides primarily in the cytoplasm and once activated it undergoes conformational changes through a process that involves dissociating from heat shock proteins. Subsequently, the ligand-bound GR complex translocate into the nucleus where it binds to hormone-responsive or glucocorticoid-responsive elements in the target cells to induce or suppress gene transcription [52–56]. It should not be surprising to note that the response to glucocorticoids in a particular cell will depend to a large degree on the specific glucocorticoid used as well as the number of hormone-responsive elements associated with that gene. This is consistent with the known variable effects of glucocorticoids on different cell types in the body. It is well known that glucocorticoid and mineralocorticoid receptors share considerable homology that allows activation of either receptor by any of the two hormones. However, the specificity of the mineralocorticoid receptor is maintained by the presence of the enzyme 11-β-hydroxysteroid dehydrogenase in renal epithelial cells which inactivates cortisol by converting it to cortisone and thus allowing only mineralocorticoids to bind to that receptor [25, 27].

The anti-inflammatory and sodium-retaining potencies of synthetic glucocorticoids are quantified in comparison to the naturally occurring molecule, cortisol, using bioassays that rely on the known effects of the hormone. For example, the anti-inflammatory effects can be measured by the ability of the drug in question to eliminate the inflammatory response to an oil injection in rats. An example of methods assessing mineralocorticoid effects includes the ability of the tested drug to lower sodium excretion in adrenalectomized rats. Other assays assess potency by evaluating the drop in eosinophil count with the administration of the drug to be tested. It is not clear that such an assay represent an accurate assessment of the anti-inflammatory potency of glucocorticoids. While the assays are useful, they are imperfect as they do not account for many other variables involved in the process, including the absorption, bioavailability, and individual differences.

Physiological and Supraphysiological Effects of Glucocorticoids

Glucocorticoids exert extensive physiological effects on many tissues throughout the body some of which are direct effects at the cellular level and others are indirect through effects on homeostatic mechanisms [12]. The fact that glucocorticoids are essential for life is best appreciated in patients with deficiency. Cortisol, the most potent endogenous glucocorticoid is one of the most important defense mechanism the body has in the early and late phases of major stresses including critical illness [2, 12, 57]. In that respect, glucocorticoids in general and cortisol in particular help maintain homeostasis through their permissive effects on catecholamine receptors in vascular smooth muscles as well as their Na-retaining properties [58–62]. In addition, this class of steroids functions as a source of energy by stimulating gluconeogenesis and facilitating the lipolytic effects of catecholamines. Glucocorticoids stimulate adipocyte differentiation and adipogenesis through activation of key

enzymes such as lipoprotein lipase. Excess glucocorticoid favors deposition of visceral rather than peripheral fat which explains the clinical picture of central obesity seen in such individuals. It is likely that the increase in visceral fat in patients with endogenous or exogenous glucocorticoid excess is due to the fact that the glucocorticoid receptor is highly expressed and also the type-1 isoenzyme of 11-β-hydroxysteroid dehydrogenase is highly expressed. The latter feature promotes the conversion of cortisol from cortisone.

The impact of excess glucocorticoids on other hormones such as growth hormone and gonadotropins is quite significant. Growth hormone secretion is suppressed with excess glucocorticoids and this becomes quite significant in children as stunted growth is commonly observed [26, 61]. Variable degrees of suppression of gonadotropin secretion are commonly observed leading to signs and symptoms of hypogonadism. This effect has deleterious consequences on bone health as it adds to the negative effects of excess glucocorticoids on the bone [26, 63]. The latter effects on the bone are due to inhibition of osteoblast function, inhibition of calcium absorption, and at the same time increased renal Ca excretion. That is why osteopenia or frank osteoporosis is commonly seen in patients with excess glucocorticoids. Excess glucocorticoids cause progressive insulin resistance that often leads to the development of frank diabetes mellitus. In contrast, deficiency of glucocorticoids leads to decreased gluconeogenesis that can lead to hypoglycemia.

Excess glucocorticoids cause catabolic changes in the skin leading to collagen breakdown, skin thinning, and easy bruisability. In addition to their effects on promoting insulin resistance in the muscle, excess glucocorticoids cause atrophy (but not necrosis) of specific fibers inducing a proximal myopathy that is one of the common manifestations in patients exposed to excessive glucocorticoids from endogenous or exogenous sources.

The brain is an important target for glucocorticoids as evidenced by the presence of glucocorticoid receptors in multiple regions. Clinical observations in patients with deficiency include lethargy and withdrawal. In contrast, patients with excess glucocorticoids often have variable degrees of euphoria, depression, and at times frank psychosis. It is highly likely that some patients with genetic predisposition and others with prior history of mental illness have more serious mental changes when exposed to excess glucocorticoids. The impact of excess glucocorticoids on memory and neurodegenerative diseases such as dementia are under investigation. The impact of excess glucocorticoids on the eyes includes higher likelihood for cataract formation and increase in intraocular pressure.

Increased appetite and consequently weight gain are common features of excess glucocorticoid exposure. The impact of excess glucocorticoids on the cardiovascular system depends to some degree on type of drug being used, its ability to retain sodium, the dose and duration of exposure, as well as the presence of preexisting conditions. With large doses of glucocorticoids salt and fluid retention, as well as hypertension are commonly observed.

Glucocorticoids also have important effects in stimulating red and white blood cells. Anemia, neutropenia, eosinophilia, and lymphocytosis are characteristic features of glucocorticoid deficiency. Perhaps their best known effects are those related

to their immunosuppressive and anti-inflammatory effects. Both of these effects are generally best appreciated with larger than physiological doses of glucocorticoids and are the basis for the most often uses of glucocorticoids therapeutically. These effects result from the suppression of leukocyte function, inhibition of synthesis, or action of lymphokines in many immune cells.

Pharmacologic and Therapeutic Uses of Glucocorticoids

The original observation by Hench where newly synthesized cortisone was used to treat a woman with Rheumatoid arthritis in 1948 was a major breakthrough that established the anti-inflammatory properties of glucocorticoids. It became obvious over the years that the anti-inflammatory effects of glucocorticoids are mediated through their respective influence on the primary and secondary immune cells. These effects are noted when glucocorticoids are used in supraphysiological or pharmacological doses. At these doses, glucocorticoids interfere with the function of leukocyte and fibroblasts and endothelial functions and inhibit leukocyte traffic and access to the site of inflammation and also suppress the production and actions of humoral factors involved in the inflammatory process such as prostaglandins and fibronectin [52]. As shown in Table 3.2, glucocorticoids are utilized in the treatment of a large number of illnesses and conditions of diverse etiologies.

Table 3.2 Examples of pharmacologic/therapeutic uses of glucocorticoids other than physiological replacement therapy

Organ/system	Specific diseases treated
Skin	Pemphigus, dermatitis
Endocrine	Pituitary tumor apoplexy, Grave's orbitopathy
Allergic reactions	Serious reactions, anaphylaxis, angioedema
Prevention of allergic reactions	For example, in patients with known allergy to contrast
Transplant rejection	Most protocols for organ transplant (kidney, liver, heart, pancreas) include glucocorticoids
Gastrointestinal/hepatic	Inflammatory bowel diseases, chronic active hepatitis
Respiratory	Obstructive airway diseases; asthma, angioedema, sarcoidosis; respiratory distress
Rheumatic/connective tissue	Practically most patients with connective tissue diseases will be treated at some point with glucocorticoids: rheumatoid arthritis, systemic lupus, temporal arteritis
Central nervous system	Increased intracranial pressure, cerebral edema from metastasis, surgery, or irradiation
Muscle diseases	Myositis, polymyalgia rheumatica, myasthenia gravis
Hematological diseases	Leukemias, lymphomas, hemolytic anemia
Kidney	Some forms of nephritis, nephrotic syndrome
Others	Septic shock unresponsive to standard therapy

The inflammatory process is often characterized by increased synthesis of inflammation mediators such as cytokines and prostaglandins. In that respect, glucocorticoids inhibit nuclear translocation and subsequently the function of pro-inflammatory transcription factors such as activator protein and nuclear factor κB that are involved in the regulation of the expression of pro-inflammatory genes [54]. The synthesis of pro-inflammatory cytokines such as interleukin-1 interleukin-6, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor is inhibited in a dose-dependent manner by glucocorticoids.

The anti-inflammatory and immunosuppressive effects of glucocorticoids are linked through multiple mechanisms/effects discussed above. In addition, glucocorticoids have an anti-mitotic action on immune system cells and reduce the survival of some of these cells. The immunological effects of glucocorticoids involve T and B lymphocytes and inhibition of monocyte differentiation into macrophages and macrophage phagocytosis.

Adverse Effects of Excess Glucocorticoids

Adverse effects of supraphysiological doses of glucocorticoid use are extremely common and are seen in the vast majority of patients. Most of these side effects have been discussed earlier under the supraphysiological effects of these drugs and are listed in Table 3.3. One important adverse event not emphasized there is the suppression of the HPA axis as a result of chronic therapy. The negative feedback of exogenously used glucocorticoids inhibits hypothalamic release of corticotropin-releasing

Table 3.3 Commonly observed adverse effects of supraphysiological doses of exogenous glucocorticoids

	Common signs/symptoms/manifestations
Skin	Easy bruising, thin fragile skin with striae especially in the abdomen, facial plethora, poor wound healing, excessive sweating, peripheral edema
Appetite, weight/blood pressure	Increased appetite, weight gain, central obesity, increased blood pressure
Glucose intolerance/diabetes mellitus	Worsening insulin resistance, hyperinsulinemia, glucose intolerance, and diabetes mellitus
Muscle power	Proximal myopathy
Bone health	Osteopenia, osteoporosis, avascular necrosis rarely
Gastrointestinal tract	Gastritis/peptic ulcer disease
Endocrine	Gonadal steroid deficiency: oligomenorrhea/amenorrhea in premenopausal, impotence in men
Eye	Cataract and glaucoma
CNS/psychiatric	Insomnia, mental changes (euphoria, depression, and at times psychosis), increased intracranial pressure
Others	Hyperlipidemia

hormone and also the pituitary from secreting ACTH, thus creating central adrenal insufficiency. The ability to suppress the HPA axis depends on the glucocorticoid used, the dose, and the timing of administration as well as duration of therapy. Longer-acting drugs such as dexamethasone given at higher than physiologic requirement (>0.375 mg/day) for greater than a month always cause suppression of the HPA axis. Prednisone or prednisolone can do the same especially if the dose is not given as a single dose in the morning but is administered 2–3 times daily. The later administration (e.g., late evening) causes HPA suppression even when the dose is lower because the effect that lately administered drug will inhibit the expected physiological rise in ACTH in the early morning hours and contribute to HPA suppression. Most commonly used diseases being treated with glucocorticoids do not require the drug administration more frequently than once a day or even better every other day.

In light of the above discussion, it is often hard to define a specific dose or duration of supraphysiological glucocorticoid administration that will definitely indicate HPA suppression. However, as discussed earlier, the timing of drug administration and the duration of biologic effects of the glucocorticoid used have a more significant impact on HPA suppression. For example, one would predict that a patient receiving a relatively small dose of prednisone such as 2.5 mg at bed time for several months to be more likely to have HPA suppression than another taking 10 mg once daily in the morning, In general, patients who appear clinically Cushingoid are likely to have suppressed HPA axis irrespective of the dose they were exposed to. The concern in such patients is that they are likely to decompensate during an acute illness if they are not treated with appropriate doses of glucocorticoids.

Approach to Minimizing Adverse Effects of Exogenous Glucocorticoids

Glucocorticoids are so important in the management of too many diseases such that they will continue to be used. However, attempts to find alternative therapy for the disease being treated is always warranted. Using the smallest dose possible, the least number of times daily, for the shortest time possible and selecting the appropriate drug would certainly be helpful. Making sure such patients are given proton pump inhibitors to minimize the chance for gastritis or ulceration is important. As discussed earlier most patients receiving chronic glucocorticoid therapy develop osteopenia or frank osteoporosis and therefore prophylactic measures should be taken to minimize that adverse event. Such maneuvers include adequate calcium and vitamin D supplementation, gonadal steroid replacement when indicated and at times the use of bisphosphonates may be necessary. The need to maintain adequate exercise can minimize the expected myopathy most of these patients develop. Even though the most commonly used glucocorticoid in treating inflammatory diseases does not have potent mineralocorticoid activity, the total dose used in many patients is high enough to result in excessive sodium retention. The concern in this setting would be in patients who have other illnesses such as congestive heart failure where

a small increase in sodium retention can cause decompensation. If glucocorticoid have to be used is such patient, it would seem reasonable to use a drug that has minimal or no mineralocorticoid activity. It is also prudent to have these patients get well-balanced meals with lower salt and adequate protein. Infections, especially fungal or opportunistic, are more likely to occur in patients on chronic glucocorticoid therapy and therefore one needs to be vigilant about any new complaints or clinical signs especially considering that such patients may not be able to mount a fever response to infections.

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Part II
Genetics and Pathophysiology

Chapter 4
Autoimmune Addison's Disease: Genetic Aetiology and Pathophysiology

Agnieszka Pazderska, Simon H. Pearce, and Anna Louise Mitchell

Introduction

Autoimmune Addison's disease (AAD) is a rare endocrine disorder with a reported prevalence of 100–210 cases per million in Caucasian populations [1–4]. Like other autoimmune diseases, it is more prevalent amongst women, with a female-to-male ratio of 1.3–3.5:1 [1], with the exception of individuals younger than 30 years of age where there is no gender difference [5]. AAD is most commonly diagnosed in individuals between their third and fifth decades of life. In European countries, the disease has a reported incidence of 4.7–6.2 per million per year. Both prevalence and incidence of AAD have been increasing in recent years raising the possibility that, as yet undefined, environmental factors may play a role in the pathophysiology of the disease [5, 6].

Historically, tuberculous adrenalitis was a frequent cause of primary adrenal insufficiency [7] and remains a problem in developing countries, but in recent decades, autoimmunity has become the commonest aetiology in developed countries [8–10], reflecting an increase generally in autoimmune conditions in the population. AAD results from destruction of the adrenal cortex by an aberrant immune response. It accounts for over 80% of cases [9–11]. Other causes of primary adrenal insufficiency can be categorised into three distinct groups: impaired steroidogenesis, defects in adrenal gland development and adrenal cortex destruction by other disease processes (Box 4.1).

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Box 4.1 Causes of primary adrenal insufficiency

Impaired steroidogenesis

- Congenital adrenal hyperplasia (*CYP21*, *CYP11B1*, *HSD3B2* and *CYP17* mutations)
- Familial glucocorticoid deficiency due to mutations in genes involved in DNA replication and mitochondrial redox homeostasis (*MCM4, NNT, TXNRD2, PRDX3, GPX1*) [12, 13]
- Smith-Lemli-Opitz syndrome (*DHCR7* mutations) [14]
- Lipoid adrenal hyperplasia due to steroidogenic acute regulatory protein (*StAR gene*) and *CYP11A1* mutations [13]
- Drug induced (mitotane, ketoconazole, metyrapone, etomidate, aminoglutethimide)

Adrenal gland dysgenesis or hypoplasia

- *DAX1* mutation
- *SF1* mutation
- ACTH receptor pathway defects (*MC2R* and *MRAP* mutations) [13]

Adrenal destruction

- Autoimmunity
- Infection, e.g. tuberculosis
- Haemorrhage
- Adrenal metastases
- Primary adrenal lymphoma
- Sarcoidosis
- Amyloidosis
- Drug induced (mitotane)
- Adrenoleukodystrophy (*ABCD1* gene mutations)

A number of observations support strong heritability of AAD; these include concordance of AAD in mono- and dizygotic twins [15–17] and clustering of AAD within families [18, 19]. In addition, AAD is frequently observed in association with other autoimmune conditions in the context of autoimmune polyglandular syndrome type 2 (APS2). APS2 is defined as AAD coexisting with autoimmune thyroid disease and/or type 1 diabetes and/or another autoimmune condition such as vitiligo or pernicious anaemia and is present in 50–65% of individuals with primary adrenal insufficiency [20–22].

Although it has been long recognised that AAD is a highly heritable disorder, the rarity and complexity of this condition make its investigation challenging [23].

Pathogenesis of AAD

AAD is characterised by selective destruction of steroid hormone-producing cells in the adrenal cortex by T-cell-mediated inflammation (adrenalitis). The steroidogenic enzymes become the autoantigens against which an autoimmune response is generated. In the disease process, all three hormone-producing cell layers, the *zona fasciculata, zona reticularis* and *zona glomerulosa*, are gradually destroyed. The primary autoantigen in AAD is the 21-hydroxylase (21-OH) enzyme, and circulating 21-OH autoantibodies can be detected in approximately 85% of subjects with AAD. They may occur years prior to the development of clinically significant steroid deficiency [24, 25]. The other targets of the autoimmune process include the 17-alpha-hydroxylase enzyme and the cholesterol side-chain cleavage enzyme [26, 27]. Autoantibodies directed against the two latter enzymes are more commonly associated with AAD occurring in the context of autoimmune polyglandular syndromes [27, 28]. Steroid 21-OH autoantibodies are predominantly of the IgG_1 isotype and target the carboxy terminal of the enzyme [29]. While the detection of these antibodies can be used to cement the diagnosis of AAD in an individual with adrenal insufficiency, their role in the pathogenesis of the disease remains unclear. Although *in vitro* studies have demonstrated that these antibodies inhibit enzymatic activity of the 21-OH enzyme by preventing its interaction with cytochrome P450 oxidoreductase, these findings have not been corroborated *in vivo* [30]. The presence of such antibodies in some individuals with no detectable reduction in steroid concentration would argue against such an interaction [31]. In addition, 21-OH antibodies can cross the placenta as IgG antibodies; however, there have been no reports of transient hypoadrenalism in offspring born to AAD mothers. This suggests that the presence of adrenal antibodies in the serum alone is insufficient to cause autoimmune adrenal insufficiency [32]. In keeping with this is the intracellular location of the steroidogenic enzymes in intact cells, precluding their direct interaction with circulating autoantibodies. Recently, it has been shown that 21-OH-specific CD4 and CD8 T cells are abundant in AAD subjects many years after diagnosis and their immunoactivation generates persistent cytolytic T-cell populations, with the potential to destroy 21-OH-expressing cells [33]. Interestingly, T-cell immune responses in AAD subjects cluster around just a few 21-OH immunodominant antigenic determinants: HLA-B8-restricted epitope $21\text{-OH}_{431\text{-}438}$, HLA-A2-restricted epitope at position $21\text{-OH}_{342\text{-}350}$ and HLA-DRB1*0401 restricted epitope at position $21\text{-OH}_{342\text{-}361}$ [33–35].

In AAD, like in other organ-specific autoimmune disorders, three stages can be identified: potential, preclinical and clinical. In the potential phase, adrenal autoantibodies are present, but adrenal steroidogenesis is normal and no clinical features of the disease can be found. It appears that adrenal autoantibodies are very rare in the general population. In a number of population studies including apparently healthy individuals, approximately 32,000 people were tested, and 21-OH

autoantibodies were detected in only 430 individuals, giving a prevalence of 13 in 100,000 [36]. Subjects with positive 21-OH antibodies have an approximately 20% cumulative risk of developing clinically apparent AAD [37]. The reported positive predictive value of adrenal antibodies for development of clinically apparent AAD varies from 0 to 90% [38–40]. These huge discrepancies can be partly explained by varying follow-up duration and differences in the populations recruited. It appears that the highest risk for development of AAD in the presence of adrenal autoantibodies occurs in paediatric populations; the reported cumulative risk ranges from 20 to 100% [40–43]. The very high risk of development of AAD in children reported in some studies is due to the inclusion of subjects with APS1 syndrome, which in itself is associated with a prevalence of AAD of up to 90%. Amongst adult individuals, higher titres of autoantibodies are associated with higher risk for development of AAD, and these individuals progress more rapidly than those with low titres [44]. However, individual responses to an ongoing autoimmune process vary hugely as illustrated by a case study of a woman with a 9-year history of hyperpigmentation, elevated ACTH concentrations and high 21-OH antibody titres but a normal cortisol response to administration of synthetic ACTH [45]. In addition, some individuals shown to have 21-OH antibodies revert to being antibody negative [37]. A study by Baker et al. suggests that this variability in controlling the autoimmune response might have a genetic basis with the HLA-B15 haplotype conferring protection from progression to AAD in antibody-positive individuals [46].

In the subclinical phase, adrenal function gradually becomes impaired, but this is not sufficient to produce overt clinical manifestations of the disease. The first biochemical evidence of impaired steroidogenesis in the adrenal cortex appears to be increased ACTH concentration followed by raised plasma renin activity, accompanied by normal or low plasma aldosterone concentration [47]. However, one group has reported that abnormalities in the plasma renin-aldosterone axis occur first [25]. The discrepancies in these observations could be related to different dietary habits, salt intake in particular, and/or treatment with medications influencing renin such as ACE inhibitors or angiotensin receptor blockers in the populations studied. Finally basal and/or stimulated cortisol concentration becomes abnormal (<550 nmol/l after 250 µg of tetracosactide—Synacthen administration). Overall it appears that the *zona glomerulosa* is the adrenal cell subtype that is most sensitive to autoimmune destruction. We postulate that this may be due to a lack of protective high glucocorticoid concentrations in the *zona glomerulosa*, which are present in the *zona fasciculata*, that can potentially inhibit local immune responses.

Finally, in the clinical phase of the disease, symptoms and signs develop; these include hyperpigmentation, fatigue, weight loss, hypotension and salt craving. This usually occurs when at least 90% of the functioning adrenocortical cells have been destroyed [48]. However, it appears that progression to the clinical phase, even in individuals with evidence of organ-specific autoimmunity and impaired steroidogenesis, is not inevitable. This is illustrated by four case reports. Three patients achieved spontaneous partial remission in established AAD [49–51], and one patient achieved long-term remission of subclinical AAD following high-dose glucocorticoid therapy for another condition [52].

Genetic Basis of APS1 Syndrome

AAD can occur in the context of a rare, monogenic syndrome known as autoimmune polyglandular syndrome type 1 (APS1) also referred to as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). The syndrome is characterised by at least two out of the following: AAD, hypoparathyroidism and mucocutaneous candidiasis [53]. These conditions usually become apparent in a specific order: fungal infection of the skin, nails and mucous membranes occurs typically in infancy, followed by hypoparathyroidism in early childhood and adrenal insufficiency predominantly in teenagers and young adults. Other APS1-associated conditions include premature gonadal failure, type 1 diabetes mellitus, hypothyroidism, alopecia, vitiligo, pernicious anaemia, autoimmune hepatitis, hypoplasia of the dental enamel and nail dystrophy [54–56]. Intrestingly, Graves' disease is very rare in the context of APS1. The syndrome is inherited in an autosomal recessive fashion and results from mutations in the autoimmune regulator (*AIRE)* gene localised on chromosome 21q22. The product of the *AIRE* gene is predominantly expressed in the thymus and concerns development and maintenance of self-tolerance [57]. Over 60 pathogenic mutations in the *AIRE* gene have been described; the majority of these result in a truncated protein product [58]. In keeping with recessive inheritance, affected individuals can be either homozygous for an identical mutation or can be compound heterozygotes. There are reports of genotype-phenotype correlations [59]. The APS type 1 syndrome is most prevalent in Iranian Jewish (1:9000), Sardinian (1 in 14,000), Finnish (1 in 25,000) and Norwegian (1 in 90,000) populations [59–62]. Recently, families with dominant inheritance of a milder APS1 phenotype have been described owing to heterozygous *AIRE* mutations that inactivate the normal *AIRE* allele (dominant-negative mutation) [63]. Affected individuals have a heterogeneous presentation with later onset and lower penetrance compared to the classical APS1 syndrome. Dominant-negative *AIRE* mutations are associated with various forms of organ-specific autoimmunity; in particular, vitiligo and pernicious anaemia are commonly present.

Genetic Basis of Human Non-APS1 AAD

Unlike APS1, isolated AAD and AAD in the context of APS2 are not inherited in a Mendelian fashion. Their pathogenesis is thought to be due to a complex interplay between genetic and environmental factors. The genetic basis for the disease is complex and involves multiple genetic susceptibility variants. It appears that no single susceptibility variant is sufficient to cause the disease and a "critical" genetic load is required to initiate the pathogenic process. A sibling recurrence risk ratio (the ratio of risk of disease manifestation, given that one sibling is affected, compared with the disease prevalence in the general population) in non-APS1 AAD is approximately 160–210, considerably higher to that seen in type 1 diabetes or Graves' disease, which have sibling recurrence risk ratios of 15 and 10, respectively [64, 65].

This suggests a very strong genetic influence in the pathophysiology of AAD. In addition, the clustering of autoimmune conditions with AAD in the context of APS2 suggests that there are common susceptibility loci for these disorders.

Molecular Genetic Studies

A number of candidate gene case-control studies have been conducted in cohorts of patients with AAD. The number of AAD cases in the cohorts studied is significantly smaller than in the genetic studies carried out in other autoimmune disorders such as type 1 diabetes or autoimmune thyroid disease because of the relative rarity of this condition. Candidate genes are selected based on underlying biological plausibility (they are either implicated in an associated autoimmune disease such as autoimmune thyroid disease or type 1 diabetes, or they are associated with rare, monogenic variants of the disease, such as APS1). To date, a number of susceptibility variants to AAD have been identified (Table 4.1). However, with the exception of MHC locus (in particular, DRB1), the susceptibility variants discovered thus far contribute only a small amount towards an individual's overall genetic susceptibility to AAD.

Autoimmunity is thought to arise as a result of aberrant responses within both the adaptive and innate immune systems. The adaptive immune system refers to antigen-specific immune responses involving antigen recognition and processing, forming "immune memory", and concerns its key cells—lymphocytes. Innate immunity refers to non-specific defence mechanisms against pathogens occurring within hours of an antigen's appearance and comprises a number of cell types. Amongst the functions of the innate immune system are pathogen recognition, production of cytokines and chemokines leading to immune cell recruitment, complement cascade activation and activation of the adaptive immune system via antigen presentation. In patients with AAD, similarly to other autoimmune diseases, aberrant responses in both innate and adaptive immunity are implicated. However, the majority of susceptibility loci identified to date encode proteins involved in antigen presentation and T-cell activation.

MHC Risk Alleles

The major histocompatibility complex (MHC) in humans, the human leukocyte antigen (HLA) complex, is located on chromosome 6p21 and comprises multiple genes involved in immune processes. Amongst those are HLA class I (HLA-A, HLA-B and HLA-C) and HLA class II (HLA-DRB1, HLA-DQB1, HLA-DQA1, HLA-DPB1 and HLA-DPA1) genes which encode antigen-presenting molecules and are the most important determinants of polygenic autoimmune disease risk. HLA proteins are expressed on the surface of antigen-presenting cells and display

Table 4.1 Case-control studies of candidate genes in patients with non-APS1 AAD

Gene or marker	Population	Number of patients	Odds ratio	P value
MHC loci				
HLA-B*08	Norway [66]	414	2.6	4×10^{-11}
HLA-DR3-DQ2	Norway [67]	94	3.6	$<10^{-7}$
HLA-DR3-DQ2	Finland, Russia, Estonia [68]	69	14.5	<0.0001
HLA-DR4-DQ8*0404	Norway [67]	94	NR	$<10^{-5}$
HLA-DR4-DQ8*0401				$<10^{-4}$
HLA-DRB1*0301	Norway [66]	414	22.13	6×10^{-20}
HLA-DRB1*0404				
Other loci				
MICA*5.1	Italy [69]	28	6.52	0.0015
	USA [70]	46	22.5	$<10^{-5}$
	Norway [66]	414	1.78	0.0003
CYP21Adel	Finland [71]	12	25.0	NR
CYP21 L10, R102, A494	Finland [72]	12	8.9	NR
CLEC16A (rs12917716)	Norway [73]	332	0.72	0.0004
	UK [73]	210	1.06	0.71
	Combined [73]	542	0.81	0.006
CYP27B1-126C > A (rs4646536)	Germany [74]	124	1.18	0.006 (genotype)
				0.3354 (allele)
	UK [75]	104	1.71	0.003
	6 European cohorts [76]	1955	0.9	0.03
VDR Fok1 (exon2)	Germany [77]	95	NR	0.0351 (genotype)
				0.3390 (allele)
FCRL3 (rs7528684)	UK [78]	200	1.61	0.0001
PTPN22 (rs2476601)	UK [79]	104	1.69	0.031
	Germany [80]	121	1.03	0.9878
	Norway [81]	302	1.39	0.016
	UK [82]	251	1.63	0.008
	Poland [82]	87	1.84	0.010
CTLA4 A > G (exon 1)	UK [83]	90	1.64	0.008
	Norway, UK, Germany, Spain and Italy [84]	1002	1.37	0.002
CTLA4 J030G > A	UK [85]	40	1.9	0.02
	Norway [85]	94	1.4	0.04
	Combined [85]	134	1.5	0.03
NLRP1 (rs12150220)	Norway [86]	333	1.25	0.007
	Poland [87]	101	1.5	0.015
PD-L1 (rs1411262)	UK [88]	315	1.33	0.032
	Norway [88]	342	1.34	0.026
	Combined [88]	657	1.32	3.03×10^{-3}

Table 4.1 (continued)

| Gene or marker	Population	Number of patients	Odds ratio	P value
STAT4 (rs4274624)	6 European cohorts [76]	1955	1.27	<0.0001
(rs10931481)		1262	1.23	0.0007
GATA3	6 European cohorts [76]	1955	0.9	0.03
NFκB1 (rs10026278)	UK [76]	309	0.69	0.0034
(rs230532)			0.65	0.00041
(rs4698861)			0.63	0.00017
Il-23A	Italy [76]	280	2.37	0.0028
GPR174 (rs3827440)	UK [89]	286	1.34	0.03
Il-2 (rs3136534)	Poland [90]	223	0.71	0.003
BACH2 (rs3757247)	UK [91]	358	1.44	1.4×10^{-6}
	Norway	317	1.41	0.0015
NFATC1 (rs754093)	Norway [92]	384	NR	0.48
	Sweden [92]	367	NR	0.033 (genotype)
				0.07 (allele)
	UK [92]	346	NR	0.15
	Combined [92]	1097	NR	0.02
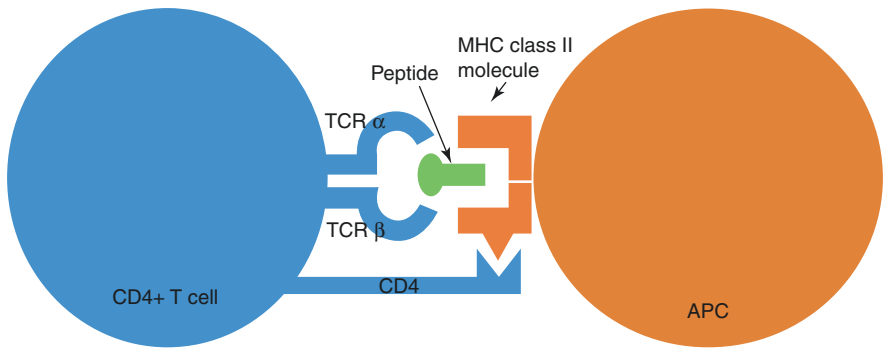
Fig. 4.1 Schematic representation of antigen presentation by MHC class II molecules to CD4+ T cell. The antigen-MHC complex is recognised by the T-cell receptor. CD4 is a co-receptor that binds to a non-polymorphic region of the MHC and assists in T-cell activation. APC, antigen-presenting cell; TCR, T-cell receptor

peptides (both self and non-self) for recognition by T cells. Aberrant activation of T cells in response to self-antigens leads to development of autoimmune disease (Fig. 4.1). Although the exact immunopathology of AAD remains to be established, it has been shown that MHC II molecule expression on adrenocortical cells is highly upregulated in the active phase of the disease [93].

Similar to other autoimmune conditions, the challenge in finding causal variants within the MHC for AAD lies in the fact that this region contains the largest number of polymorphisms in the entire genome [94] and that there is strong and extensive

LD amongst alleles throughout this locus [95]. A number of variants within the MHC class II genes are associated with several autoimmune conditions. In particular, a strong association between autoimmunity and allelic variability in HLA-DR and DQ molecules, which present exogenous antigens for recognition by CD4+ helper (Th) cells, exists. Allelic polymorphism at these loci results in variant proteins that allow self-peptides to enter the antigen-binding groove more readily. The association between HLA class II molecules and AAD has been recognised for three decades [96]: in a seminal study by Maclaren and co-workers, susceptibility to AAD was linked to HLA-DR3 and HLA-DR4 alleles. Subsequently, these findings were replicated in a number of AAD cohorts [66, 67, 71, 97, 98]. A particularly high-risk genotype for AAD has been identified as DR3-DQ2/DR4-DQ8 [67]. To date, the HLA class II alleles DRB1*0301 and DRB1*0404 have been shown to confer the highest risk for AAD with odds ratios (OR) of 2.9 and 3.3, respectively [66]. An especially large risk increment occurs in compound heterozygotes possessing both these haplotypes (OR = 22). The DRB1 alleles occur in strong linkage disequilibrium (LD) with other alleles associated with AAD: DQB1*02 and DQB1*0302 (OR 1.8 and 1.5, respectively) [66]. In addition, a number of haplotypes conferring protection from AAD have been also discovered: DRB1*0401-DQ8 [67] and DRB1*0403 [99]. Thus far, none of the susceptibility loci identified are specific to AAD with the possible exception of HLA-DRB1*0404. The possible explanation for this is that peptides from 21-OH might bind particularly well and be presented to autoreactive T cells by this HLA class II molecule [35].

Polymorphisms in the *CYP21A2* (cytochrome P450, family 21, subfamily A, polypeptide 2) gene, which encodes the 21-OH enzyme and is located within MHC class III region, have been associated with AAD. *CYP21A2* is 600 kb away from the HLA-DRB1 locus; therefore, the association of its polymorphisms and AAD has been attributed to long-range LD with MHC class II alleles [72]. A recent study confirmed that no specific variants of *CYP21A2* are associated with AAD. Instead, *CYP21A2* polymorphisms are in LD with the high-risk haplotype *HLA-DRB1* locus and do not contribute to the disease susceptibility independently [100].

The genes that appear to be independently associated with AAD susceptibility include HLA-B (OR 2.6 for HLA-B*08) and MHC class I-related chain A (*MICA*) (OR 1.8 for MICA*5.1) [66]. Homozygosity for the MICA*5.1 allele in the presence of the high-risk HLA genotype DR3-DQ2/DR4.4-DQ8 confers extreme risk for AAD development [101].

Non-MHC Risk Alleles

The *CIITA* gene encodes a protein functioning as a HLA class II transactivator, the master control factor for MHC class II expression. Mutations in *CIITA* result in a severe monogenic immunodeficiency disease known as bare lymphocyte syndrome. Allelic variability in this gene has been associated with conditions such as
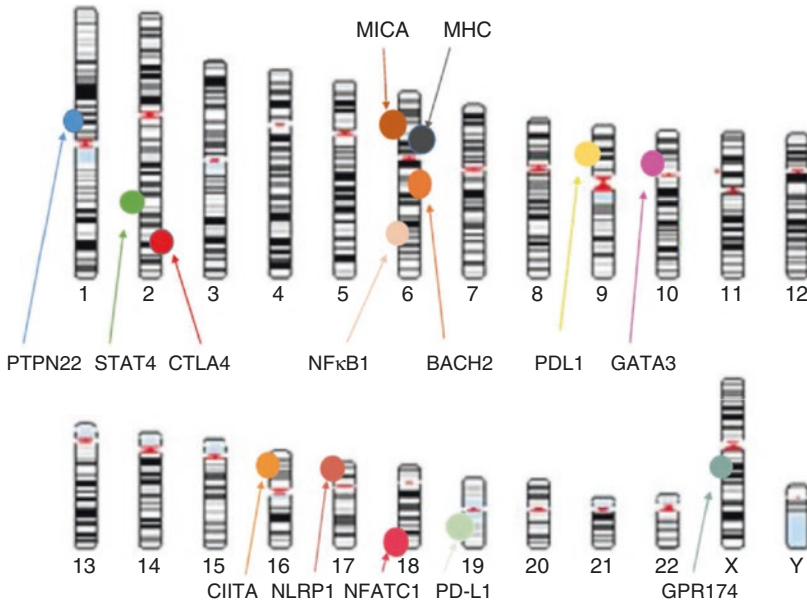
Fig. 4.2 Schematic representation of genes associated with autoimmune Addison's disease that are implicated in T-cell proliferation and activation

rheumatoid arthritis [102], systemic lupus erythematosus [103] and coeliac disease [104], amongst others. Polymorphisms in the promoter, as well as in intron 3, of this gene have been reported to be associated with AAD susceptibility [73, 105] Although the mechanism by which these polymorphisms confer disease susceptibility remains unknown, it has been hypothesised that they could influence the levels of tissue selectivity of HLA class II expression (Fig. 4.2).

T-Cell Proliferation, Differentiation and Activation Genes

Activation of T lymphocytes, the key cells of the adaptive immune system, requires simultaneous engagement of the T-cell receptor by MHC class II peptides and costimulatory molecules. The cytotoxic T-lymphocyte-associated antigen 4 (*CTLA4*) gene located on chromosome 2q33 encodes CD152, a costimulatory molecule which acts as a vital negative regulator of T-cell activation and proliferation [106]. CTLA4 competes with costimulator CD28 for binding to B7 on antigen-presenting cells. The critical role of this molecule is demonstrated in *CTLA4* knock-out mice which die prematurely, at an age of 2–3 weeks, due to severe lymphoproliferation, lymphocytic infiltration and destruction of major organs [107]. In humans, mutations in the *CTLA4* gene result in an immune dysregulation syndrome [108]. *CTLA4* gene polymorphisms have been linked with susceptibility to a number of autoimmune diseases including autoimmune thyroid disease [109, 110],

type 1 diabetes mellitus [111, 112], rheumatoid arthritis [113] and coeliac disease [114]. The association between *CTLA4* gene variants and AAD has been reported in a number of studies in different European populations [83–85, 115, 116]. The most commonly described *CTLA4* polymorphisms in AAD populations are non-synonymous polymorphism in exon 1 region of *CTLA4* gene +49 A → G (*Ala17Thr*) [83, 84, 115], (AT)n dinucleotide repeat polymorphism within the 3′ untranslated region [116] and G or A alleles of the JO30 SNP downstream of this gene [85]. Polymorphisms in the latter are postulated to be the causative variants affecting the relative amount of the soluble and membrane-bound CTLA4 and therefore enabling CD28 to access more of its ligand, resulting in T-cell activation [117]. In contrast to this hypothesis, a number of studies have demonstrated that individuals with auto-immune conditions have, in fact, increased serum levels of soluble CTLA4 isoforms [118, 119] suggesting that the complexity of CTLA4-CD28 interaction and signal-ling is incompletely understood. Another mechanism of negative immune regula-tion by CTLA4-positive cells is the ability of CTLA4 to capture and degrade CD80 and CD86 (ligands shared with the stimulatory receptor CD28) from antigen-presenting cells [120].

Another key autoimmunity gene, *PTPN22* on chromosome 1p13, encodes a tyro-sine phosphatase which is a crucial regulator of immune homeostasis, inhibiting T-cell receptor (TCR) signalling. The association of a missense SNP (1858C > T) in *PTPN22*, encoding an arginine to tryptophan substitution at amino acid 620, has been identified in type 1 diabetes [121], rheumatoid arthritis [122], systemic lupus erythe-matosus [123] and Graves' disease [79]. For a number of autoimmune diseases, this allelic variant ranks as the most important non-MHC single gene contributor to dis-ease susceptibility. A number of studies have implicated this variant in susceptibility to AAD [79, 81, 82]. The functional effect of 1858 C > T polymorphism has not been fully elucidated. Some studies suggest that this variant results in increased suppres-sion of TCR signalling [124, 125], while others suggest the opposite [126, 127]. One study suggested that proteolytic binding and cleavage of Arg620Trp is increased with consequent reduction in LYP levels in T and B cells leading to lymphocyte and den-dritic cell hyperresponsiveness and autoimmunity [127].

Programmed death ligand-1 (PD-L1, CD274, B7-H1) is a costimulatory mole-cule binding the PD-1 moiety of T cells, leading to downregulation of cytokine production and T-cell activation, thereby inducing immune tolerance. PD-L1 vari-ants have been implicated in susceptibility to AAD, although the effect on risk is very modest (OR 1.34 for the allele with the strongest association) [88].

Interleukin-2 (Il-2) and its receptor are important determinants of the immune response. Il-2 is a potent T-cell growth stimulator and influences T-cell differentia-tion, in particular formation of the regulatory T cell (T_{reg}) lineage. T_{reg} cells are cru-cial in maintaining self-tolerance due to their ability to suppress autoreactive T cells which escape negative selection in the thymus. *IL2RA* gene encodes the alpha sub-unit (CD25) of the IL2 receptor, a unique subunit conferring high affinity to IL2. Polymorphic variants of *IL2* (4q27) and *IL2RA* (10p15.1) genes have been associ-ated with type 1 diabetes and rheumatoid arthritis [128–130]. The C minor allele in *IL2* conferred protection from AAD in a Polish cohort. However, there was no

association found between *IL2* polymorphisms and Norwegian patients with AAD [73]. Similarly, an association between *IL2RA* polymorphisms and susceptibility to AAD was found in Norwegian subjects, but this finding has not been replicated in British or Polish series [73, 90]. Although there is known genetic heterogeneity between various AAD populations, the association between AAD and *IL2* or *IL2RA* needs further replication in larger cohorts.

STAT4 (signal transducer and activator of transcription 4) on chromosome 2q32 encodes a transcription factor which is implicated in Th-1 and Th-17 differentiation and activation. Polymorphisms of *STAT4* have been shown to be associated with rheumatoid arthritis [131–133], systemic lupus erythematosus [122] and type 1 diabetes [134]. A meta-analysis by Mitchell et al. revealed a significant association between *STAT4* polymorphisms and AAD in European populations [76]. The allelic variability identified in AAD maps to intron 3 of the *STAT4* gene and is in moderate-to-strong linkage disequilibrium with *STAT4* polymorphisms identified in the other autoimmune conditions. The exact mechanism by which polymorphisms in this gene lead to autoimmune disease remains unknown.

The *GATA3* gene on chromosome 10p14 encodes a C2C2-type zinc finger transcription factor which regulates a number of steps in T-cell development and differentiation. In particular, this transcription factor has been shown to be the Th2 lineage master regulator [135] and could therefore contribute to T-cell dysregulation present in autoimmune disease. A recent meta-analysis demonstrated an association between *GATA3* polymorphisms and AAD in European cohorts [76]. The minor G allele at *rs3802604* was protective for AAD (OR 0.9). This finding is, however, in contrast to the known association of the same allele with susceptibility to rheumatoid arthritis [136] possibly reflecting immunopathogenic differences between these two conditions.

The *BACH2* gene on chromosome 6q15 plays a vital role in CD^{4+} T-cell differentiation; in particular it is crucial in the formation of T$_{reg}$, which are the key cells in maintaining immune tolerance. Polymorphisms at the *BACH2* locus have been associated with type 1 diabetes, generalised vitiligo, autoimmune thyroid disease, Crohn's disease and coeliac disease [137–141]. Recently, an association between the minor T allele at *rs3757247* in the *BACH2* locus and susceptibility to AAD was described in UK and Norwegian cohorts [91].

The first linkage study in multiplex AAD families implicated regions on chromosomes 6 (corresponding to the *HLA* region), 7, 9 and 18 in the susceptibility to AAD [92]. A follow-on study, looking at 64 SNPs underlying the linkage peaks on chromosomes 9 and 18 conducted in case-control cohorts from the UK, Norway and Sweden, revealed nominal association with three independent SNPs in chromosome 18 and AAD. One of these encodes a non-synonymous variant (*pCys751Gly*) in exon 9 of the *NFATC1* (nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1) gene. Upon T-cell activation, family members of NFAT translocate to the nucleus where they can activate target genes and as such play a central role in gene transcription during the immune response [142]. NFATC1 has been shown to play a role in the regulation of PD-1 expression, a cell surface receptor functioning as an immune checkpoint and reducing T-cell activation [143].

The *GPR174* (*G protein-coupled receptor 174*) gene at Xq21.2 consists of one exon encoding a protein which belongs to the G protein-coupled receptor superfamily and is
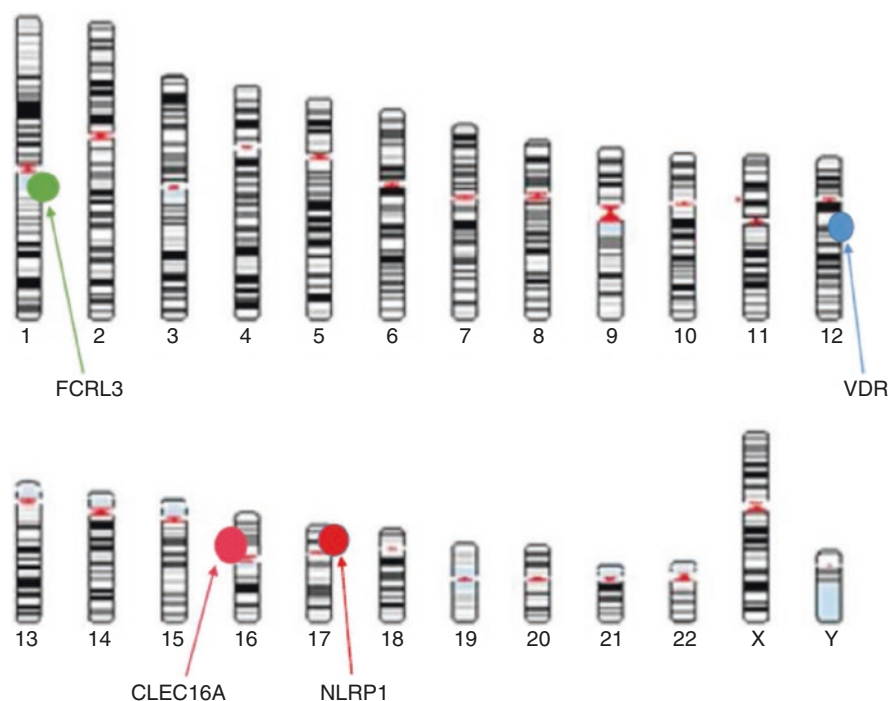
Fig. 4.3 Schematic representation of genes found to be associated with autoimmune Addison's disease which are implicated in proliferation and activation of B cells and antigen-presenting cells

involved in immune cell signal transduction. Recently a lysophosphatidylserine (LysoPS) was found to be a ligand for GPR174, and the interaction between the two has been shown to stimulate an increase in the intracellular cyclic adenosine monophosphate (cAMP) [144]. cAMP has been previously demonstrated to be a potent negative regulator of T-cell immune function [145]. These findings offer a plausible link between *GPR174* polymorphisms and autoimmunity. Polymorphisms in *GPR174* have been found to be associated with Graves' disease [146] [147]. A serine to proline non-synonymous variant in *GPR174* has been associated with AAD [89]. The localisation of *GPR174* on chromosome X and its role in AAD autoimmunity are particularly interesting given the gender bias observed in this disorder, although it is unlikely that a single gene is responsible for the higher susceptibility of females to develop AAD (Fig. 4.3).

Genes Implicated in B Lymphocyte and Antigen-Presenting Cell Proliferation and Activation

Vitamin D has been recognised for its effects on the immune system. On a molecular level, the active form of vitamin D, 1,25-dihydroxyvitamin D, leads to reduced expression of HLA class II molecules on endocrine cells and inhibits T-cell proliferation [148, 149]. 1,25-Dihydroxyvitamin D is also implicated in innate immunity

by inhibiting differentiation of dendritic cells which are potent antigen-presenting cells [150]. Polymorphisms in the vitamin D receptor (*VDR*) gene, located on chromosome 12q12-14, have been studied in a number of autoimmune conditions including type 1 diabetes and autoimmune thyroid disease with conflicting results [151–154]. Although specific genotypes in VDR have been associated with AAD risk in a relatively small German cohort, no such association was found for an individual VDR allele [77]. Additional studies are required to confirm VDR as a susceptibility locus for AAD. In contrast to this, a promoter polymorphism in the *CYP27B1* gene (*−1260C > A*) has been shown to be associated with AAD in two independent cohorts [74, 75]. More recently, an intronic SNP, *rs4646536*, in *CYP27B1*, was associated with AAD in a large meta-analysis of several European cohorts [76]. CYP27B1 hydroxylase catalyses the conversion of 25-hydroxyvitamin D to its active form, 1,25-dihydroxyvitamin D. A promoter polymorphism in *CYP27B1* might affect enzyme transcription and thus the rate of final hydroxylation of 1,25-dihydroxyvitamin D.

The Fc receptor-like 3 gene (*FCRL3*) located on chromosome 1q21 encodes an orphan cell surface receptor belonging to the immunoglobulin receptor superfamily, expressed predominantly on B lymphocytes. A polymorphism in the *FCRL3* promoter region (*FCRL3_3*C*) has been implicated in susceptibility to rheumatoid arthritis, autoimmune thyroid disease and systemic lupus erythematosus in Asian cohorts [155]. Contrary to the findings in other autoimmune conditions, a study of a UK AAD cohort found that the *FCRL3_3*C* variant confers protection from the disease [78]. The allele most associated with disease risk in this cohort was found to be *FCRL3_3*T* (OR = 1.61). Based on functional studies of this locus, *FCRL3_3*T* is predicted to result in lower promoter activity. Such contradictory findings for one haplotype conferring both protection and disease susceptibility in different populations illustrate the complexity of the genetic underpinning of polygenic autoimmune diseases including AAD.

NLRP1 (nuclear localisation leucine-rich-repeat protein 1) is a regulator of the innate immune response. NLRP1 belongs to the NOD-like receptor family and participates in recognising microbial products, such as lipopolysaccharide, and assembly of inflammasomes, cytoplasmic protein complexes mediating pro-inflammatory responses via cytokine activation [156]. Polymorphisms of the *NLRP* gene have been reported to confer risk for a number of autoimmune conditions including vitiligo [157], type 1 diabetes [86], coeliac disease [158] and rheumatoid arthritis [159]. A coding variant of *NLRP1* (*Leu155His*) has been associated with AAD in two European cohorts [86, 87]. Surprisingly, different alleles were found to confer risk of AAD in Polish (minor allele A) and Norwegian (major allele T) populations.

The *CLEC16A* (*C-type lectin domain family 16*) gene encodes a protein of unknown function but which is almost exclusively expressed in immune cells such as dendritic cells, B lymphocytes and natural killer cells. This makes it a plausible susceptibility gene for autoimmunity. A polymorphism in the *CLEC16A* gene (intronic SNP *rs12917716*) was found to be associated with AAD in a Norwegian cohort with an OR of 0.71. Comparable effects of *CLEC16A* SNPs were previously demonstrated in cohorts of subjects with type 1 diabetes (OR 0.65 to 0.83) [160, 161].

Processes Affecting Gene Expression

Gene expression can be altered by both common copy number variation (CNV) and epigenetic modification such as DNA methylation. As a result, different phenotypes can develop despite similar genetic profiles.

CNV in the human genome has been recently identified as a source of genetic diversity and has been shown to influence disease susceptibility [162]. Recently, CNVs in two genes, *UGT2B28* and *ADAM3A,* have been found to be associated with AAD [163]. However, the mechanism by which this association confers susceptibility to the disease remains unknown.

Abnormal DNA methylation is commonly observed in autoimmune disorders. It has been suggested that hypermethylation (addition of methyl groups to oligonucleotides by DNA methyltransferases) of promoter regions silences genes, whereas intronic hypermethylation is involved in gene activation. DNA methylation has been shown to be one of the mechanisms involved in transcriptional control of genes such as *FOXP3*, *Interferon Gamma* and *AIRE* which in turn influence T-cell differentiation and function. A recent study identified multiple hypomethylated gene promoter regions in DNA isolated from CD4 T cells from AAD subjects [164]. A multitude of differently methylated regions have been localised in genes implicated in immune modulation and autoimmunity suggesting that this epigenetic modification plays a role in the immunopathogenesis of this disease. This is likely to be an area of research going forward.

Genetics of Canine Addison's Disease

Autoimmune hypocortisolism is highly prevalent in a number of dog breeds including collies, poodles, terriers and retrievers. Canine hypoadrenalism shares some susceptibility loci with human AAD including MHC (DLA, dog leucocyte antigen), *PTPN22*, *NLRP1* and *AIRE* [165]. In addition, allelic variability in IL-16 and GC has also been implicated in canine Addison's disease. Similar to humans, most of the allelic variability associated with the increased risk pertains to genes implicated in T-cell receptor pathways.

Environmental Factors in Pathophysiology of AAD

We have recently suggested a potential seasonal periodicity, with excess risk for development of AAD in individuals born in winter months and a protective effect when born in summer. Exposure to seasonal viral infection in the perinatal period and vitamin D exposure related to UVB radiation intensity are the postulated environmental factors underpinning this association [166].

Another interesting concept is that of physical or psychological stress as a trigger of autoimmune processes. In fact, many retrospective studies have found that a high proportion of patients with various autoimmune conditions reported emotional stress prior to disease onset [167]. However, data pertaining to the role of stress in the pathophysiology of AAD are lacking.

Summary

Our current concept of AAD aetiopathogenesis is that it results from an interplay between as yet unidentified environmental factors and genetic susceptibility loci. The susceptibility genes discovered to date encode proteins that are involved in the activation and regulation of antigen-specific T cells; however, these have only a modest effect in terms of disease risk contribution and are commonly associated with other autoimmune disorders. Further work is required to gain a better understanding of the genetic architecture of this interesting autoimmune condition.

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Chapter 5
Genetics and Pathophysiology of Congenital Adrenal Hyperplasia

Selma Feldman Witchel

Abbreviations

17-OHP	17-hydroxyprogesterone
21-OHD	CAH due to 21-hydroxylase deficiency
ACTH	Adrenocorticotropic hormone
CAH	Congenital adrenal hyperplasia
CRH	Corticotropin-releasing hormone
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
NC-21-OHD	Nonclassic 21-hydroxylase deficiency
SULT2A1	Steroid sulfotransferase

Introduction

The virilizing congenital adrenal hyperplasias are a family of autosomal recessive disorders affecting adrenal steroidogenesis that are characterized by excessive adrenal androgen production. The most common form is 21-hydroxylase deficiency (21-OHD) due to mutations in the 21-hydroxylase (*CYP21A2*) gene. The other virilizing forms are 3β-hydroxysteroid dehydrogenase and 11β-hydroxylase deficiencies associated with mutations in the 3β-hydroxysteroid dehydrogenase (*HSD3B2*) and 11β-hydroxylase (*CYP11B1*) genes, respectively. Another form of CAH associated

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with genital ambiguity is oxidoreductase deficiency (PORD), which is associated with mutations in the cytochrome P450 oxidoreductase (*POR*) gene. *POR* encodes a flavoprotein that serves as an electron donor for cytochrome P450 steroidogenic enzymes such as 21-hydroxylase. Congenital lipoid adrenal hyperplasia (CLAH) is associated with mutations in the steroidogenic acute regulatory protein (StAR) gene, undervirilization of male fetuses, and absence of circulating steroid hormones. Mutations in 17α-hydroxylase/17,20-lyase (*CYP17A1*) are associated with undervirilization in males, absent puberty in females, and hypertension. Mutations in the aromatase (*CYP19A1*) gene interfere with the conversion of androgens to estrogens and are characterized by maternal virilization during puberty, virilization of female fetuses, failure of epiphyseal fusion, tall stature, and hyperandrogenic symptoms in adolescent and adult females. This chapter will focus on 21-hydroxylase deficiency because it is the most common form of congenital adrenal hyperplasia. A brief outline of other defects in steroidogenesis is provided in Table 5.1.

The clinical features associated with CAH comprise a spectrum reflecting the consequences of the specific mutation. In the case of 21-OHD, the continuum ranges from salt-losing and simple virilizing forms to the milder forms. Collectively,

Table 5.1 Disorders of steroidogenesis. Gene, gene location, and typical phenotypes are listed. In general, severity of phenotype correlates with genotype

Gene	Location	Phenotype	Characteristic laboratory findings
CYP21A2 Classic forms	6p21.33	Ambiguous genitalia with virilization of females with continued postnatal virilization if undiagnosed Normal male genitalia at birth Acute adrenal insufficiency with salt-losing crises	Increased 17-OHP, P4, androstenedione, and ACTH Increased PRA
CYP21A2 Nonclassic forms	6p21.33	Premature pubic hair, tall stature, irregular menses, acne, and infertility	Increased 17-OHP, P4, androstenedione, and ACTH
HSD3B2	1p12	Ambiguous genitalia with virilization of females Ambiguous genitalia with undervirilization of male infants Acute adrenal insufficiency with salt-losing crises	Increased 17-Preg, DHEA Increased PRA in classic salt-losing forms
CYP11B2	8q24.3	Ambiguous genitalia with virilization of females with continued postnatal virilization if undiagnosed Variable hypertension	Increased 11-deoxycortisol, DOC, androstenedione, and ACTH
StAR	8p11.23	Undervirilization of male infants Acute adrenal insufficiency with salt-losing crises	All steroid hormones are low or absent
CYP17A1	10q24.3	Undervirilization of males Delayed/absent puberty in females Variable hypertension	Increased DOC and ACTH Low 17α-hydroxylated steroids Decreased PRA

Table 5.1 (continued)

Gene	Location	Phenotype	Characteristic laboratory findings
POR	7q11.23	Ambiguous genitalia in males and females Antley-Bixler skeletal anomalies, i.e., craniosynostosis, radiohumeral synostosis, midface hypoplasia, and femoral bowing Infertility	Increased 17-OHP, P4, and ACTH Decreased DHEA, androstenedione, testosterone Normal electrolytes
CYP19A1	15q21.2	Virilization of female infants Maternal virilization during pregnancy Delayed puberty with hypogonadotropic hypogonadism and multicystic ovaries in females Delayed/failed epiphyseal fusion Osteopenia/osteoporosis Impaired glucose tolerance/insulin resistance Decreased sperm number and impaired motility	Increased androgens and P4 Increased LH and FSH
CYB5	18q22.3	Undervirilization of male infants because cytochrome b_5 is requisite cofactor for P450c17	Decreased testosterone Methemoglobinemia

Key: *17-OHP* 17-Hydroxyprogesterone, *P4* progesterone, *DHEA* dehydroepiandrosterone, *PRA* plasma renin activity

the salt-losing and simple virilizing forms are considered to be the classic forms. The mild form is also known as the late-onset or nonclassic form (NCAH). This classification system is somewhat contrived because disease severity is better represented as a continuum based on residual enzyme activity. The incidence of the classic forms ranges from 1:5000 to 1:15,000 with variation among ethnic/racial backgrounds [1]. The prevalence of 21-OHD is lower among African-Americans than Caucasians in the United States [2]. Incomplete ascertainment muddies accurate determination of the incidence of NCAH. However, available data indicate that NCAH may occur in 1:1000 with increased frequency among Hispanics, Yugoslavs, and Ashkenazi Jews [3].

Pathophysiology

In these disorders, the loss of cortisol negative feedback inhibition leads to increased hypothalamic corticotrophin-releasing hormone (CRH) and pituitary adrenocorticotropic hormone (ACTH) secretion. The excessive ACTH secretion leads to accumulation of steroid hormone intermediates proximal to the deficient enzyme and hyperplasia of the zona fasciculata and zona reticularis.
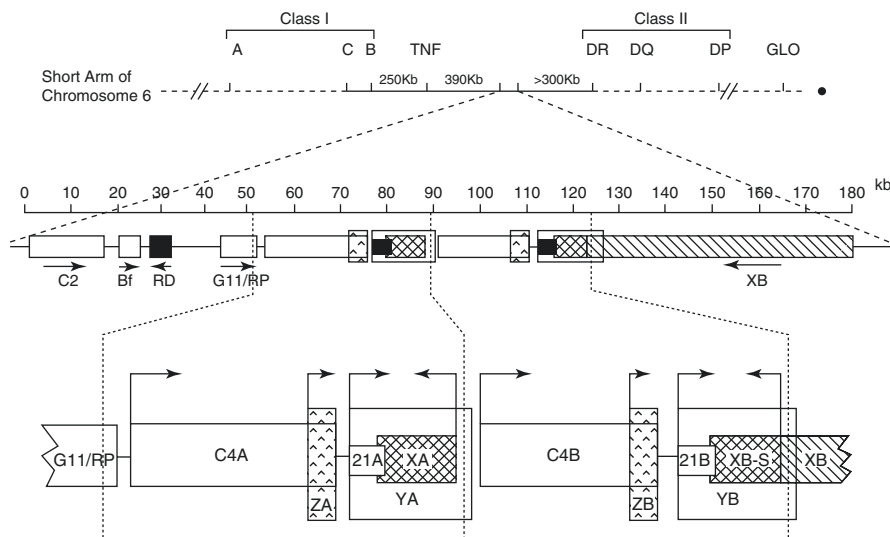
Fig. 5.1 Pathways of adrenal steroid hormone synthesis particularly relevant for 21-OHD. *CYP11A1* cytochrome P450 cholesterol side-chain cleavage, *StAR* steroidogenic acute regulatory protein, *CYP17A1* 17α-hydroxylase/17,20-lyase, *HSD3B2* 3β-hydroxysteroid dehydrogenase type 2, *P450oxido* P450-oxidoreductase, *CYB5A* cytochrome b₅, type A, *SULT2A1* sulfotransferase 2A1, *CYP21A2* 21-hydroxylase, *CYP11B1* 11β-hydroxylase, *CYP11B2* aldosterone synthase, *AKR1C3* 17β-hydroxysteroid dehydrogenase type 5, *SRD5A* 5α-reductase

In the classical pathway of adrenal steroidogenesis (Fig. 5.1), cholesterol is converted to pregnenolone by the P450 side-chain cleavage enzyme encoded by *CYP11A1*. Most steroidogenic enzymes are cytochrome P450 enzymes which acquired their family name because they absorb light at 450 nm when reduced with carbon monoxide [4]. In the zona glomerulosa, pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase type 2 encoded by *HSD3B2*. Progesterone is converted to dexoxycorticosterone by 21-hydroxylase and subsequently to aldosterone by aldosterone synthase encoded by *CYP11B2*. Aldosterone secretion is regulated by the renin-angiotensin system and serum potassium concentrations.

In the zona fasciculata, pregnenolone is hydroxylated by the enzyme 17α-hydroxylase/17,20-lyase encoded by *CYP17A1* to 17-hydroxypregnenolone, which is converted to 17-hydroxyprogesterone (17-OHP) by 3β-hydroxysteroid dehydrogenase type 2. Subsequently, 17-OHP is converted by 21-hydroxylase to 11-deoxycortisol, which is then converted by 11β-hydroxylase to cortisol. In the zona reticularis, the enzyme 17α-hydroxylase/17,20-lyase converts 17-hydroxypregnenolone to dehydroepiandrosterone (DHEA), which is subsequently converted to androstenedione by 3β-hydroxysteroid dehydrogenase type 2. DHEA can undergo sulfation by steroid sulfotransferase, SULT2A1, to form DHEAS.

Thus, the substrates immediately proximal to 21-hydroxylase, progesterone and 17-OHP, are elevated in patients with 21-OHD. Unfortunately, the pathophysiology of CAH is more complex than would be predicted for an autosomal recessive disorder in

which the expression of the defective protein is limited to the adrenal cortex. This complexity is likely due to genetic variants at other loci which influence steroid metabolism and steroid responsiveness. More recently described alternative pathways affecting steroid hormone metabolism may also influence the clinical manifestations.

In the alternative "backdoor" pathway, 17-OHP is sequentially converted by 5α-reductase and the 3α-reductase activities of AKR1C2/4 to generate 5α-pregnane-3α,17α-diol-20-one (pdiol) that is subsequently converted to dihydrotestosterone (DHT) [5]. This alternative pathway bypasses testosterone as an intermediate. Urinary concentrations of metabolites indicative of increased flux through the alternative pathway are higher in affected individuals, particularly infants [6]. This pathway may contribute to the androgen excess responsible for prenatal virilization of affected female fetuses [7].

Under normal circumstances, the direct conversion of 17-OHP to androstenedione is not significant in humans. Yet, when 17-OHP accumulates in 21-OHD, it is metabolized by this alternative pathway [8]. Defective 21-OHD also promotes accumulation of other steroid hormone intermediates such as 21-deoxycortisol, 16α-hydroxyprogesterone, 11-ketoandrostenedione, and 11-ketotestosterone [9]. The enzyme 17β-hydroxysteroid dehydrogenase type 5 also known as aldo-keto reductase 1C3 (AKR1C3) can convert DHEA and androstenedione to androstanediol and testosterone, respectively [10]. It has been suggested that 11β-hydroxyandrostenedione, 11-ketoandrostenedione, 11β-hydroxytestosterone, and 11-ketotestosterone (11KT) are specific markers for adrenal-derived C-19 androgen hormones [11]. Regarding androgenic potency, 11β-hydroxytestosterone and 11-ketotestosterone have similar but slightly lower androgenic activity than testosterone using an in vitro cell-based luciferase reporter assay [12].

Clinical Features

Consequences of cortisol deficiency include poor cardiac function, poor vascular response to catecholamines, and increased secretion of antidiuretic hormone [13]. For 21-OHD, complete loss of function mutations abrogate aldosterone synthesis leading to hyponatremia due to impaired urinary sodium reabsorption. The hyponatremia leads to hypovolemia, elevated plasma renin levels, and, eventually, shock if not promptly recognized and treated. In the absence of aldosterone, potassium cannot be excreted efficiently resulting in hyperkalemia [14]. In 21-OHD, the elevated 17-OHP and progesterone concentrations exacerbate the mineralocorticoid deficiency because both hormones have antimineralocorticoid effects and, in vitro, interfere with aldosterone-mediated mineralocorticoid receptor transactivation [15]. In addition, the lack of prenatal cortisol exposure disrupts adrenomedullary development and can be associated with epinephrine deficiency and hypoglycemia [16].

Female infants with classical 21-OHD, either salt-losing or simple virilizing, generally present in the neonatal period with ambiguous genitalia. In some instances, the diagnosis of genital ambiguity has been suspected based on prenatal ultrasound

findings. For affected female infants, the external genital findings can range from a nearly male appearance with penile urethra and bilateral undescended testes to minimal clitoromegaly. The most common physical findings in affected girls include clitoromegaly, fused rugated labia majora, and a single perineal orifice. The extent of prenatal virilization can lead to misassignment of gender at birth. Occasionally, the minimally virilized girl may not be identified until progressive clitoromegaly prompts a medical evaluation.

Affected 46,XX female infants with 21-OHD have normal female internal genitalia. The uterus can be identified on ultrasound. The ovaries may be too small to be visualized on ultrasound. Despite excessive prenatal androgen exposure, ovarian position is normal, Mullerian structures persist, and the Wolffian ducts regress. The Mullerian structures develop normally to form the fallopian tubes, uterus, and upper vagina. Virilized girls may have incomplete separation of the urethra and vagina resulting in a urogenital sinus and a single perineal orifice.

Apart from hyperpigmentation, external genital development is normal in boys with 21-OHD. Whereas girls are usually detected due to genital ambiguity, boys with salt-losing CAH appear well in the immediate newborn period. Infants with CAH tend to feed poorly and fail to regain their birth weight. Typically, they develop vomiting, hypotension, hyponatremia, and hyperkalemia in the first 10–14 days of life. Prior to implementation of newborn screening, affected boys typically presented with hyponatremic dehydration, hyperkalemia, and shock with the potential for a fatal outcome.

Pubarche refers to the development of pubic hair, axillary hair, apocrine body odor, and acne. Pubarche is the physical manifestation of adrenarche which reflects adrenal pubertal maturation and increased production of adrenal C19 steroids. Children with simple virilizing or NCAH often present with premature development of pubic hair (premature pubarche). Premature pubarche is defined as the presence of pubic hair, axillary hair, or apocrine odor developing before 8 years in girls and 9 years in boys. Additional features in children include tall stature, accelerated linear growth velocity, and advanced skeletal maturation. Clitoromegaly may develop in girls. Boys manifest phallic enlargement with prepubertal-sized testes. In a multicenter study, children less than 10 years of age most often presented with premature pubarche [17]. Among children with premature pubarche, the diagnosis of CAH should be considered when basal 17-OHP, androstenedione, and testosterone concentrations are elevated and/or bone age is advanced [18]. Nevertheless, CAH is an uncommon cause of premature adrenarche [19].

Symptoms of milder, late-onset, or nonclassic 21-OHD (NC-21-OHD) include hirsutism, irregular menses, chronic anovulation, acne, and infertility. Hirsutism, defined as excessive growth of coarse terminal hairs in androgen-dependent areas in women, has been reported to be the most common presenting feature among women [20, 21]. Hirsutism reflects the apparent sensitivity of the pilosebaceous unit/hair follicle to both circulating androgen and local androgen concentrations. Importantly, the extent of the hirsutism correlates poorly with circulating androgen concentrations [22].

Acne can occur among patients with NC-21-OHD but is rarely the primary clinical manifestation. Consideration should be given to further evaluation for patients with severe cystic acne refractory to oral antibiotics and retinoic acid treatment.

Severe androgenic alopecia accompanied by marked virilization in older previously undiagnosed women has been described [23].

The nature of the symptoms leads to an ascertainment bias favoring diagnosis in affected women. Men with NC-21-OHD are typically identified through family studies. Individuals with NC-21-OHD usually do not have elevated ACTH concentrations. Some have an overresponsive ACTH-stimulated glucocorticoid response, possibly reflective of subtle adrenal hyperplasia [24].

Due to the similar clinical features, it may be difficult to distinguish women with NC-21-OHD from those with polycystic ovary syndrome (PCOS) [25, 26]. Women with NC-21-OHD tend to have higher 17-OHP and progesterone concentrations than women with PCOS [27]. Insulin resistance, obesity, polycystic ovary morphology, and elevated LH/FSH ratios tend to be more common among women with PCOS. However, none of these features clearly differentiate women with NCAH from those with PCOS [28]. Anti-Mullerian hormone concentrations do not discriminate women with NCAH from those with PCOS [29].

Family studies have demonstrated that not all individuals with genotypes consistent with NC-21-OHD develop symptoms of androgen excess. Curiously, in a study of 145 probands with 21-OHD, 4% of parents were identified to have undiagnosed or cryptic NC-21-OHD [30]. Apart from infertility among the women, these individuals had achieved normal adult heights and did not report episodes of adrenal insufficiency [30].

One uncommon feature is an adrenal myelolipoma. These adrenal mass lesions consist of myeloid, erythroid, and megakaryocytic cell lines and appear as hyperechoic masses on ultrasound and fat-containing masses on CT scan. Typically, these lesions are benign, but larger lesions are at risk for hemorrhage or rupture. MRI signal characteristics depend on the composition of the lesion.

Hypothalamic-Pituitary-Gonadal (HPG) Axis and Reproductive Concerns

Oligo-amenorrhea, chronic anovulation, and infertility are common presenting complaints for women with NC-21-OHD and can occur in women with classic 21-OHD despite adequate hormone replacement therapy. Hence, women with 21-OHD can develop a secondary "PCOS" phenotype [31]. The specific molecular mechanisms responsible for the altered hypothalamic-pituitary-adrenal (HPO) axis function accompanied by apparent ovarian androgen excess are unclear. Increased circulating concentrations of adrenal androgens and progestins likely influence HPO axis function.

Additional features affecting female reproduction include vaginal stenosis with dyspareunia, impaired sensation, changes in cervical mucous, poor self-esteem, and disinterest in having children. Impaired quality of life and risk for depression have been reported to be higher in women with 21-OHD [32]. Potential contributing factors include engaging in high-risk behaviors, perception of being different from

other women, and perceived lack of autonomy [33]. Reproductive outcomes for women with CAH can be greatly improved by adequate suppression of progesterone and 17-OHP to promote ovulation and implantation of the fertilized ovum; this may require optimizing both glucocorticoid and mineralocorticoid therapies [34]. Occurrence of miscarriages is higher among untreated women with NC-21-OHD [20, 21]. The consequences of prenatal androgen exposure on the developing female brain are being explored [35].

Quality of life (QoL) for women with CAH has been a long-standing concern for women with CAH. One series reported later sexual debut, fewer pregnancies and children, and increased incidence of homosexuality; these outcome measures were related to type of surgical correction and the severity of their mutations [36]. Girls with CAH are reported to prefer more masculine toys, more male-dominant occupations, rougher sports, and non-heterosexual orientation [37]. Another series of 24 women, who answered a questionnaire, reported that 87.5% of women indicated that CAH had not interfered with their social relationships [38]. Regarding gender identity and sexual orientation, 25% of women indicated that they had occasionally wished to be a man, and 62% reported having heterosexual orientation at all times [38]. This area of investigation has been confounded with several factors including small numbers of subjects, lack of control subjects, changes in surgical techniques over time, and variability of age at the time of surgical correction.

Careful consideration regarding surgery is urged for girls with genital ambiguity. Only experienced surgeons/urologists should perform feminizing genitoplasty and vaginal reconstruction [39]. During adolescence, the adequacy of the vaginal introitus for the use of tampons and sexual intercourse should be assessed. For girls with vaginal stenosis, dilatation is often helpful.

Gonadal adrenal rest tumors, predominantly testicular adrenal rests (TARTs), occur in up to half of men with 21-OHD. These tumors arise from adrenal cells that descend with the testes during testicular development. TARTs are not malignant but can compress the rete testis and seminiferous tubules culminating in testicular atrophy and obstructive azoospermia. Ultrasound and MRI are helpful to detect TARTs less than 2 cm because small lesions are generally not palpable. TARTs may be present in childhood and adolescence and have rarely been described in men with NC-21-OHD [40, 41]. Although TARTs have been attributed to poor adherence with hormone replacement therapy, pathogenesis of TARTS may be more complicated [42]. In affected males, the elevated adrenal C19 steroid secretion can suppress gonadotropin secretion resulting in hypogonadotropic hypogonadism and subsequent oligospermia. Ovarian adrenal rest tumors (OARTs) have been infrequently reported in affected women.

Molecular Genetics

The *CYP21A2* gene is located in a complex genetic region at chromosome 6p21.3 where it lies in close proximity to a highly homologous pseudogene, *CYP21A1P*. *CYP21A2* and *CYP21A1P* are arranged in tandem repeats with the *C4A* and *C4B* genes, which encode complement component 4. The tenascin (*TNX*) and serine

threonine nuclear protein kinase (*RP*) genes are also mapped to this region. These four genes, *RP*, *C4*, *CYP21*, and *TNX*, form a unit known as RCCX. Most alleles carry two RCCX units in which one has *CYP21A2* and the other has *CYP21A1P* (Fig. 5.2).

To date, over 200 *CYP21A2* mutations have been reported (http://www.hgmd. cf.ac.uk; www.cypalleles.ki.se). Yet, despite the large number of reported mutations, approximately ten mutations account for the majority of affected alleles. Most mutations result from gene conversion events in which the functional gene acquires deleterious *CYP21A1P* sequences or from misalignment during meiosis that can give rise to duplication or deletions of the RCCX unit. Haplotypes with three or four RCCX units have been described [43]. Another example of misalignment is a *CYP21A1P/CYP21A2* chimera in which a portion of the *CYP21A1P* gene is fused to a portion of the *CYP21A2* gene [44]. Rarely, CAH can be associated with uniparental disomy [45]. The de novo mutation rate is approximately 1%.

Most affected individuals are compound heterozygotes with different mutations on each allele. Mutations range from complete loss of function to mild missense mutations. Estimates of in vitro 21-hydroxylase activity range from <1% for mutations associated with salt-losing CAH to 2–10% for simple virilizing CAH and to 30–50% for NCAH. Genotype, residual enzyme activity, and phenotype generally correlate such that an individual's phenotype reflects their milder mutation. Patients with classical salt-losing CAH usually carry complete loss of function mutations on both alleles. Patients with simple virilizing CAH typically have a complete loss of function mutation on one allele and the I172N or intron 2 splicing mutation on their other allele. Patients with NCAH often carry different mutations with at least one allele carrying a mild missense mutation such as V281 L. Approximately 25–50% of individuals with NCAH are reported to have mild mutations on both alleles [46–
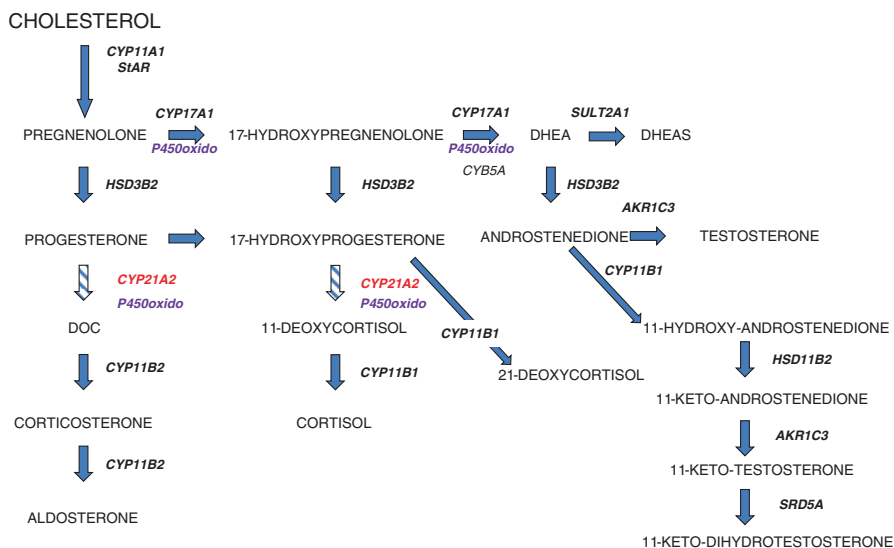

Fig. 5.2 Genetic organization of *CYP21A2* and *CYP21A1P*. This figure illustrates the location of the *C4A*, *CYP21A2*, *C4B*, and *CYP21A1P* genes on the short arm of chromosome 6

48]. Mutations associated with NCAH include V281L, P453S, and R339H. The P30L mutation is often detected in patients with NCAH but is typically associated with more severe androgen excess [49].

As noted above, the *CYP21A2* locus is quite complex which precludes molecular genetic analysis as the first-line diagnostic test. Molecular genetic testing is also confounded by the possibility of multiple mutations on a single allele and the presence of different *CYP21A2* mutations in one family. Multiple genetic testing strategies such as PCR-based mutation detection methods, sequencing, and multiplex ligation-dependent probe amplification may be needed to accurately interrogate and segregate the mutations in an affected individual. In some instances, it may be necessary to perform genetic analyses on the parents to segregate the specific maternal and paternal mutations and confirm that mutations are on opposite alleles. Despite these potential obstacles, genetic analysis can be a useful adjunct to newborn screening [50].

As noted above, one RCCX unit contains *CYP21* and *TNX* genes (Fig. 5.2). TNXB encodes tenascin-X, which is an extracellular matrix glycoprotein involved in collagen organization and matrix integrity. Mutations in *TNXB* are associated with Ehlers-Danlos syndrome. Several distinct alleles have been characterized with loss of *CYP21A2* and specific *TNXB* alleles. Patients have been described to have monoallelic or biallelic *TNXB* variants. The severity of the Ehlers-Danlos syndrome is dependent on the *TNXB* genotype. Patients with CAH will benefit from evaluation for features associated with Ehlers-Danlos syndrome such as hypermobile joints and skin laxity [51].

Diagnosis

An elevated 17-OHP concentration provides confirmation of the diagnosis of 21-OHD deficiency. Most affected infants have random 17-OHP values >5000 ng/dl (150 nmol/L) [52]. For infants, additional laboratory evaluation can include electrolytes, plasma renin activity, progesterone, and androstenedione concentrations. Pelvic ultrasound imaging and chromosome analyses are recommended for virilized female infants.

For individuals with symptoms suggestive of NC-21-OHD, an early morning basal 17-OHP has been suggested as an effective screening test. Armengaud et al. reported 100% sensitivity and 99% specificity with a threshold value of 200ng/dl (6 nmol/L) to diagnose NC-21-OHD in children with premature pubarche [19]. A bone age X-ray should be obtained to assess for acceleration of skeletal maturation.

Blood samples for 17-OHP determinations should be obtained in the follicular phase for reproductive-aged cycling women because the 17-OHP concentration may be elevated during the luteal phase. In this situation, Escobar-Morreale et al. recommended using a basal 17-OHP of 170 ng/dl (5.1 nmol/L) as the "cut point" for women [53]. Nevertheless, for any age group, an ACTH stimulation test may be warranted to complete the evaluation for 21-OHD. For an ACTH stimulation test, following collection of a basal blood sample, 0.25 mg synthetic ACTH (Cortrosyn) is administered by intravenous or intramuscular routes; a second blood sample is

collected at 30 and/or 60 min. In addition to 17-OHP, cortisol should be measured especially among individuals with NC-21-OHD to assess the adequacy of cortisol secretion. To differentiate 21-OHD from other disorders of steroidogenesis, determination of progesterone, 17-hydroxypregnenolone, 11-deoxycortisol, DHEA, deoxycorticosterone, and androstenedione may be warranted [54].

In general, *CYP21A2* mutations on both alleles will be identified when ACTH-stimulated 17-OHP concentrations are greater than 1500 ng/dl (45 nmol/L). However, some individuals with diagnostic genotypes have ACTH-stimulated 17-OHP values between 1000 and 1400 ng/dl (30–45 nmol/L). Individuals with 21-OHD have elevated 21-deoxycortisol concentrations, but commercial availability of this hormone assay is limited. Liquid chromatography-tandem mass spectroscopy (LC-MS/MS) has demonstrated elevated 17-OHP, 21-deoxycortisol, 16α-hydroxyprogesterone, and progesterone; these steroids comprise sensitive and specific biomarkers to accurately identify patients with CAH due to 21OHD [9]. As noted above, the 11oxo-C19 steroids, 11β-hydroxyandrostenedione, 11-ketoandrostenedione, 11β-hydroxytestosterone, and 11-ketotestosterone (11KT), are elevated in 21-OHD [11].

Newborn Screening

Newborn screening (NBS) for 21-OHD was initiated in the late 1970s using filter paper whole-blood 17-OHP measurements of whole-blood 17-OHP [55]. All 50 states and many countries have developed NBS programs. To minimize false-positive results, blood samples should be collected after 48 h of life. Automated time-resolved dissociation-enhanced lanthanide fluoroimmunoassays (DELFIA) are often used for 17-OHP determinations. The major goals of NBS are to identify infants with salt-losing 21-OHD, to prevent misidentification of affected females, and to decrease the morbidity and mortality associated with acute adrenal insufficiency [56]. Nevertheless, false-positive screening results occur among preterm, stressed, or heterozygous infants. Cross-reactivity with sulfated steroids and 16α-hydroxyprogesterone is another reason for false-positive results. Decreased 11β-hydroxylase activity in the neonate may be another confounder contributing to false-positive testing [57]. Birth weight and gestational age cut points have been developed to minimize recalls for false-positive tests. False-negative 17-OHP results leading to delayed diagnoses have been reported for both newborn girls and boys [58].

Treatment

Treatment needs to be focused on the individual's symptoms. In other words, treatment should not be initiated merely to decrease abnormally elevated hormone concentrations. For children and adolescents, treatment goals include normal linear

growth velocity, normal rate of skeletal maturation, appropriately timed spontaneous pubertal development, and positive self-esteem. Treatment goals for adolescent and adult women include normal menstrual cyclicity, fertility, and prevention of further hirsutism and acne. Maintenance of fertility is also a concern for adult males with classic CAH. Healthcare should ideally be provided in a multidisciplinary setting with endocrinologists, pediatricians/internists, surgeons/urologists/gynecologists, behavioral health specialists, and nurse educators [59].

Laboratory goals include androstenedione and testosterone concentrations that are appropriate for age, gender, and stage of puberty. Normalization of 17-OHP and progesterone concentrations generally indicates excessive hormone replacement therapy.

Hydrocortisone (Cortef®) is the preferred glucocorticoid replacement in infants, children, and adolescents. The usual dosage ranges from 6 to 15 mg/m^2/day generally administered three times per day (for a 1.75 m^2 individual, 7.5 mg in the morning, 5 mg in the afternoon, and 10 mg before bed are equivalent to 12.8 mg/m2/day). Some clinicians advocate reverse circadian dosing with the highest dose in the evening. However, the larger bedtime dose may not adequately suppress the early morning ACTH rise, and some individuals complain of insomnia with a higher bedtime dose. Hydrocortisone dose equivalence greater than 17 mg/m^2/day during childhood (> 30 mg per day for a 1.75 m^2 individual) was associated with greater compromise of adult height [60]. Prednisone and dexamethasone have longer half-lives such that less frequent dosing is needed; these medications may be considered for use in the adult patient. Some adult patients with classic CAH are well controlled on combinations of hydrocortisone and small doses of prednisone or dexamethasone at bedtime [61]. Some women with CAH experience persistent hyperandrogenic anovulation and benefit from taking oral contraceptives. Cosmetic hair removal including shaving, waxing, electrolysis, laser therapies, and topical eflornithine cream may be helpful.

Several factors should be contemplated regarding the use of glucocorticoid replacement therapy for patients with NC-21-OHD. Many patients with NC-21-OHD will not require daily glucocorticoid replacement to maintain their health. Indeed, the vast majority of men with NC-21-OHD are generally asymptomatic and do not benefit from treatment. Older adolescent and adult women can be treated with oral contraceptives to decrease the ovarian contribution to androgen excess. Children and adolescents with NC-21-OHD may have extremely advanced skeletal maturation and may benefit from glucocorticoid replacement therapy. For patients with NC-21-OHD, daily or stress-dose glucocorticoid treatment may be indicated only when ACTH-stimulated cortisol is less than 18 mg/dl (500 nmol/L). Some women and men with NC-21-OHD may benefit from short-term hydrocortisone or prednisolone therapy to treat infertility [62]. As noted above, suppression of progesterone can improve fecundity in women with CAH [34]. Hence, therapy for patients with NC-21-OHD needs to be individualized and may vary according to the patient's specific current needs.

The synthetic hormone, 9α-fludrocortisone acetate, is used for mineralocorticoid replacement with the goal of achieving a plasma renin activity that is within normal limits for age. Due to their salt-poor diet, transient pseudohypoaldosteronism, and

immature kidneys, infants typically require higher mineralocorticoid replacement during the first few months of life. Some infants may require additional salt supplementation.

Stress dosing is necessary for significant illnesses, surgery, or life-threatening stress. Tripling the usual daily dose is the semi-arbitrary guideline for stress dosing. If the individual is unable to take or tolerate oral medications, parental hydrocortisone should be administered as follows: < 12 months of age, 25 mg; 1–4 years of age, 50 mg; and > 4 years of age, 100 mg. All individuals on glucocorticoid treatment require instruction regarding oral stress doses and administration of parental hydrocortisone. All patients with CAH should wear medical alert identification badges/jewelry.

Treatment of CAH is often challenging because of the difficulty inherent in balancing overtreatment and undertreatment. Parameters that influence optimal dosing include variation in absorption from the gastrointestinal tract, CBG concentrations, and cortisol half-life in the circulation [63]. For this reason, novel therapies are being explored. One approach has been the development of a time-released glucocorticoid preparation, Chronocort® [64]. Continuous glucocorticoid replacement using a subcutaneous pump has been tried [65]. In a short clinical trial, abiraterone acetate, which inhibits *CYP17A1*, has been used in conjunction with replacement hydrocortisone treatment [66].

Prenatal Treatment

To prevent prenatal virilization of the external genitalia of affected females, prenatal dexamethasone treatment was explored starting in the 1980s [67, 68]. Dexamethasone has been used because it is not inactivated by 11β-hydroxysteroid dehydrogenase type 2 and can cross the placenta. Whereas this treatment appears to be efficacious to decrease virilization of the external genitalia, numerous safety concerns have arisen. To be effective, dexamethasone treatment must be started within 6–7 weeks of conception. Yet, genetic diagnosis by chorionic villus biopsy cannot be safely done until 10–12 weeks. Thus, all at-risk pregnancies are treated even though only one in eight fetuses is predicted to be an affected female and seven of eight fetuses are unnecessarily exposed to prenatal dexamethasone treatment.

Clinical outcome studies have demonstrated increased social anxiety, low birth weight (LBW), failure to thrive, developmental delay, mood disturbance, and poor school performance. Hirvikoski et al. reported a significant negative effect on short-term memory/verbal working memory in children unaffected with CAH who had been treated with dexamethasone during the first trimester of fetal life; however, long-term memory and learning, as well as full-scale IQ, were comparable to untreated controls [69]. Early prenatal dexamethasone exposure has been reported to affect cognitive functions in healthy unaffected girls [70].

In another treatment paradigm, antenatal GCs are used in infants at risk for preterm delivery. In this situation, structural changes in the brain characterized by cortical thinning specifically in the rostral anterior cingulate cortex in children 6–10 years of age were reported in term infants. This area of the brain is important for emotional regulation [71].

Data available in additional clinical outcome studies and using animal models raise significant concern about the use of prenatal dexamethasone and urge that it not be used except in research studies under the guidance of the appropriate Institutional Review Board [72]. One novel approach has utilized cell-free DNA that is found in the maternal circulation. Identification of the *SRY* gene accompanied by sequencing of the *CYP21A2* gene has been used to identify affected females who might benefit from treatment [73]. The drawback of this approach is that the results must be quickly obtained to guide treatment decisions. Another option is preimplantation genetic diagnosis, which allows selection of unaffected embryos for reimplantation [74].

Outcome

Data reporting outcome on older populations of patients with CAH are disappointing. Treatment regimens vary widely with use of diverse glucocorticoid preparations, differing dosages, and dissimilar regimens regarding diurnal dosing. Medical issues identified in adults in the CaHASE study from the United Kingdom included osteopenia, osteoporosis, short stature, obesity, hypertension, and infertility [75, 76]. Data accrued through the NIH Natural History Study showed poor outcomes associated with highly variable treatment attributed to generally poor adherence to medical management [77]. Bachelot et al. reported their outcome experience regarding adult patients followed at a single medical center; they found obesity, abnormal bone mineral density, adrenal tumors, TARTs, and menstrual irregularity were common [78]. Analysis of patients with CAH enrolled in the Swedish CAH register revealed increased cardiovascular and metabolic morbidity especially obesity [79]. The common theme for these disappointing outcome studies is poor medical supervision and suboptimal management in adult patients. Patients seem to be lost for follow-up after transitioning from pediatric to adult healthcare.

Future Directions

In the interval of time since the initial description of CAH by Luigi de Crecchio, much has been learned about the pathophysiology and molecular genetics of this common autosomal recessive disorder [80]. Nevertheless, better diagnostic tools and improved hormone replacement regimens would greatly benefit our patients [81]. Finally, as more 21OHD patients are adults than children, the focus of research needs to shift to transition of care, long-term complications, and reproductive health.

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Chapter 6
Primary Aldosteronism: From Genetic Causes to Clinical Guidelines

Kazutaka Nanba, Hirotaka Shibata, and William E. Rainey

Introduction

Since the first description of primary aldosteronism (PA) by Dr. Jerome W. Conn in 1955 [1], significant progress has been made in the diagnosis and management as well as in determining pathogenesis of PA. PA is characterized clinically by hypertension and often hypokalemia due to excess production of aldosterone from the adrenal gland. PA is now recognized as the most common form of secondary hypertension with an estimated prevalence of 6–10% in hypertensive population [2–7] and 17–23% in resistant hypertension [8–10]. There are several subtypes of PA. Aldosterone-producing adenoma (APA) and idiopathic hyperaldosteronism (IHA) are the major subtypes, comprising 35% and 60% of all cases, respectively [2]. Rarer subtypes are unilateral adrenal hyperplasia, aldosterone-producing adrenocortical carcinoma, and familial hyperaldosteronism (FH). Recent discovery of somatic and germline mutations underlying PA has provided insights into the mechanism causing the dysregulation of adrenal aldosterone production. The mutations in genes such as inwardly rectifying potassium channel (*KCNJ5*) [11], P-type ATPase gene family (*ATP1A1* and *ATP2B3*) [12], L-type voltage-gated calcium channel (*CACNA1D*) [13, 14], and T-type voltage-gated calcium channel (*CACNA1H*) [15] have been identified in APA and/or familial PA and are known to alter the pathway involved in regulation of aldosterone production. Understanding the physiological role and regulation of aldosterone production is essential to elucidate pathophysiology of PA.

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Aldosterone Biosynthesis

The adrenal gland can be divided into two functionally distinct tissues: adrenal cortex and medulla. The adrenal cortex is composed of three zones, namely, zona glomerulosa (ZG), zona fasciculata (ZF), and zona reticularis (ZR). Each zone is morphologically and functionally distinct and responsible for synthesizing different hormones. Aldosterone is the primary mineralocorticoid involved in maintaining fluid and electrolyte balance. Aldosterone biosynthesis occurs almost solely in the adrenal ZG. Aldosterone is derived through the successive actions of four enzymes [16]. Cholesterol side-chain cleavage (CYP11A1), 21-hydroxylase (CYP21), and aldosterone synthase (CYP11B2) are members of cytochrome P450 family of enzymes. CYP11A1 and CYP11B2 are localized to the inner mitochondrial membrane, while CYP21 is found in the endoplasmic reticulum. The fourth enzyme, type 2 3β-hydroxysteroid dehydrogenase (HSD3B2), is a member of the short-chain dehydrogenase family and is localized in the endoplasmic reticulum. In the first reaction, cholesterol is converted to pregnenolone by mitochondrial CYP11A1. This represents the rate-limiting reaction for all steroid-producing tissues and requires the transport of cholesterol from the cytoplasm to the mitochondrial outer membrane, followed by movement from the outer to the inner mitochondrial membrane where CYP11A1 is located [17]. This step is acutely regulated through the expression and phosphorylation of steroidogenic acute regulatory protein (StAR) [18–21]. Pregnenolone passively diffuses into the endoplasmic reticulum and is converted to progesterone by HSD3B2. Progesterone is hydroxylated to deoxycorticosterone by CYP21. Deoxycorticosterone can be converted to aldosterone by three successive oxidation reactions (11β- and 18-hydroxylation, followed by 18-oxidation), which in humans can be mediated by a single enzyme, CYP11B2. Although the last step of cortisol production also involves the 11-hydroxylation of 11-deoxycortisol to cortisol by 11β-hydroxylase (CYP11B1), this enzyme only poorly catalyzes the 18-hydroxylation reaction and does not catalyze 18-oxidation, thus preventing synthesis of aldosterone in the zona fasciculata (ZF). In humans, functional zonation relies in part on the localized expression of two cytochrome P450 enzymes, specifically CYP11B2 and 17α-hydroxylase (CYP17). Expression of CYP11B2 is limited to the ZG, and this effectively prevents production of aldosterone in the other adrenocortical zones [22]. On the other hand, CYP17 diverts pregnenolone and progesterone away from the pathway leading to aldosterone and into that leading to cortisol, explaining the reason for the lack of expression of CYP17 in the ZG [23].

Regulation of Aldosterone Production

Angiotensin II (Ang II), potassium (K$^+$), and adrenocorticotrophic hormone (ACTH) are the main physiological agonists which regulate aldosterone secretion. The regulation of aldosterone biosynthesis is divided into two main phases in the steroidogenic pathway [24, 25]. Acutely (minutes after a stimulus), aldosterone production
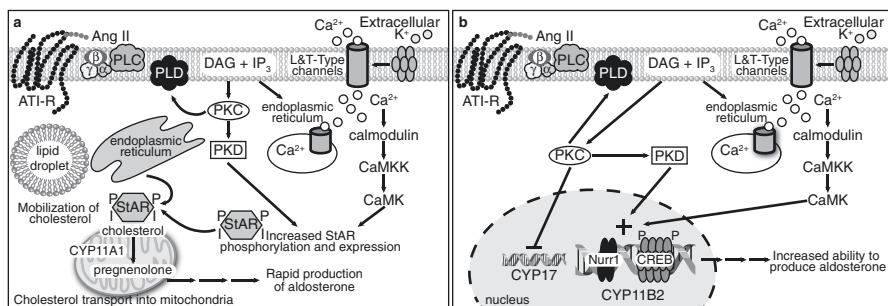
Fig. 6.1 Acute and chronic regulation of aldosterone production. (**a**) The acute actions of Ang II and extracellular potassium (K+) on adrenal glomerulosa cell aldosterone production. Ang II binds type 1 Ang II receptors (AT1R) activating phospholipase C (PLC) to release diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP$_3$). IP$_3$ binds the IP$_3$R on the endoplasmic reticulum, releasing calcium and raising cytosolic calcium concentrations. DAG activates protein kinase C (PKC) and protein kinase D (PKD) and phospholipase D (PLD). Ang II also activates PLD in part through PKC. Small increase in extracellular K+ depolarizes the glomerulosa cell, activating voltage-dependent L- and T-type calcium channels, increasing Ca^{2+} influx. Ca^{2+}/calmodulin-dependent protein kinases (CaMK) and PKD increase StAR and its phosphorylation leading to increased cholesterol movement into the mitochondria. Within the mitochondria, cholesterol is converted to pregnenolone by P-450 side-chain cleavage (CYP11A1) which is then metabolized to aldosterone. (**b**) The chronic actions of Ang II and K+ on adrenal glomerulosa cell aldosterone production. Ang II binds AT1R to activate PLC activity, which releases DAG and IP$_3$. DAG activates PKC and PKD, and IP$_3$ causes the release of intracellular calcium and the activation of CaMK kinase (CaMKK) and CaMK. Small increases in extracellular K+ also depolarize the glomerulosa cell, increasing calcium influx. PKC activation inhibits the transcription of 17α-hydroxylase (CYP17), while CaMK and PKD increase transcription of aldosterone synthase (CYP11B2). This occurs through increased expression and phosphorylation of specific transcription factors that include NURR1 and CREB. The increase in CYP11B2 raises the capacity to produce aldosterone

is controlled by rapid signaling pathways that increase the movement of cholesterol into the mitochondria where it is converted to pregnenolone. This has been called the "acute rate-limiting step" and is mediated by increased expression and phosphorylation of StAR [18–21] (Fig. 6.1a). Chronically (hours to days), the overall capacity to produce aldosterone relies on CYP11B2 [26] (Fig. 6.1b). In vitro studies have defined the part of the intracellular signals involving Ang II-directed expression of CYP11B2 [27, 28]. The Ang II receptor type 1 (AT1R) couples to several signaling pathways in ZG cells including activation of phospholipase C, which increases intracellular calcium and diacylglycerol [29–35]. The second messengers activate calmodulin/calmodulin-dependent protein kinases (CaMK) and protein kinase C, respectively [16]. CaMK activity is important in mediating aldosterone secretion, as inhibition of this enzyme decreases Ang II-induced aldosterone secretion [36–39]. Recent study suggests that CaMK kinases (CaMKK) also play a pivotal role in the calcium signaling cascade regulating adrenal aldosterone production [40]. On the other hand, K+ increases calcium through activation of voltage-sensitive L- and T-type calcium channels, resulting in the influx of calcium from extracellular sources [16]. As with Ang II, this influx is required for the response to potassium,

since inhibition of calcium influx abolishes potassium-stimulated aldosterone secretion [38, 41–43]. Both Ang II and K$^+$ share calcium signaling as the primary regulator of aldosterone production. The key role of calcium signaling is further supported by human adrenal gene mutations that cause aldosterone excess through the disruption of calcium signaling as a major cause of the dysregulation of aldosterone production [11–15, 44, 45].

ACTH is also able to stimulate aldosterone production acutely both in vivo and in vitro. The adrenal cortex including ZG expresses high levels of the melanocortin receptor 2 (MC2R). ACTH binds to MC2R and activates adenylate cyclase via the heterotrimeric G protein, Gs [16]. Adenylate cyclase produces cyclic AMP (cAMP) which stimulates the activity of cAMP-dependent protein kinase or protein kinase A. PKA induces the expression and phosphorylation of StAR leading to increased cholesterol delivery to the inner mitochondrial membrane [46]. In addition to stimulating cAMP-induced PKA activation, ACTH is able to promote calcium influx likely through PKA-mediated phosphorylation of L-type calcium channels [47, 48], increasing cytosolic Ca^{2+} concentration and enhancing adenylate cyclase production and aldosterone production [49, 50].

ZG capacity to produce aldosterone is dependent on CYP11B2 expression which apparently results from increased transcription of the gene [51]. Activation of transcription seems to rely on the activation of transcription factors that bind to a cAMP response element (CRE) found in the proximal region of CYP11B2 promotor [52]. In addition, both Ang II and K$^+$ rapidly induce the expression of the nuclear hormone receptor NURR1, which also binds the promotor and activates *CYP11B2* transcription [53, 54]. The expression of NURR1 is also increased in adrenal aldosterone-producing tumors and may play a role in the increase in tumor CYP11B2 expression [55].

Primary Aldosteronism

Introduction

Many studies have been conducted to understand the pathophysiology of PA since Dr. Conn's first description of a patient with APA. After describing the disease, Dr. Conn initially thought that APA could be the cause of up to 20% of patients with arterial hypertension [56]. Subsequently, many physicians undertook a search for adrenal APAs in hypertensive patients and were disappointed when the prevalence of PA appeared to be much lower than expected [57, 58]. This led to the characterization of PA as a rare disease, which at the time was estimated to represent only about 1% of the hypertensive population [59]. However, with the application of aldosterone to renin ratio (ARR) [60] as a screening test for PA, the estimate of the prevalence of PA increased dramatically [61]. The prevalence of PA appears to increase according to the severity of the hypertension [62], and in those patients with resistant hypertension, the prevalence of PA is approximately 20% [8]. It is

Table 6.1 Subtypes of primary aldosteronism

Subtype	Frequency	Treatment
APA	30–50% of PA	Adrenalectomy
IHA	50–70% of PA	MRA
Unilateral adrenal hyperplasia	<5% of PA	Adrenalectomy
Aldosterone-producing adrenocortical carcinoma	<0.5% of PA	Adrenalectomy
Familial forms		
FH-I or GRA	<1% of PA	Dexamethasone or MRA
FH-II	5% of PA	Adrenalectomy or MRA
FH-III	<1% of PA	Bilateral adrenalectomy or MRA
Familial forms due to *CACNA1D* or *CACNA1H* mutations	Extremely rare	MRA, CCB

APA aldosterone-producing adenoma, *IHA* idiopathic hyperaldosteronism, *FH-I* familial hyperaldosteronism type I, *GRA* glucocorticoid remediable aldosteronism, *FH-II* familial hyperaldosteronism type II, *FH-III* familial hyperaldosteronism type III, *MRA* mineralocorticoid receptor antagonists, *CCB* calcium channel blocker

now recognized that there are several subtypes in PA. APA and IHA are the most common subtypes of PA, and unilateral adrenal hyperplasia, aldosterone-producing adrenocortical carcinoma, FH, and ectopic aldosterone-producing adenoma or carcinoma are known as much less common forms of PA [2]. The currently described PA subtypes and their prevalence are summarized in Table 6.1.

Diagnosis

In clinical practice, early detection of PA and targeted treatment for excess aldosterone is essential to prevent cardio- and cerebrovascular complications. According to the Endocrine Society Clinical Practice Guidelines for diagnosis and treatment of PA published in 2016, the diagnosis of PA is a three-step process, comprising (1) case detection, (2) confirmatory testing, and (3) subtype classification [63]. The Endocrine Society Guidelines [63] state the categories of patients with a higher prevalence of PA, who should be screened for the disease:

– Sustained blood pressure above 150/100 mmHg on each of three measurements obtained on different days
– Hypertension (blood pressure > 140/90 mmHg) resistant to three conventional antihypertensive drugs (including a diuretic)
– Controlled blood pressure (<140/90 mmHg) on four or more antihypertensive drugs
– Hypertension and spontaneous or diuretic-induced hypokalemia
– Hypertension and adrenal incidentaloma
– Hypertension and sleep apnea

– Hypertension and a family history of early-onset hypertension or a cerebrovascular incident at a young age (<40 years)
– Hypertension and a first-degree relative with PA

In addition to those listed above, there are several studies supporting a positive relationship between aldosterone excess and diabetes mellitus and metabolic syndrome. Fallo et al. [64], in a large population of 466 hypertensives, reported a higher prevalence of metabolic syndrome in PA patients compared to essential hypertensives (41.1 vs. 29.6%) and a higher prevalence of hyperglycemia (27.0% vs. 15.2%). Moreover, diabetes mellitus was found to be more prevalent in patients with PA than in 338 matched controls (23 vs. 10%) in the German Conn's registry population [65]. Considering these findings, hypertensive patients with metabolic syndrome and type 2 diabetes with resistant hypertension are also strong candidates to be considered in the screening for PA [66].

The ARR is currently the most reliable means available for screening for PA [63]. However, there are some issues on the measurement and its interpretation. The most significant confounding factor affecting renin and aldosterone measurements is concurrent or recent use of antihypertensive drugs [67]. In particular, current recommendations suggest withdrawing mineralocorticoid receptor (MR) antagonists and potassium-wasting diuretics for 4 weeks before ARR testing [63]. In addition, the patient ideally should not be taking several common antihypertensive medications that include angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARB), beta-blockers, and dihydropyridine calcium channel blockers (CCBs) for at least 2 weeks prior to the test [68, 69]. The complete cessation of all antihypertensive treatment is usually unnecessary because there are substitute medications such as non-dihydropyridine CCB, verapamil, and alpha-blockers that have a minimal effect of the ARR [63]. In some patients with severe PA, treatment with MR antagonists cannot be safely discontinued; in this setting, PA-related testing can be pursued as long as renin is suppressed [63]. With regarding to the interpretation of ARR, there is no general consensus on ARR cutoff values, and individual centers use different standards and assays, with a subsequent wide variation in the sensitivity and specificity of the test. Another limitation of ARR is that in the presence of very low renin levels, the ARR may be elevated even when plasma aldosterone is also low which is inconsistent with PA [63]. To avoid this problem, some investigators include a plasma aldosterone concentration of >15 ng/dl within screening criteria [2].

The Endocrine Society Guidelines recommend that patients with a positive ARR undergo confirmatory testing. However, the choice of the test remains a matter of debate, and there is not enough evidence to recommend one over the others. Four different confirmatory tests have been included in the Endocrine Society Guidelines [63]: (1) oral sodium loading with urinary aldosterone, (2) saline infusion with plasma aldosterone, (3) fludrocortisone suppression with plasma aldosterone, and (4) captopril challenge with plasma aldosterone and plasma renin activity. Furosemide upright posture with measurement of plasma renin activity is also in used in Japan [68]. A preliminary study by Ahmed et al. [70] suggests that a seated saline infusion test may be superior to standard recumbent saline infusion test in

terms of sensitivity for detecting PA, especially posture-responsive forms, and may represent a reliable alternative to fludrocortisone suppression test. Although these confirmatory tests have variable characteristics in terms of sensitivity, specificity, and reliability, the choice of confirmatory test is commonly determined by considering cost, patient compliance, laboratory routine, and local expertise [63].

For patients with biochemically confirmed PA, it is important to differentiate unilateral and bilateral disease in order to direct the patient toward appropriate therapy: unilateral adrenalectomy for APA and medical treatment with MR antagonists for IHA. As an initial study in subtype testing, all patients should undergo adrenal computed tomography (CT) to rule out the rare, but often fatal, adrenocortical carcinoma [63]. In the absence of cancer, all patients for whom unilateral adrenalectomy is feasible and desired by the patient should undergo adrenal vein sampling (AVS). AVS is highly recommended according to a recent meta-analysis [71], demonstrating that imaging studies show poor sensitivity and specificity for defining the source of aldosterone overproduction. However, there are some drawbacks in AVS. AVS is a technically challenging, invasive, and poorly standardized procedure [72]. During AVS, the blood sample is often obtained near the orifice of the vein and is potentially diluted with other blood, introducing an error in the measurement of aldosterone levels. For this reason, it is necessary to measure cortisol concentrations simultaneously to correct for any dilution. Three AVS protocols are discussed in the Endocrine Society Guidelines [63]: (1) sequential or simultaneous unstimulated bilateral AVS, (2) sequential or simultaneous bilateral AVS followed by bolus cosyntropin-stimulation, and (3) continuous cosyntropin infusion with sequential bilateral AVS; however, there is not enough evidence to recommend one of these protocols over the others.

In patients with an onset of confirmed PA earlier than 20 years of age and in those who have a family history of PA or stroke at a young age (<40 years), the Endocrine Society Guidelines recommend genetic testing for FH-1 (glucocorticoid remediable aldosteronism) which is inherited in an autosomal dominant fashion and is responsible for 1% of cases of PA [63]. In very young patients with PA, genetic testing for germline mutations in the *KCNJ5* gene is also recommended for FH-III [63].

Treatment

Treatment options for PA depend on the subtype of PA (Table 6.1).

Unilateral PA: Unilateral adrenalectomy is the treatment choice for unilateral PA because patients have resolution of hypokalemia in nearly all cases and improvement or cure of arterial hypertension in up to 60–70% of patients postoperatively. If a patient is unable or unwilling to undergo surgery, medial treatment including an MR antagonist is recommended [63]. Factors that have been reported to predict cure after adrenalectomy are positive preoperative response to spironolactone [73, 74], short duration of hypertension [73, 75–77], family history of hypertension in one or no first-degree relative [77], preoperative use of two or fewer antihypertensive agents [77], higher ARR, and 24-h urinary aldosterone secretion [73, 77].

Bilateral PA: Medical treatment with an MR antagonist is recommended to treat bilateral PA. Currently there are two alternatives in MR antagonists: spironolactone and eplerenone. Spironolactone is the drug most studied for treatment of PA, although it is a nonselective aldosterone antagonist. The starting dose for spironolactone should be 12.5–25 mg/d in a single dose. The lowest effective dose should be found by gradually titrating upward, if necessary, to a maximum dose of 100 mg/day [63]. This medication can have some side effects in men that include erectile dysfunction and gynecomastia. Eplerenone is a newer selective MR antagonist without antiandrogen and progesterone agonist effects [78]. The starting dose for eplerenone is 25 mg twice daily. In patients with stage III chronic kidney disease, clinicians should administer MR antagonists with caution because of the risk of hyperkalemia. Administering MR antagonists in patients with stage IV renal disease should be avoided [63]. Amiloride (Midamor), an epithelial sodium channel antagonist, also has some advantages although less efficacious than spironolactone. Being a potassium-sparing diuretic, amiloride can effectivly treat both hypertension and hypokalemia in patients with PA [63].

Molecular Mechanisms Underlying Sporadic and Familial PA

Over the last five years, the use of next-generation sequencing (NGS) has dramatically increased our understanding of the genetic causes of PA. Taking advantage of these next-generation technologies, substantial efforts have been directed at defining the pathogenic and molecular mechanisms responsible for autonomous aldosterone overproduction in both sporadic and familial forms of PA. The use of NGS has resulted in the identification of several genes underlying hereditary forms of PA and/or APA, such as *KCNJ5* [11], *ATP1A1*, *ATB2B3* [12], *CACNA1D* [13, 14], and *CACNA1H* [15].

KCNJ5 Mutations

The *KCNJ5* gene encodes the inward-rectifying K$^+$ channel GIRK4, a member of the G-protein activated K$^+$ channel subfamily. This potassium channel is expressed at the plasma membrane of different cell types, forming a homotetramer or a heterotetramer with GIRK1 (encoded by *KCNJ3*) [79]. Mutations in the *KCNJ5* gene have been implicated in the pathogenesis of both FH-III and sporadic APAs [11]. In vitro studies demonstrate that the mutations in *KCNJ5*, which are located near or within the GYG motif of the ion selectivity filter of the channel, result in pathological sodium permeability, cell membrane depolarization, opening of voltage-gated calcium channels, and increases in intracellular calcium concentration, which in turn activate the transcription of *CYP11B2* and increase aldosterone production [11, 80, 81] (Fig. 6.2a). FH-III is a rare inherited form of PA [82]. The disorder is transmitted in an autosomal dominant fashion. In most cases, with the exception of the families carrying the p.G151E mutation [83] and the p.Y152C mutation [81], affected
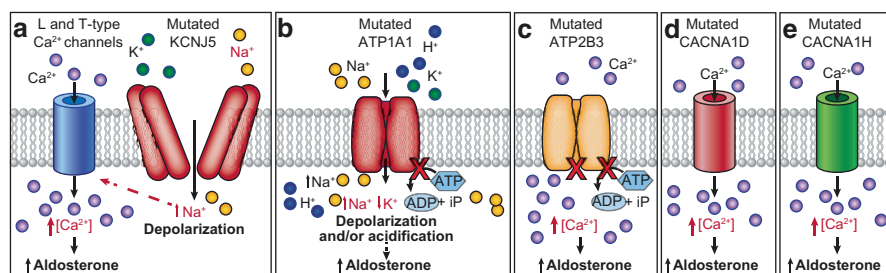
Fig. 6.2 Molecular mechanisms causing autonomous aldosterone production in adrenal cells harboring somatic or germline mutations. (**a**) The G protein-activated inward rectifier potassium channel GIRK4 which is encoded by the *KCNJ5* gene physiologically keeps the cell in a hyperpolarized state. The mutations located near or within the ion selectivity filter of the channel cause a loss of ion selectivity, increased sodium conductance, cell membrane depolarization, and opening of voltage-gated calcium channels, leading to increased intracellular calcium concentration. (**b**) *ATP1A1* gene encodes the α1-subunit of the Na$^+$/K$^+$ ATPase. The Na$^+$/K$^+$ ATPase plays a key role in maintenance of the resting membrane potential and cellular excitability. Mutations in *ATP1A1* gene affecting the ion binding capacity disrupt this function leading to cell membrane depolarization and/or cytosolic acidification. (**c**) Ca^{2+}-ATPase which is encoded by the *ATP2B3* gene pumps calcium out of the cell. Loss of this function leads to an increased intracellular calcium concentration. (**d**) Ca$_v$1.3 is the α1-subunit of an L-type voltage-gated calcium channel. Mutations in *CACNA1D* gene cause activation of the channel at a lower depolarized state, suppress the channel's inactivation, or directly increase the current flux of calcium. (**e**) The *CACNA1H* gene encodes the α1-subunit of the T-type, low voltage-dependent calcium channel Ca$_v$3.2. Mutated *CACNA1H* displays impaired channel inactivation and activation at more hyperpolarized potentials, resulting in increased intracellular calcium levels

members present with early onset and particularly severe forms of PA, with profound hypokalemia and severe resistant hypertension, requiring bilateral adrenalectomy to control blood pressure.

Choi et al. [11] originally demonstrated two recurrent *KCNJ5* somatic mutations (p.L168R and p.G151R) in 8 out of 22 sporadic APAs. Several centers have investigated the prevalence of *KCNJ5* mutations in APAs and have observed variations based on sex and race. In particular, *KCNJ5* mutations are much more common in East Asians compared to that seen in Europeans (70–75% vs. 35–40%) [84, 85]. A recent meta-analysis including 13 studies for a total of 1636 APA patients showed an association of *KCNJ5* mutations for females as well as for subjects with pronounced hyperaldosteronism, larger tumor size, and younger age at diagnosis [86]. The reasons for this gender dimorphism remain to be elucidated. In vitro studies demonstrated that overexpression of mutant *KCNJ5* (p.G151R and p.L168R) in the HAC15 human adrenocortical cells increased *CYP11B2* transcript levels as well as *CYP11B1* compared with cells expressing wild-type *KCNJ5* [80]. A significant increase in transcriptional regulators of *CYP11B2*, NURR1 and NOR-1, was also observed in HAC15 cells expressing the mutant *KCNJ5* [80]. Less common somatic mutations which locate near or within the ion selectivity filter of the channel have also been identified [14, 87–94].

The utility of hybrid steroids such as 18-oxocortisol and 18-hydroxycortisol for PA subtype classification has long been studied [95–102]. Recent research has

revealed that patients with APA harboring a *KCNJ5* mutation are much more likely to have elevated 18-oxocortisol levels compared with other genotypes [102]. In vitro studies also confirmed that a *KCNJ5* mutation in HAC15 cells increased 18-oxocortisol production [45, 103]. Therefore there remains potential of hybrid steroid measurement to differentiate IHA from APA in PA patients of East Asian decent where 70% of APA have *KCNJ5* somatic mutations [100]. However, in Europeans the use of this particular steroid would be limited because of the lower percentage of patients with *KCNJ5* mutations. APAs with *KCNJ5* somatic mutations tend to be composed of ZF-like lipid-rich clear cells with high expression of CYP17 and CYP11B1 as well as CYP11B2, while *ATP1A1*, *ATP2B3*, and *CACNA1D* mutated APAs present more frequently ZG-like compact cells with high expression of CYP11B2 [14, 104–106], supporting the findings of increased hybrid steroid production in *KCNJ5* mutants.

ATP1A1 and ATP2B3 Mutations

The *ATP1A1* gene encodes the α1-subunit of the Na^+/K^+ ATPase, while the *ATP2B3* gene encodes the plasma membrane Ca^{2+} ATPase isoform 3. Physiologically, the Na^+/K^+-ATPase exchanges 3 cytoplasmic Na^+ ions for 2 extracellular K^+ ions for each ATP molecule hydrolyzed, thereby sustaining appropriate ion gradients to allow maintenance of the resting membrane potential. The plasma membrane Ca^{2+} ATPase isoform 3 transports cytoplasmic calcium ions out of the cell, thus playing a key role in regulating intracellular calcium homeostasis. Somatic mutations in *ATP1A1* and *ATP2B3* have been identified by Beuschlein et al. [12] in three and two out of nine sporadic APAs, respectively. Similarly, Azizan et al. [14] identified somatic mutations in *ATP1A1* in four out of ten sporadic APAs which are composed of ZG-like compact cells. In a large cohort of APAs ($n = 474$), collected through the European Network for the Study of Adrenal Tumors (ENS@T), somatic mutations in *ATP1A1* and *ATP2B3* have been reported in 5.3% and 1.7% of APAs, respectively [107], while the prevalence of these somatic mutations seems to be lower in East Asian populations [90].

Cell-based studies demonstrated that *ATP1A1* mutations lead to cell membrane depolarization, increased *CYP11B2* transcription, and aldosterone production through two different mechanisms: (1) impaired ATPase activity due to a loss of pump function and (2) inward ouabain-sensitive Na^+ current or proton-dependent current (Fig. 6.2b) [12, 87]. The intracellular mechanism responsible for the upregulation of *CYP11B2* and autonomous aldosterone overproduction in adrenocortical cells carrying an *ATP1A1* mutation is believed to be via an increase in intracellular calcium concentration, as occurs in APAs harboring a mutation in *KCNJ5*. However, a recent study reported no increase in intracellular calcium concentration in H295R adrenocortical cells expressing *ATP1A1* mutations [108]. Of note, cytosolic pH measurement revealed an acidification of mutant *ATP1A1*-expressing cells due to a pathological H^+ leak, and the possible contribution of cellular acidification to aldosterone overproduction that was supported by the demonstration that cytosolic acid-

ification with Na^+-acetate stimulated *CYP11B2* transcription and aldosterone production in untransfected H295R cells [108]. A recent in vitro study showed that a mutation in *ATP2B3* (p.L425_V426del) promotes aldosterone production through at least two different mechanisms: (1) a reduced Ca^{2+} export due to loss of the physiological pump function and (2) an increased Ca^{2+} influx due to opening of depolarization-activated Ca^{2+} channels as well as a possible Ca^{2+} leak through the mutated pump (Fig. 6.2c) [109].

CACNA1D Mutations

Calcium channels mediate the entry of calcium ions into excitable cells and are involved in a variety of cellular processes, including muscle contraction and hormone release. Adrenal glomerulosa cells express both L-type and T-type voltage-gated calcium channels [16]. The *CACNA1D* gene encodes the voltage-dependent L-type calcium channel subunit alpha-1D ($Ca_v1.3$) that contains four homologous repeats (I-IV). Azizan et al. [14] identified seven different somatic *CACNA1D* mutations, while Scholl et al. [13] identified four mutations in sporadic APAs without *KCNJ5* mutations. Somatic mutations in the *CACNA1D* gene have been identified in around 8–11% of APAs from Western countries and unlike in *KCNJ5*, *ATP1A1*, and *ATP2B3*, these *CACNA1D* mutations have been observed throughout the gene affecting all four homologous repeats [13, 14, 107]. Electrophysiological experiments revealed channel activation at less depolarized potentials, leading to increased Ca^{2+} influx, which is associated with enhanced aldosterone production (Fig. 6.2d) [13].

Germline de novo *CACNA1D* mutations (p.G403D and p.I770M) have been identified in two subjects affected by a clinical syndrome characterized by PA, seizures, and severe neurological impairment [13]. Interestingly, in one of the two affected subjects, blood pressure was normalized by the administration of the CCB, amlodipine, raising the possibility that CCBs could represent an effective and targeted treatment for those PA patients carrying a mutation in this gene.

CACNA1H Mutations

The *CACNA1H* gene encodes the pore-forming alpha subunit of a T-type, low voltage-activated calcium channel ($Ca_v3.2$). A germline p.M1549V mutation was identified by exome-sequencing in five out of forty unrelated subjects diagnosed with hypertension and PA by the age of 10 [15]. The expression of the p.M1549V mutation in HEK293T cells resulted in impaired channel inactivation and activation at more hyperpolarized potentials, resulting in increased intracellular Ca^{2+} concentrations (Fig. 6.2e). A recent in vitro study showed that inhibition of CACNA1H channels with the T-type CCB, mibefradil completely abrogated the effects of mutant *CACNA1H* in the HAC15 cells on *CYP11B2* expression, suggesting the potential efficacy of CCB for the treatment of a subset of patients with PA [110].

Conclusions

PA is the most common cause of endocrine-related hypertension and is frequently not detected as a result of inadequate screening. Due to the increased risk for cardio-vascular complications in PA pateints, early diagnosis and treatment is essential. As research in this field continues, it is likely that a better understanding of the genetic causes of PA will provide streamlined diagnostic methods and novel targeted therapies.

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Chapter 7
Pathophysiology and Genetic Landscape of Adrenocortical Tumors and Hyperplasias

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Introduction

Tumors arising from the adrenal cortex comprise a large spectrum of benign or malignant pathologies including hyperplasias, adenomas, and carcinomas. They can be caused by sporadic somatic or germline genetic mutations. They can be solitary lesions or part of hereditary syndromes [1]. Bilateral hyperplasias can present as micronodular or macronodular lesions [2]. They can be silent or secreting variable levels of hormonal excess. Little was known about the genetic landscape of adrenocortical tumors until the last decade when advances in genomics technologies gave rise to a new type of molecular classification and a better understanding of the pathophysiology of adrenocortical tumors. Genomic approaches were applied at different levels ranging from gene expression, miRNA expression to DNA methylation and chromosomal structure (copy number alterations, loss of heterozygosity), and finally to the whole-genome DNA sequence [3]. This chapter will focus on the molecular causes and pathophysiology of each type of adrenocortical tumors.

Adrenocortical Adenomas (ACA)

Although the majority of ACA are nonsecreting (or secreting at very low levels), ACA are classified based on the type of hormone excess. Mineralocorticoids which are produced by zona glomerulosa (ZG) cells result in primary aldosteronism (PA),

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glucocorticoids secreted by zona fasciculata cells (ZF) are responsible for Cushing's syndrome (CS), and sex steroids mainly androgens from zona reticularis (ZR) cells can cause virilizing syndromes. Genetic defects have been identified in most of these syndromes highlighting the importance of genetic counseling to affected patients and their family members [1].

Cushing's Syndrome

In primary adrenal causes of CS, cAMP signaling pathway appears to play a fundamental role in regulation of metabolism, cell replication, differentiation, and apoptosis in adrenal tissues; this implies that any defect in this pathway leading to its activation would result in cell proliferation and excess hormone production [4]. In normal physiology, the production of corticotropin-releasing hormone (CRH) in the hypothalamus and of ACTH by the corticotroph cells is suppressed by excess secretion of cortisol. The binding of ACTH to its specific melanocortin type 2 receptor (MC2R) regulates cortisol secretion; MC2R is a seven-transmembrane domain receptor that belongs to the family of G-protein-coupled hormone receptor (GPCR) [5, 6]; it is expressed on ZF cells that interacts with MC2R-associated proteins [7] and induces the dissociation of Gs-α subunit, which generates cAMP from ATP by activation of adenylate cyclase (AC) [8]. The second messenger cAMP and its effector PKA are key regulators of adrenocortical cells. PKA is a prototypical serine/threonine kinase consisting of a dimer of two regulatory (with four known isoforms RIα, RIβ, RIIα, RIIβ) and two catalytic subunits (with four isoforms Cα, Cβ, Cγ, Prk) [9]. They constitute a tetramer in its inactive holoenzyme form [10] where two cAMP molecules are needed to bind to specific domains of the R subunits of PKA, thereby dissociating the tetramer and releasing the C subunit (PRKACA) from its inactivating regulatory subunits; activated PRKACA phosphorylates different intracellular targets, including the transcription factor CREB. The latter activates the transcription of cAMP-responsive element-containing genes in the nucleus including cholesterol transporters and steroidogenic enzymes, which stimulates acutely cortisol synthesis and chronically cellular proliferation [11, 12]. Specific phosphodiesterases (PDEs) are responsible of the degradation of the intracellular cAMP in order for the two regulatory and catalytic subunits of PKA to be reassembled to return to their inactive state [13] (Fig. 7.1).

Primary adrenal causes account for 20–30% of overt endogenous hypercortisolism and include unilateral adrenal adenomas (10–20%), carcinomas (5–7%), or rarely bilateral adrenal hyperplasias (BAH) (<2%) [14]. BAH can be macronodular (nodules >1 cm) or micronodular (nodules <1 cm) [2]. Micronodular subtype includes the pigmented form of primary pigmented nodular adrenocortical disease (PPNAD) and the nonpigmented form of micronodular adrenocortical disease (MAD) [13, 14]. PPNAD presents either as isolated disease or as part of Carney complex (CNC).
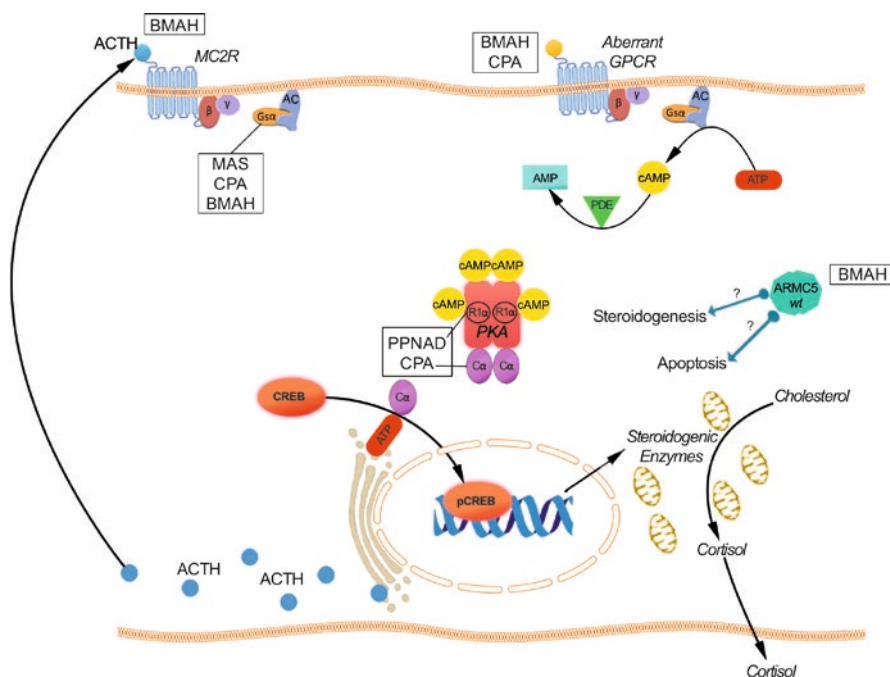
Fig. 7.1 Schematic representation of the cAMP signaling pathway involved in the control of cortisol secretion in primary adrenal Cushing's syndrome. The binding of ACTH to MC2R leads to dissociation of Gs-α subunit and activation of adenylate cyclase generating cAMP from ATP. The binding of cAMP to specific domains of the regulatory subunits of protein kinase A (R1 α) dissociates the tetramer, thereby releasing the catalytic subunit (C α), which phosphorylates different intracellular targets, including the transcription factor CREB; the latter activates the transcription of cAMP-responsive element-containing genes in the nucleus including cholesterol transporters and steroidogenic enzymes. Specific PDEs are responsible of the degradation of the intracellular cAMP in order for the two R1 α and C α subunits of PKA to be reassembled to return to their inactive state. Genetic defect in this pathway leading to its constitutive activation can underlie tumor development and excess hormone production. BMAH or CPA cells can express several functional aberrant GPCR. Activation of these receptors by their natural ligands induces the activation of intracellular cascade similar to the one activated normally by the binding of ACTH to MC2R, thereby stimulating the release of both cortisol and locally produced ACTH (in BMAH tissues) which also triggers cortisol production through autocrine and paracrine mechanisms involving the MC2R. ARMC5 is a new indirect or direct regulator of steroidogenesis and apoptosis. ARMC5 inactivating mutations induce a decreased steroidogenic capacity and a protection against cell death. *ARMC5* Armadillo repeat-containing 5, *BMAH* bilateral macronodular adrenal hyperplasia, *CPA* cortisol-producing adenoma, *GPCR* G-protein-coupled hormone receptors, *MC2R* melanocortin type 2 receptor, *PDE* phosphodiesterases, *PPNAD* primary pigmented nodular adrenocortical disease

Cortisol-Producing Adenomas (CPA)

Somatic mutations in the gene encoding the catalytic subunit of PKA (*PRKACA*) represent the most frequent mechanism of cortisol-secreting adenomas [15] (Table 7.1). They occur in patients diagnosed with CS at a younger

Table 7.1 Molecular mechanisms implicated in adrenocortical tumors

	CPA	PPNAD	BMAH	PA	ACC
A. Genetic alterations					
1. cAMP/PKA signaling pathway	GNAS^a PRKAR1A^b (allelic losses) PRKACA^a (missense or insertion) PDE8B^b PDE11A^b	– – **PRKAR1A^b (LOH)** PRKACA^c PDE8B^b	MC2R^a (missense) GNAS^a – PRKACA^c PDE8B^b PDE11A^b		PRKAR1A^b
2. Armadillo proteins	CTNNB1^a AXIN2^a	CTNNB1^a	**ARMC5^b (LOH, nonsense or missense)**	ARMC5^b	**CTNNB1^a**
3. Other	–	–	MEN1^b, FAP^b, FH^b, EDNRA, DOTL1, HDAC9, PRUNE2	**KCNJ5**, CACNA1D, CACNA1H, ATP1A1, ATP2B3, chimeric 11β-hydroxylase/aldosterone synthase gene	IGF2, TP53, ZNRF3, TERT, RPL22, TERF2, CCNE1, NF1
B. Abnormal protein expression	GPCR PRKAR1A – – –	– – PRKACA Glucocorticoid receptor Estrogen receptor	**GPCR** PRKAR1A – – –	GPCR	

The most frequent mechanisms are highlighted in bold

PPNAD primary pigmented nodular adrenocortical disease, *BMAH* bilateral macronodular adrenal hyperplasia, *PA* primary aldosteronism, *ACC* adrenocortical carcinoma

[a] Activating mutation

[b] Inactivating mutation

[c] Gene duplication (complex genomic rearrangements resulting in copy number gain leading either to micronodular or macronodular hyperplasia depending on the extent of gene amplification)

age (45.3 ± 13.5 vs. 52.5 ± 11.9 years) [16] with a female predominance [17]. The first two mutations identified in a cohort of ten cortisol-producing adrenal adenomas were shown to inhibit the binding of the R subunit making the Cα subunit constitutively active [15]. A combination of biochemical and optical assays, including fluorescence resonance energy transfer in living cells, showed that neither mutants can form a stable PKA complex, due to the location of the mutations at the interface between the catalytic and the regulatory subunits [18]. The most common mutation p.Leu206Arg was present in 37% of these adrenal tumors [15]. It consists of substitution of a small hydrophobic leucine with a large positively charge hydrophilic arginine at position 206. It is located in the active cleft of the C subunit, and it inactivates the site where the regulatory subunit RIIβ binds, leading to cAMP-independent PKA activation. The second mutation (Leu199_Cys200insTrp) involves the insertion of a tryptophan residue between the amino acid 199 and 200 and was present in one case only. Later, two novel mutations were identified in a study of 22 adrenal adenomas with CS with p.Cys200_Gly201insVal and p.Ser213Arg + p.Leu212_Lys214insIle-Ile-Leu-Arg being found in three and one adenomas, respectively. They indirectly interfere with the formation of a stable PKA holoenzyme by impairing the association between the catalytic and the regulatory subunits [19]. Other groups confirmed the presence of these mutations in unilateral adrenal adenomas with overt hypercortisolism at a rate of 23–65% [16, 17, 19–21]. However, they are seldom present in adenomas with mild cortisol secretion, which might justify why subclinical CS rarely becomes overt CS [15, 19, 20]. These observations suggest that subclinical CS may have a different genetic etiology than overt CS rather than being a part of the same pathophysiological spectrum [22]. Isolated somatic *GNAS* mutations can also occur in 5–17% of cortisol-secreting adenomas [16, 21, 23]. Finally, Bimpaki et al. demonstrated that cortisol-secreting adrenal adenomas could have functional abnormalities of cAMP signaling, independently of their *GNAS*, *PRKAR1A*, *PDE11A*, and *PDE8B* mutation status most probably due to epigenetic events or other gene defects [24] (Table 7.1). Although *β-catenin* (*CTNNB1*) mutations are mainly observed in larger and nonsecreting adrenocortical adenomas, suggesting that the Wnt/β-catenin pathway activation is associated with the development of less differentiated tumors, Bonnet et al. described *CTNNB1* mutations in 6 and 8 out of 19 and 46 subclinical and overt cortisol-producing tumors, respectively [25]. Recently Goh et al. identified *CTNNB1* mutations as responsible for 16% of the cortisol-secreting adenomas [16]; they were also noted by other groups in some cases of adrenal adenomas with CS or SCS [17, 26, 27] (Table 7.1). Exome sequencing of 74 cortisol-secreting adenomas without initial identification of *PRKACA* mutations identified several mutations in cAMP/PKA pathway (including three novel *PRKACA* mutations), in the WNT/β-catenin pathway, and in the Ca^{2+}-signaling pathway [28].

Primary Bilateral Macronodular Adrenal Hyperplasia (BMAH)

In contrast to somatic mutations causing cortisol-secreting adenomas, very rare germline complex genomic rearrangements in the chromosome 19p13.2p13.12 locus, resulting in copy number gains that includes *PRKACA* gene, rarely caused either micronodular or macronodular hyperplasia depending on the extent of gene amplification [15, 29, 30]. *MC2R* mutations are extremely rare causes of adrenal hyperplasia or tumor formation [31, 32]. In only two patients with BMAH, constitutive activation of the *MC2R* with consequent enhanced basal receptor activity resulted either from impaired receptor desensitization secondary to a C-terminal *MC2R* mutation (F278C) [33] or from synergistic interaction between two naturally occurring missense mutations in the same allele of the *MC2R*: substitution of Cys 21 by Arg (C21R) and of Ser 247 by Gly (S247G) [34] (Table 7.1).

Somatic activating mutations of the *Gs-α* subunit of heterotrimeric G protein also termed *gsp* mutations (*GNAS*) were the first identified in a particular bilateral nodular form of primary adrenal CS [35, 36]. It occurred in a mosaic pattern in some fetal progenitor cells during early embryogenesis resulting in the constitutive activation of the cAMP pathway in cells of various tissues which developed from the affected progenitor cells. This mutation was identified initially in the McCune-Albright syndrome (MAS) where a minority of patients develops nodular adrenal hyperplasia and CS among other more common manifestations such as *café au lait* spots and bone fibrous dysplasia or other endocrine tumors causing ovarian precocious puberty, acromegaly, or hyperthyroidism [35, 37]. In MAS patients with CS, *GNAS* mutations are found in the cortisol-secreting nodules, whereas the internodular adrenal cortex which is not affected by the mutation becomes atrophic as ACTH becomes suppressed. Isolated somatic *GNAS* mutations can also occur in rare cases of BMAH [38, 39] without any other manifestations of MAS. This suggests that the somatic mutation in MAS occurs at an early stage of embryogenesis in cells which are precursors of several tissues. In isolated BMAH, the somatic mutation probably occurs in mosaic pattern in more differentiated adrenocortical progenitor cells only which will migrate to generate bilateral macronodular adrenal glands; a somatic *GNAS* mutation giving rise to a unilateral adenoma occurs later in life in a single committed zona fasciculata cell [16, 21, 23].

Inactivating germline mutations in *ARMC5* gene were first described in apparently sporadic cases of primary BMAH [40] (Table 7.1). Armadillo proteins form a large family of proteins that are characterized by the presence of tandem repeats of a 42-amino acid motif with each single ARM-repeat unit consisting of three α-helices [41]. The most well-known protein of this family is β-catenin, which is crucial in the regulation of development and adult tissue homeostasis through its two independent functions, acting in cellular adhesion in addition to being a transcriptional co-activator. Deregulation in the Wnt/β-catenin signaling pathway is involved in the pathogenesis of adrenocortical adenomas and carcinomas. Armadillo repeat-containing 5 (*ARMC5*) is a novel Armadillo (ARM) repeat-containing gene and encodes a protein of 935 amino acids; its peptide sequence

reveals two distinctive domains: ARM domain in the N-terminal and a BTB/POZ in the C-terminal (Bric-a-brac, Tramtrack, Broad complex/Pox virus, and Zinc finger) [42].

The bilateral nature of macronodular hyperplasia as well as its long and insidious onset motivated the search for a genetic predisposition that could result in earlier diagnosis and better management to avoid bilateral adrenalectomy. Single-nucleotide polymorphism arrays, microsatellite markers, and whole-genome and Sanger sequencing were applied to genotype leucocyte and tumor DNA obtained from patients with BMAH. The search for the responsible genes was conducted in apparently sporadic and familial cases [40, 43–47]. The initial germline mutation in the *ARMC5* gene, located at 16p11.2, was detected in 18 out of 33 apparently sporadic tumors, 55% of cases of BMAH with Cushing's syndrome [40]. Further studies in sporadic cases found that the prevalence of germline ARMC5 mutations was closer to 25% [43, 45, 46]. Inactivation of *ARMC5* is biallelic, one mutated allele being germline and the second allele being a somatic secondary event that occurs in a macronodule; these findings are consistent with its role as a potential tumor suppressor gene according to Knudson's two-hit model [40, 43]. Correa et al. demonstrated that *ARMC5* has an extensive genetic variance by Sanger sequencing 20 different adrenal nodules in the same patient with BMAH [48]. They found the same germline mutation in the 20 nodules (p.Trp476* sequence change) but uncovered 16 other mutation variants in the 16 nodules. This suggests that the germline mutation is responsible for the diffuse hyperplasia, but second somatic hits are required to enhance adrenal macronodular formation [40, 48]. In the first large BMAH family studied, a heterozygous germline variant in the *ARMC5* gene (p.Leu365Pro) was identified in all 16 affected Brazilian family members as well as other mutations in two of three other families [43]. Interestingly, only two mutation carriers had overt CS, and the majority had subclinical disease, and one carrier had no manifestations despite being 72 years old. In addition, in one third of the affected individuals, only unilateral adrenal lesion was present as progression of the full-blown disease, needing many years and requiring the occurrence of additional somatic mutations in several macronodules. This raised the question of the prevalence of *ARMC5* mutation in apparently unilateral incidentalomas in the general population. Recent screening of sporadic cases of patients with bilateral incidentalomas revealed a low frequency (1 out of 39 patients) of *ARMC5* mutation [49].

Other families with BMAH have also been identified with *ARMC5* mutations or alterations [44, 47, 50–52]. In all cases the pattern of inheritance is autosomal dominant. A germline deletion rather than mutation of *ARMC5* was reported in a family presenting with vasopressin-responsive SCS and BMAH [52]. By applying droplet digital polymerase chain reaction, the mother and her son had germline deletion in exon 1–5 of *ARMC5* gene locus. Furthermore, Sanger sequencing of DNA from the right and left adrenal nodules as well as peripheral blood of the son revealed the presence of another germline, missense mutation in *ARMC5* exon 3 (p.P347S) [52].

The presence of *ARMC5* mutation in patients with BMAH and aberrant GPCR has been reported, but the relationship has not been well established yet. The most frequent aberrant responses were to upright posture, isoproterenol, vasopressin, and metoclopramide tests [40, 45, 51]. In contrast, none of the patients with food-dependent CS carried *ARMC5* mutations [40, 45]. *ARMC5* inactivation decreases steroidogenesis, and its overexpression alters cell survival, which could argue why relatively inefficient cortisol overproduction is seen despite massive adrenal enlargement [2, 53, 54]. Despite this, the index cases operated for Cushing's syndrome and *ARMC5* mutation carriers presented more severe CS than cases operated for Cushing's syndrome without *ARMC5* mutation; carrier patients had a more severe clinical phenotype and biochemical profile as well as larger adrenals on imaging with a higher number of nodules [45, 46]. *ARMC5* mutations appear to be the most frequent genetic alteration in BMAH with 61 different mutations, 27 germinal and 30 somatic, found all along the protein in different domains. Thus, genetic counseling and screening for these mutations are highly encouraged in family members of patients with BMAH even without the evidence of a clinical disease [42, 43, 54]. As *ARMC5* appears to be a tumor suppressor gene and is widely expressed in many tissues other than the adrenal, it is of interest to examine whether mutation carriers could develop other tumors. In a few families with BMAH, the occurrence of intracranial meningiomas was described, and a somatic *ARMC5* mutation was found in a meningioma of a patient with familial BMAH with a germline *ARMC5* mutation suggesting the possibility of a new multiple neoplasia syndrome [44]. Finally, *ARMC5* mutations have been identified in primary aldosteronism where 6 patients of 56 (10.7%, all Afro-Americans) had germline mutations in the *ARMC5* gene; among these 6 patients, 2 suffered from BMAH [55].

Several other gene mutations have been reported in patients with CS mainly presenting with BMAH including the multiple endocrine neoplasia type 1 (*MEN1*), familial adenomatous polyposis (*APC*), and type A endothelin receptor (*EDNRA*) [39, 53, 56, 57]. Furthermore, somatic mutations other than *ARMC5* have also been found in patients with BMAH such as the *DOT1L* (DOT1-like histone H3K79 methyltransferase) and *HDAC9* (histone deacetylase 9) genes; these two nuclear proteins are involved in the transcriptional regulation; however, their mutations were found at a much lower frequency than *ARMC5* [17] (Table 7.1).

In addition to genetic alterations, the abnormal expression of several proteins was found to regulate steroidogenesis in cortisol-secreting tumors and hyperplasia [58]. In particular, the aberrant adrenocortical expression of various G-protein-coupled receptors (GPCR) can be increased such as the ectopic ones for glucose-dependent insulinotropic peptide (GIPR), β-adrenergic receptors (B-AR), vasopressin AVP (V2-V3 R), serotonin (5-HT7R), glucagon (GCGR), and angiotensin II (AT1R). Other eutopic receptors can be overexpressed such as those for vasopressin (V1R), luteinizing hormone/human chorionic gonadotropin (LHCGR), or serotonin (5-HT$_4$R) [59]. Five systematic studies have demonstrated abnormal expression of more than one type of GPCR in 80% of patients with BMAH. Multiple

responses within individual patients occurred with up to four stimuli in 50% of the patients; AVP and 5-HTR4 agonists were the most prevalent hormonal stimuli triggering aberrant responses in vivo [39, 60–63]. In addition, more recently, local paracrine production of ACTH was identified in clusters of BMAH cells, and ACTH production was stimulated by aberrant GPCR [64]. Thus, cortisol production is controlled by aberrant GPCR as well as by ACTH produced within the BMAH adrenocortical tissue, amplifying the effect of the aberrant receptors' ligands. The percentage of aberrant responses in patients with unilateral adenoma and mild CS or SCS was similar to those in BMAH patients [63]. However it was less frequent in patients with unilateral adenomas and overt CS [62] most probably due to higher prevalence of *PRKACA* mutations in these patients [15].

Micronodular Adrenal Hyperplasia

PRKAR1A is an adrenocortical tumor suppressor gene according to in vitro and transgenic mouse studies. Its inactivation leads to ACTH-independent cortisol secretion by the resulting bilateral micronodules [9, 65]. PKA activation due to *PRKAR1A* mutations results either from reduced expression of the RIα subunits or from impaired binding to C subunits [66] (Table 7.1). Loss of RIα is sufficient to induce ACTH-independent adrenal hyperactivity and bilateral hyperplasia and was demonstrated for the first time in vivo in an adrenal cortex-specific *PRKAR1A* KO mouse model referred to as AdKO. Pituitary-independent CS with increased PKA activity developed in AdKO mice with evidence of deregulated adrenocortical cell differentiation, increased proliferation, and resistance to apoptosis. Moreover, RIα loss led to regression of adult cortex and emergence of a new cell population with fetal characteristics [65]. In vitro and in vivo models of PPNAD (AdKO mice) showed that PKA signaling increased mTOR complex 1, leading to increased cell survival and possibly tumor formation [67]. Tumor-specific loss of heterozygosity (LOH) involving the 17q22-24 chromosomal region harboring PRKAR1A and inactivating mutations of *PRKAR1A* are responsible for CS in isolated or familial PPNAD and CNC [66, 68–70]. They are found in more than 60% of patients with CNC and in up to 80% of CNC patients who develop CS from PPNAD [70, 71]. Furthermore, somatic allelic losses of the 17q22-24 region and inactivating mutations in *PRKAR1A* were identified in 23% and 20% of adrenocortical tumors, respectively [72]. Although *PRKAR1A* mutations are not found in BMAH, somatic losses of the 17q22-24 region and PKA subunit and enzymatic activity changes show that PKA signaling is altered in BMAH similarly to what is found in adrenal tumors with 17q losses or *PRKAR1A* mutations [73]. CS presenting in persons younger than 30 years of age with bilateral, small (usually 2–4 mm in diameter), black-pigmented adrenal nodules are all characteristics of PPNAD. A distinctive feature of PPNAD compared to BMAH is the presence of atrophy in the internodular adrenal tissue. CNC is a familial autosomal variant that includes PPNAD among other tumors such as atrial myxomas, peripheral nerve tumors, breast/testicular tumors, and GH-secreting pituitary tumors along with skin

manifestations [74]. Patients with CS due to *PRKAR1A* mutations tend to have a lower BMI with evidence of increased PKA signaling in periadrenal adipose tissue, which is in concordance with the role of PKA enzyme in the regulation of adiposity and fat distribution [75].

Phosphodiesterases (PDE) play a role in the hydrolysis of cAMP. There are two types of PDE8 enzymes coded by two distinct genes, *PDE8A* and *PDE8B*, which are highly expressed in steroidogenic tissues such as the adrenal, the ovaries, and the testis as well as in the pituitary, thyroid, and pancreas [76, 77]. Genetic ablation of *PDE8B* in mouse models or long-term pharmacological inhibition of PDE8s in adrenocortical cell lines were shown to increase the expression of steroidogenic enzymes such as StAR and p450scc (CYP11A); furthermore, they potentiated ACTH stimulation of steroidogenesis by increasing cAMP-dependent PKA activity [78]. A *PDE8B* missense mutation (p.H305P) was described in a young girl with isolated micronodular adrenocortical disease (iMAD), which is a nonpigmented micronodular hyperplasia without *PRKAR1A* [79]. HEK293 cells transfected with the *PDE8B* mutant gene exhibited higher cAMP levels than with wild-type *PDE8B*, indicating an impaired ability of the mutant protein to degrade cAMP [79]. Other inactivating mutations in phosphodiesterase 11A isoform 4 gene (*PDE11A*) and 8B (*PDE8B*) have been also described in adrenal adenomas, carcinomas, and BMAH [14, 23, 24, 78, 80–82].

In a Carney complex patient without Cushing's syndrome but with skin pigmentation, acromegaly, and myxomas, gene triplication of chromosome 1p31.1, including *PRKACB*, which codes for the catalytic subunit beta (Cβ) resulted in increased PKA activity (Fig. 7.1). It is likely that whereas the loss of RIα leads to the full Carney complex phenotype, the gain of function in Cα leads to adrenal adenomas and Cushing's syndrome, while in this case, amplification of Cβ resulted in certain nonadrenal manifestations of CNC [83]. Finally, somatic *CTNNB1* mutations were also found in 2 out of 18 patients with PPNAD (11%). In both cases, the mutations occurred in relatively larger adenomas that had formed in the background of PPNAD [27, 84].

A distinctive feature of PPNAD is the paradoxical increase in urinary-free cortisol during the 6-day dexamethasone suppression test (Liddle test), which was found in 69–75% of two small series of patients with PPNAD [85, 86]. The glucocorticoid receptor (GR) is largely overexpressed in PPNAD nodules [87], which mediates stimulation of PKA catalytic subunits [85]. In a patient with PPNAD, who increased cortisol secretion during pregnancy and oral contraceptive use, β-estradiol (E2) stimulated cortisol secretion in a dose-response manner [88] via activation of overexpressed estrogen receptors ERα and G-protein-coupled receptor 30 (GRP30) [89].

Recently, it was shown that PPNAD tissues overexpress the 5-HT synthesizing enzyme tryptophan hydroxylase type 2 (Tph2) and the serotonin receptors, types 4, 6, and 7, leading to formation of an illicit stimulatory serotonergic loop whose pharmacological inhibition in vitro decreases cortisol production. *PRKAR1A* mutations led to PKA activation and induction of serotonin and fuctional aberrant serotonin receptors partly regulating cortisol excess [90].

Primary Aldosteronism (PA)

Potassium homeostasis and maintenance of intravascular volume are controlled by the binding of free aldosterone to the mineralocorticoid receptor in epithelial cells, resulting in increased intestinal and renal Na^+ and Cl^- absorption and reabsorption, respectively [91]. Aldosterone excess in PA results in sodium retention and hypertension and can also result in hypokalemia [92]. Under resting physiological conditions, the strongly negative resting membrane potential of ZG cells is maintained by the concentration gradient of K^+ between the intracellular and extracellular space which is generated by the activity of the Na^+, K^+-ATPase. Angiotensin II and increased K^+ lead to cell membrane depolarization which opens voltage-dependent Ca^{2+} channels. Furthermore, angiotensin II acts through the angiotensin II type 1 receptor (AT1R) inducing Ca^{2+} release from the endoplasmic reticulum. Consequently, the increase in intracellular Ca^{2+} concentration activates the calcium signaling pathway, which triggers activation of CYP11B2 transcription [92].

The mechanisms implicated in the pathophysiology of PA are not entirely clarified. Gene rearrangement or mutations in ion channel genes regulating intracellular ionic homeostasis and cell membrane potential were described in sporadic and familial cases of PA [93–96]. On the other hand, several hormones seem to activate variable levels of eutopic or ectopic aberrant receptors [97], and autocrine and paracrine regulatory mechanisms [98] can increase aldosterone secretion in PA independently from the suppressed RAS.

Genetic Alterations in PA

Bilateral adrenal hyperplasia (BAH) and aldosterone-producing adenoma (APA) are the two most common subtypes of PA (BAH in 50–70% of the cases and APA in 30–50%); less frequent causes include primary (unilateral) adrenal hyperplasia (5%), aldosterone-producing adrenocortical carcinoma (<1%), familial hyperaldosteronism (1%), and ectopic aldosterone-producing adenoma or carcinoma (<0.1%) [99]. Familial hyperaldosteronism (FH) type 1, previously known as glucocorticoid-remediable aldosteronism (GRA), is suspected in young patients whose relatives suffer from cerebrovascular accidents and who present with PA that is relieved by dexamethasone [100]. It is an autosomal dominant disease whereby the promoter of the chimeric 11β-hydroxylase/aldosterone synthase gene belongs to the 5′ end of CYP11 B1 (11β hydroxylase) and drives the expression of the 3′ end of CYP11 B2 (aldosterone synthase) ectopically in ZF cells under the main regulation by ACTH [101]; the diagnosis in these is used to be based on the drop in aldosterone secretion following dexamethasone administration by more than 80% or to <4 ng/dL [102]; however, nowadays genetic analysis is the main diagnostic tool. FH type 2 is more frequent (1.2–6%) than FH type 1 (≤1%); it is diagnosed in a PA patient who has a first-degree relative (parent/sibling/offspring) with established PA but without FH type 1 gene rearrangement. To date no culprit gene has been identified but linkage

analysis has mapped FH type 2 to chromosome 7p22 [103]. FH type 3 and FH type 4 are caused by germline mutations in *KCNJ5* [104] and *CACNA1D/CACNA1H* genes, respectively, encoding a potassium channel GIRK4 and voltage-gated calcium channels [105, 106], respectively (Table 7.1).

Somatic mutations in *KCNJ5* were identified in almost 30–40% of APA with higher prevalence in the Japanese population [107]. *KCNJ5* can alter channel selectivity allowing enhanced Na^+ conductance; Na^+ influx results in cell depolarization, the activation of voltage-gated Ca^{2+} channels, aldosterone production, and cell proliferation [94, 108]. Mutations in *CACNA1D* gene result in channel activation and less depolarized potentials causing increased Ca^{2+} influx, aldosterone production, and cell proliferation in affected ZG cells [96, 105]. Mutations in *CACNA1H* gene were discovered in children with PA; they result in impaired channel inactivation and activation at more hyperpolarized potentials, producing increased intracellular Ca^{2+} and aldosterone excess [106]. Mutations in *ATP1A1* gene (encoding the Na^+/K^+ ATPase α subunit) and *ATP2B3* gene (encoding the plasma membrane Ca^{2+} ATPase) were identified in 5.2% and 1.6%, respectively, of patients in a series of APA [93] (Table 7.1). The pathophysiology of progression from normal adrenal to APA and the causes of diffuse bilateral hyperplasias, either as BAH or in mild form adjacent to the dominant aldosteronoma, are still unknown. In fact, these somatic mutations appear to be as independent events since different mutations in the genes described above are found in different aldosterone-producing nodules from the same adrenal [109]. Nishimoto et al. found that aldosterone-producing cell clusters (APCCs), which are nests of cells below the adrenal capsule with an increased expression of CYP11B2, are common in normal adrenals. They protrude into cortisol-producing cells that are usually negative for CYP11B2 expression. He also speculated that APCCs could be a precursor for APA because they harbor a different mutational spectrum compared to APA [110]. Moreover, nodule formation and excess aldosterone production constitute two dissociated events, complying with the two-hit hypothesis for APA formation [111, 112] because no mutations of any of the above ion channel genes were found in BAH or in ZG hyperplasia adjacent to the dominant aldosteronomas [93, 108, 113]; the first hit may result from a somatic mutation in one of the genes described above, at least in approximately 60% of cases, causing a unilateral aldosteronoma or a dominant nodule adjacent to ZG hyperplasia. Possible causes of the second hit that results in dysregulation in cellular proliferation/apoptosis accelerating adenoma formation could be due either to dysregulation of PKA pathway or to gene mutations such as *ARMC5* [55] or to activation of the Wnt/β-catenin pathway [114, 115]. The activation of the latter pathway could be mostly related to downregulation of a negative regulator of this pathway, named SFRP2 caused by a high methylation of SFRP2 promoter [116].

Aberrant Expression or Function of G-Protein-Coupled Receptors or Their Ligands

Adrenal production of aldosterone in APA and BAH was found to be under the influence of aberrant G-protein-coupled receptors (GPCR) and their ligands, as demonstrated by in vivo and in vitro studies [117, 118]. Whether these aberrant

regulatory secretory mechanisms and the overexpression of GPCR in PA are secondary to unknown proliferative mechanisms or are primary and at least partially responsible for the abnormal proliferation, the initiation of diffuse BAH is currently unclear. Yet, they obviously play a role in aldosterone production in PA which is relatively autonomous from the suppressed RAS but regulated by the ligands of various aberrant hormone receptors.

In zona glomerulosa cells, binding of ACTH or other hormones to their specific GPCR activates the cAMP/PKA pathway [8], the cAMP response element-binding protein (CREB), and the StAR expression. This induces a slow but sustained calcium influx through the L-type calcium channels. The subsequent increase in intracellular calcium activates calcium/calmodulin-dependent protein kinase and steroidogenesis [119, 120].

Aldosterone secretion is regulated mainly by angiotensin II and potassium and to a lesser extent by ACTH and serotonin. However, the latter two play an increased role in PA most probably due to the overexpression and function of melanocortin-2 receptor (MC2R) and serotonin receptor (5-HT$_4$ R), respectively [99]. An acute ACTH administration resulted in an exaggerated increase in plasma aldosterone concentration in PA patients with APA or BAH compared to normal individuals. Endogenous ACTH can be an important aldosterone secretagogue in PA as demonstrated by the diurnal increase in aldosterone in early morning and its suppression by dexamethasone. Screening using a combination of dexamethasone and fludrocortisone test reveals a higher prevalence of PA in hypertensive populations compared to the aldosterone to renin ratio [99]. RT-PCR or transcriptome studies demonstrated eutopic overexpression of MC2R in resected aldosteronomas as compared to cortisol-secreting adenomas, nonfunctional adenomas, and adrenocortical carcinomas [121–123]. Levels of MC2R mRNA were increased by a mean of 3.9-fold in 15 adrenal tumors tissues (14 APA and 1 BAH) compared to normal adrenal [97]. However, great variability was noticed in the level of expression in each tumor as four had lower levels than normal (0.3–0.7-fold), while those with increased expression varied between 1.4- and 20.6-fold compared to normal. As most patients with BAH are not operated, there is almost no data on MC2R expression in BAH, but in the only case with BAH studied by this group, MC2R was 20-fold increased. The variable level of MC2R overexpression in each APA or in the adjacent zona glomerulosa hyperplasia may explain the discordant results of adrenal vein sampling between basal levels and post ACTH administration in the determination of source of aldosterone excess [124].

A higher stimulation of aldosterone was seen in most PA patients following the administration of serotonin 5-HT$_4$ agonists as compared with the physiological moderate increase in normal individuals [97, 125, 126]. Conversely, nonspecific inhibitors of 5- HT$_4$ produced only minor and transient inhibition of aldosterone secretion in APA [127, 128], whereas specific 5-HT$_4$R antagonists were potent inhibitors of basal and cisapride-induced aldosterone secretion [126]. In the immediate vicinity of ZG cells, chromaffin cells, endothelial cells, nerve terminals, and cells of the immune system can secrete various factors to control aldosterone production [129]. Moreover, aldosterone secretion can be stimulated by the local release of 5-HT$_4$ by perivascular mast cells (MC) which can activate 5-HT$_4$R

expressed in ZG cells [130], specially that the density of MC was found to be increased in APA tissues compared with normal adrenals [131]. Finally, a role of MC in tumorigenesis was suggested [132, 133]. Consequently, a paracrine loop of regulation of aldosterone production appears to be present as the 5-HT$_4$R have been found to be overexpressed in the majority of APA (but variable as MC2R) [97, 98, 134], and the ligand may be locally overexpressed also.

The expression of ectopic receptors which are usually not expressed at significant levels in normal ZG cells include those for glucose-dependent insulinotropic peptide (GIPR) [134], luteinizing hormone/human chorionic gonadotropin (LH-hCG R) [97, 134–140], β-adrenergic receptors (β-AR) [141], vasopressin (V1-AVPR) [97, 134, 142, 143], glucagon (glucagon receptor), TRH (TRH R) [97, 137, 144], and endothelin-1 ET$_A$ and ET$_B$ receptors [145]. Co-expression of multiple aberrant GPCR was also noted; renin-independent stimulation of aldosterone secretion was observed in vivo following mixed meal, oral glucose, or administration of GIP, vasopressin, and tegaserod in a patient with unilateral source of PA [134]. Furthermore, co-secretion of aldosterone and cortisol due to aberrant expression of GPCR was reported; in a patient with BMAH and β-AR-aberrant expression, isoproterenol stimulated both cortisol and aldosterone production [141].

Teo et al. described heterozygous activating somatic *CTNNB1* (β-catenin) mutations in tumors of three women with APAs, two who presented during pregnancy and one after menopause [146] (Table 7.1). All three expressed aberrant human chorionic gonadotropin (hCG) and GnRH receptors at levels 100-fold higher than in other APAs mainly due to the high levels of endogenous hCG during pregnancy and of GnRH and LH after menopause. It was demonstrated that the *CTNNB1* mutation led to activation of the WNT pathway; it was suggested that could be the cause of dedifferentiation of gonadal progenitor cells present in the adrenal tissues with increased expression of gonadal receptors [146]; however other authors did not find such a correlation between *CTNNB1* mutation and aberrant LH receptor [147, 148].

Adrenocortical Carcinomas

Adrenocortical carcinoma is a rare but frequently aggressive malignancy with poor therapeutic response when it becomes metastatic. Regulatory mechanisms of cortisol production in adrenocortical carcinomas remain not fully elucidated. Decreased activity of steroidogenic enzymes translates into elevated urinary metabolites of several androgens or glucocorticoid precursors [149]. Its molecular causes are still largely unknown; recent comprehensive reviews of its clinical presentation, genetic predisposing and predictive factors, investigation, and therapy have been published recently [150, 151]. Here we will simply review recent molecular studies that identified novel genes as potential drivers involved in sporadic adrenocortical tumorigenesis, in addition to the previously known insulin-like growth factor 2 (*IGF2*), β-catenin (*CTNNB1*), and *TP53* [152, 153] (Table 7.1). β-Catenin gain-of-function mutations are evident in approximately 25% of both

benign and malignant sporadic adrenocortical neoplasms [153]. ACC may be associated with Beckwith-Wiedemann, familial adenomatous polyposis coli, and Li-Fraumeni syndromes [1]. One genomic profiling effort of ACC led by a French consortium has revealed novel candidate driver genes such as *ZNRF3* and *TERT* and identified molecular profiles in subgroups of patients with variable clinical outcomes [154, 155].

A recent comprehensive pangenomic Cancer Genome Atlas (TCGA) group study expanded the list of ACC driver genes to include *PRKAR1A*, *RPL22*, *TERF2*, *CCNE1*, and *NF1* (Table 7.1). Copy number analysis revealed frequent occurrence of massive DNA loss followed by whole-genome doubling (WGD), which was associated with aggressive clinical course, suggesting WGD is a predictive of disease progression which is also associated with increased TERT expression, decreased telomere length, and activation of cell-cycle programs. Subtype analysis identified three ACC subtypes with distinct clinical outcome and molecular alterations which could be captured by a 68-CpG probe DNA methylation signature, proposing a strategy for clinical stratification of patients based on molecular markers [156].

Conclusion

Over the past decades, the emergence of genomics has led to significant progress in the genetic landscape of adrenocortical tumors resulting in a new subclassification and understanding of molecular mechanisms leading to APA, CPA, BMAH, and ACC. The main altered signaling pathways could become therapeutic targets, which increase our hope that one day targeted molecular therapies would complement surgery as the treatment of choice for these lesions. Two major beneficial applications of genetically analyzing adrenal tumors include genetic counseling, for example, for the *ARMC5* mutations in BMAH, and a better prognostication, adjuvant or advanced disease therapy of ACC based on targeted measurements of a few discriminant molecular alterations. Future collaborative studies on an even larger number of patients with various relatively rare adrenal tumors phenotypes and genotypes will provide further better personalized therapies.

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Chapter 8
Pheochromocytomas and Paragangliomas: Genetics and Pathophysiology

Lauren Fishbein

Introduction

Pheochromocytomas and paragangliomas (PCC/PGL) are rare neuroendocrine tumors of the autonomic nervous system which occur in 2–8 per million people and are the cause of at least 0.2–0.6% of all patients with hypertension [1]. These numbers may be even higher as one autopsy study found that only 44% of PCC were diagnosed before death [2]. In addition, up to 7–10% of true adrenal incidentalomas are PCC [3, 4]. PCC/PGL are associated with high morbidity secondary to mass effect, hormonal oversecretion, and/or metastatic disease. The hypersecretion of catecholamines and metanephrines from most PCC/PGL leads to cardiovascular complications including hypertension, stroke, and even death. Although most tumors are benign, some become metastatic and are associated with a poor prognosis. Interestingly, up to 40% of PCC/PGL are associated with a germline mutation in a wide range of susceptibility genes making them the most common solid tumor associated with inherited mutations. Highly relevant in vitro and in vivo model systems are lacking; however, integrative genomic studies have identified several clues to the pathophysiology of PCC/PGL.

Chromaffin Tissue and Catecholamine Synthesis

PCC/PGL are tumors of the autonomic nervous system (ANS). The ANS controls the involuntary functions of the body, such as in glands, cardiac muscle and smooth muscle in the lungs, gastrointestinal track, and urogenital track. The ANS is divided

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into the sympathetic nervous system which controls the "flight or fight" response and the parasympathetic nervous system which controls the body homeostasis at rest. When activated, the sympathetic nervous system decreases smooth muscle tone and contractility and increases heart rate, whereas the parasympathetic nervous system slows heart rate, increases intestinal activity, and relaxes sphincter muscles [5].

The ANS is derived from chromaffin cells of embryonic neural crest origin. During normal development, neural crest cells migrate out to form both the sympathetic and parasympathetic ganglia as well as the adrenal medulla [6]. Tumors that occur in the adrenal medulla are termed pheochromocytomas (PCC), and tumors that form in the extra-adrenal ganglia throughout the body are called paragangliomas (PGL). Most head and neck PGL (HNPGL) are derived from parasympathetic ganglia, while PCC and the majority, but not all, of the other extra-adrenal PGL are derived from sympathetic ganglia.

Chromaffin cells are structurally similar to postsynaptic sympathetic neurons. The splanchnic nerve of the sympathetic nervous system releases acetylcholine which binds to the nicotinic acetylcholine receptors in the adrenal medulla signaling the release of catecholamines [7]. The catecholamine synthesis pathway begins with the amino acid tyrosine being converted to DOPA by tyrosine hydroxylase, the rate limiting step in catecholamine production (Fig. 8.1). The last step in the synthesis pathway is the conversion of norepinephrine to epinephrine by phenylethanolamine N-methyltransferase (PNMT). The PNMT enzyme is upregulated by cortisol. Therefore, the normal adrenal gland secretes 80% epinephrine and only 20% norepinephrine because the surrounding high circulating cortisol levels from the zona fasciculata layer in the adrenal cortex upregulate PNMT [7]. In contrast, PMNT is low in extra-adrenal ganglia which are not exposed to such high circulating cortisol levels. Epinephrine and norepinephrine have a very short half-life of only a few
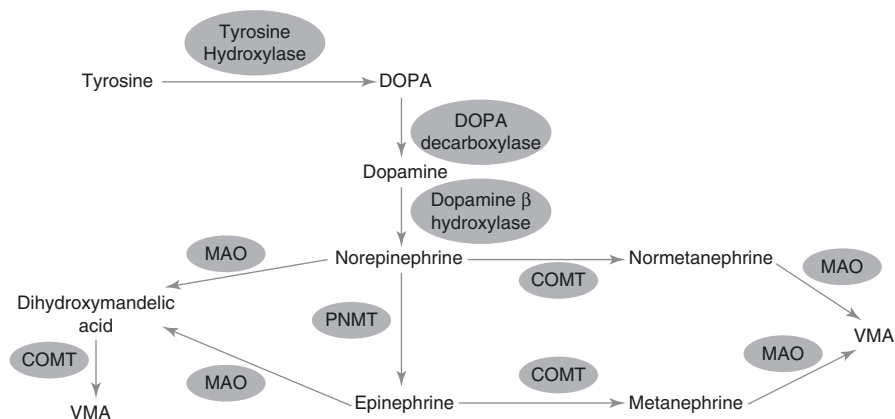
Fig. 8.1 Catecholamine and metanephrine synthesis pathway. *PNMT* phenylethanolamine N-methyltransferase, *COMT* catechol-O-methyltransferase, *MAO* monoamine oxidase, *VMA* vanillylmandelic acid

minutes when circulating in the blood. These hormones are degraded to metanephrine and normetanephrine, respectively, through methylation by catechol-O-methyltransferase (COMT) (Fig. 8.1).

Consequences of Catecholamine Hypersecretion

Catecholamines and metanephrines bind to the seven transmembrane spanning G-protein coupled α- and β-adrenergic receptors and signal through the inositol trisphosphate (IP3) and cyclic adenosine monophosphate (cAMP) second messenger pathways to regulate many cardiovascular, bronchial, and metabolic processes [7]. The hypersecretion of catecholamines and metanephrines leads to numerous symptoms and signs associated with constitutive activation of these pathways including the classic triad of headaches, palpitations, and diaphoresis (Table 8.1) [8–10]. Hypertension, whether sustained, episodic, orthostatic, or relative, is a common consequence of catecholamine over secretion. Surprisingly, a significant number of patients may be asymptomatic, complicating the diagnosis. Severe cardiovascular complications from PCC/PGL include sudden death, arrhythmias, myocardial infarction, and reversible dilated or hypertrophic cardiomyopathy [11]. Acute cardiomyopathy from catecholamine surges occurs in 11% of patients [12]. Given all of these consequences of hormonal hypersecretion, there is high morbidity and mortality when the PCC/PGL go unrecognized or untreated.

Table 8.1 Clinical signs and symptoms of PCC/PGL

Sign/symptom	Range percentage (%)
Classic	
Hypertension (sustained or episodic)	67–94
Palpitations/tachycardia	34–58
Headache	33–52
Sweating	31–49
Anxiety	24–35
Tremors	11–26
Nonclassic	
Nausea	6–22
Pain (abdomen/back)	15–21
Shortness of breath	9–17
Weight loss	6–17
Vertigo	17
Absence of symptoms	8–10
Rare	
Hyperglycemia	
Fatigue	
Syncope	
Nausea/vomiting	

Adapted from Mannelli et al. [9], Kopetschke et al. [8], and Wachtel et al. [10]

Metastatic PCC/PGL

Another factor associated with increased morbidity and mortality in patients with PCC/PGL is the presence of metastatic disease. Up to 25% of PCC/PGL are malignant (~10% of PCC and ~20% of PGL) [13] defined by the presence of distant metastases where chromaffin tissue is not normally present [1]. Metastatic disease can have a long latency period and occur even up to 20 years after initial diagnosis. The prognosis for patients with metastatic disease is poor with only a 50% 5-year survival rate [13]. Predicting which tumors will become metastatic has proven difficult. Single histopathologic features alone such as vascular or capsular invasion are not helpful in predicting metastases; therefore, identifying biomarkers of malignant potential is an important focus of research.

Two histopathologic scoring systems taking into account multiple features have been developed to try to predict malignant potential [14, 15]. The first system is the Pheochromocytoma of the Adrenal Gland Scaled Score (PASS) based on a retrospective analysis of 100 adrenal PCC comparing histopathologic features to the clinical behavior over 10 years of follow-up [15]. Points are assigned to each histopathologic finding such as large nesting patterns, high cellularity, presence of necrosis or vascular or capsular invasion, and high number of mitoses or atypical mitoses, for a maximum score of 20 (Table 8.2). This study suggests that tumors with a PASS less than four were always clinically benign, whereas tumors with a PASS of four or higher had the potential to become metastatic. There are several limitations to this system. It was not developed for PGL, and it is unknown if the same criteria apply. Most importantly, the PASS has extremely high inter-observer and intra-observer variability [16], making it unreliable, and consequently, most centers do not use it.

The second scoring system is the Grading System for Adrenal Pheochromocytoma and Paraganglioma (GAPP) which scores both PCC and PGL to a maximum of 10 points based on many similar histopathology features as the PASS and includes additional points for the Ki67 proliferation index and biochemical secretion (Table 8.2) [14, 17]. In the GAPP scoring system, tumors are divided into well-differentiated (scores 0–2), moderately differentiated (scores 3–6), and poorly differentiated (scores 7–10) categories similar to the grading system for pancreatic neuroendocrine tumors (PNETS). In a cohort of 163 PCC/PGLs, the GAPP distinguished between benign and malignant PCC/PGL with mean scores ±SEM of 2.08 ± 0.17 and 5.33 ± 0.43, respectively [17]. While the GAPP is promising, it is likely subject to similar intra- and interobserver variability as the PASS. In addition, the GAPP has not been validated by any independent groups and, therefore, cannot yet be applied clinically.

To date, the only reliable predictor of an increased risk of malignancy is a germline *SDHB* mutation, and about half of patients with metastatic disease are *SDHB* mutation carriers [13, 18]. In addition, tumors larger than 4–5 cm and/or secreting methoxytyramine, a dopamine metabolite (not yet clinically available in most centers), are associated with an increased risk of malignancy [13, 19]. Unfortunately, the current treatment options for patients with widely metastatic PCC/PGL are lim-

Table 8.2 Histopathology scoring systems for predicting metastatic potential in PCC/PGL

Histopathologic feature	Pheochromocytoma of the Adrenal Gland Scaled Score; points	Grading System for Adrenal Pheochromocytoma and Paraganglioma; points
Histology pattern	• Large nests or diffuse growth = 2	• Zellballen = 0
		• Large or irregular nests = 1
		• Pseudorosettes = 1
Cellularity	• High = 2	• Low: < 150 cells/HPF = 0
		• Moderate: 15–200 cells/HPF = 1
		• High: > 250 cells/HPF = 2
Necrosis	• Present = 2	• Absent = 1
		• Present = 2
Invasion	• Vascular invasion = 1	• Absent – vascular or capsular = 0
	• Capsular invasion = 1	• Present – vascular or capsular = 1
Proliferation	• Mitotic figures = 2 (> 3/10 HPF)	• Ki67 index < 1% = 0
		• Ki67 index 1–3% = 1
	• Atypical mitoses = 2	• Ki67 index > 3% = 2
Other features	• Extension to adipose tissue = 2	• Epinephrine elevated with or without norepinephrine = 0
	• Cell spindling = 2	
	• Cellular monotony = 2	• Norepinephrine and/or dopamine but without epinephrine = 1
	• Nuclear pleomorphism = 1	
	• Nuclear hyperchromasia = 1	• Non-secreting = 0
Maximum score	**20**	**10**
Histological grade	Not applicable	• Well differentiated (scores 0–2)
		• Moderately differentiated (scores 3–6)
		• Poorly differentiated (scores 7–10)

HPF high-powered fields
Adapted from Thompson [15] and Kimura et al. [17]

ited and none are curative. As the molecular differences between clinically benign and aggressive tumors are investigated, researchers may identify additional biomarkers for malignant transformation as well as potential therapeutic targets.

PCC/PGL Genetics

For a long time, it was taught that PCC/PGL are the "tumor of tens" where 10% of tumors are associated with hereditary conditions. It is now known that the percentage of tumors from patients with germline mutations is much larger [18, 20]; and in addition, genomic studies have identified some common somatic mutations that drive tumorigenesis. In fact, researchers have identified the driver mutation (germline and somatic) in at least 60% of PCC/PGL [20]. However, there is still much to

learn about the pathogenesis of disease in the remaining subset of tumors with no identified driver mutation and much to understand with regard to the differing rates of malignant transformation even among tumors with similar driver mutations.

Germline Genetics

A unique feature of PCC/PGL is the extensive list of susceptibility genes which when mutated lead to an increased risk of tumor development. Up to 40% of patients with a PCC/PGL have a germline mutation in a susceptibility gene, making this tumor type the most common solid tumor to be associated with cancer risk genes [18, 20]. The American Society for Clinical Oncology suggests that clinical genetic testing be offered for any cancer syndrome which has at least a 10% risk of having an inherited gene mutation [21, 22]. Therefore, it has been recommended that all patients with PCC/PGL be offered clinical genetic testing [23] to help guide screening, treatment, and surveillance for patients and their family members who might be mutation carriers. Recognition of the diverse cancer syndromes associated with PCC/PGL, and the wide range of genetic mutations causing them, has greatly increased our understanding of the pathophysiology of PCC/PGL (Table 8.3).

Table 8.3 Genetic syndromes associated with PCC/PGL

Syndrome	Gene	Inheritance	Associated symptoms/signs
Neurofibromatosis Type 1	*NF1*	Autosomal dominant	Diagnostic clinical criteria (at least two)
			• Cutaneous neurofibromas (at least two)
			• Plexiform neurofibromas
			• Café au lait spots (at least six, greater than or equal to 0.5cm in prepubertal patients and to 1.5 cm in postpubertal patients)
			• Lisch nodules (benign iris hamartoma)
			• Inguinal or axillary freckling
			• Long bone dysplasia
			• Optic gliomas
			• First degree relative with NF1
			Increased risk but not part of diagnostic criteria
			• PCC (possibly bilateral)
			• Malignant peripheral nerve sheath tumors
			• Juvenile myelomonocytic leukemia

Table 8.3 (continued)

Syndrome	Gene	Inheritance	Associated symptoms/signs
von Hippel-Lindau	*VHL*	Autosomal dominant	• Hemangioblastomas of the CNS (including retina)
			• Endolymphatic sac tumors
			• Epididymal cystadenomas
			• PCC (possibly bilateral)
			• Renal cell carcinomas
			• Renal cysts
			• Pancreatic neuroendocrine tumors
			• Pancreatic cysts
Multiple Endocrine Neoplasia Type 2	*RET*	Autosomal dominant	Classic Type 2A
			• Medullary thyroid cancer
			• PCC (possibly bilateral)
			• Hyperparathyroidism
			Type 2B
			• Medullary thyroid cancer
			• PCC (possibly bilateral)
			• Marfanoid habitus
			• Mucocutaneous neuromas
			• Gastrointestinal ganglioneuromas
Hereditary PGL syndrome	*SDHA*	Autosomal dominant (*SDHD*, *SDHAF2* – paternal inheritance)	• EAPGL (*SDHB*, *SDHD*, *SDHC*, *SDHA*)
	SDHB		• HNPGL (*SDHD*, *SDHC*, *SDHAF2*, rare *SDHB*)
	SDHC		• PCC (*SDHB*, *SDHD*, rare *SDHC*)
	SDHD		• Renal cell carcinoma (clear cell)
	SDHAF2		• GI stromal tumors
			• Pituitary adenoma rare
Familial PCC/PGL syndrome	*MAX*	Autosomal dominant	• PCC (bilateral)
Familial PCC/PGL syndrome	*TMEM127*	Autosomal dominant	• PCC
			• EAPGL and HNPGL possible
			• Renal cell carcinoma (clear cell) rare
Polycythemia PGL syndrome	*EPAS1*	Autosomal dominant (often somatic mosaic)	• Polycythemia
			• PCC/PGL
			• Somatostatinoma
Hereditary Leiomyomatosis and Renal Cell Cancer syndrome	*FH*	Autosomal dominant	• Cutaneous and uterine leiomyomas
			• Renal cell carcinoma (type 2 papillary)
			• PCC/PGL rare

PCC pheochromocytoma, *PGL* paraganglioma, *EAPGL* extra-adrenal paraganglioma, *HNPGL* head and neck paraganglioma

Genetic Syndromes Associated with PCC/PGL

Neurofibromatosis Type 1

Neurofibromatosis type 1 (NF1), also called von Recklinghausen's disease, is an autosomal dominant cancer syndrome occurring in 1 in 2800 individuals and caused by loss of function mutations in the *Neurofibromatosis Type 1 (NF1)* tumor suppressor gene [24]. *NF1* is located on chromosome 17q11.2 and encodes the large 2818 amino acid protein neurofibromin. The most well-defined role of neurofibromin is as a GTPase-inactivating RAS and, thereby, regulating both the PI3K/mTOR (mammalian target of rapamycin) and mitogen-activated protein kinase (MAPK) pathways to control cell proliferation and differentiation [25]. When *NF1* is mutated, there is uncontrolled activation of these pathways leading to tumor formation.

Traditionally, NF1 is diagnosed based on clinical criteria rather than genetic testing due to the large size of the gene and lack of mutational hotspots. Up to 50% of patients with NF1 have *de novo* mutations, and there is variable penetrance and expressivity for the disease even in those family members with the same mutation [26]. The clinical diagnosis is made when patients have two or more of the following characteristics: six or more café au lait spots of varying size based on pubertal status, two or more neurofibromas or one plexiform neurofibroma, Lisch nodules (benign iris hamartomas), optic glioma, skeletal dysplasia (thinning of the long bones), or a first degree family member with NF1 (Table 8.3) [24]. Although not part of the diagnostic criteria, patients with NF1 also are at increased risk of developing PCC, malignant peripheral nerve sheath tumors, and juvenile myelomonocytic leukemia. The prevalence of PCC in patients with NF1 varies from 1 to 15% depending on case detection testing [27–29]. Guidelines for treating patients with NF1 recommend screening for PCC in individuals with hypertension [24]. PCCs in NF1 are usually unilateral, but can be bilateral, and often secrete both epinephrine and norepinephrine. The mean age of diagnosis of PCC in patients with NF1 is 43 years old [30], similar to those with sporadic disease. Although PCCs are rare in individuals with NF1, the rate of malignancy is elevated to approximately 12% [30].

von Hippel-Lindau Disease

von Hippel-Lindau disease (vHL) is another autosomal dominant cancer syndrome occurring in 1 in 36,000 individuals per year and caused by loss of function mutations in the *von Hippel-Lindau (VHL)* tumor suppressor gene [31]. *VHL* is located on chromosome 3p25-26 and encodes two isoforms of the von Hippel-Lindau protein (pVHL), an E3 ubiquitin ligase: one full-length 213 amino acid protein and one smaller protein which lacks the first 53 amino acids [32]. Under normoxic conditions, pVHL binds to a hydroxylated proline residue on Hypoxia-Inducible Factor α (HIFα) and marks it for proteasomal degradation by ubiquitination [33]. Under

hypoxic conditions, or if pVHL is inactivated by mutations, pVHL will not bind to HIFα which is then free to dimerize with HIFβ, translocate to the nucleus, and act as a transcription factor for downstream genes involved in the hypoxia signaling pathway [32].

vHL is diagnosed based on clinical features and by identifying the *VHL* gene alteration through clinical genetic testing. Patients with vHL develop a wide range of benign and malignant tumors including renal cysts and clear cell renal cell carcinomas (RCC), pancreatic cysts and PNETS, hemangioblastomas of the central nervous system, endolymphatic sac tumors, and PCC (Table 8.3) [34]. Up to 20% of all patients with vHL will develop a PCC with the mean age of diagnosis at 30 years, and PCC may be the presenting sign of disease [35]. Interestingly, there are strong genotype-phenotype correlations between PCC and *VHL* mutations [36–41]. Patients with vHL type 1 have truncating gene mutations or deletions, and these patients have a low risk of PCC and a higher risk of RCC. On the other hand, patients with vHL type 2 have missense mutations in *VHL* and carry a high risk of PCC or RCC depending on the location of the missense mutation. Missense mutations on the surface of pVHL portend a higher risk of PCC than missense mutations in the core of the protein [42]. Mutations that disrupt the interaction between pVHL and HIF are associated with RCC, while mutations in other parts of pVHL are associated with PCC [37, 40, 43, 44]. This suggests that increased HIF activity is related to renal cell carcinoma formation, while other HIF-independent functions of pVHL may be causative for PCC development. However, by cDNA microarray analysis, *VHL*-associated PCC display a pseudohypoxia gene expression pattern as expected to occur secondary to increased HIF activity [45], so the exact mechanism by which the genotype-phenotype correlations exist remains to be elucidated.

PCC in vHL can be unilateral or bilateral, and there are rare cases of extra-adrenal PGL [46, 47]. Interestingly, vHL-associated PCC tend to secrete norepinephrine despite forming in the adrenal medulla. The reason for this deviation in the usual adrenal medullary hormonal secretion pattern is due to decreased PNMT expression secondary to promoter hypermethylation, leading to a decreased conversion of norepinephrine to epinephrine [48, 49]. The malignant PCC rate in patients with vHL is about 5% [34]. Guidelines suggest that patients with high-risk *VHL* mutations be screened annually for PCC starting at age 5 [50].

Multiple Endocrine Neoplasia Type 2

Multiple Endocrine Neoplasia Type 2 (MEN2) is the third classic autosomal dominant cancer syndrome associated with PCC/PGL risk. MEN2 occurs in 1 in 30,000 people and is caused by activating mutations in the *RET* (*Rearranged During Transfection*) proto-oncogene. *RET* is on chromosome 10q11.2 and encodes the 860 amino acid RET protein, a transmembrane tyrosine kinase required for neural crest development [51]. When bound by ligand, the RET protein dimerizes, autophosphorylates, and signals through the PI3K pathway to regulate proliferation and

apoptosis [52]. Activating mutations in *RET* cause constitutive upregulation of PI3K pathway leading to tumor development.

The MEN2 diagnosis is suspected based on clinical features and confirmed by mutation testing of the *RET* gene. Patients with MEN2 are divided into two groups per the most recent guidelines, MEN2A and MEN2B [53] (Table 8.3). Classical MEN2A is defined by medullary thyroid cancer (MTC) (95% of patients), primary hyperparathyroidism (15–30% of patients), and PCC (50% of patients). MEN2A also includes three additional subtypes: (1) patients with MTC and cutaneous lichen amyloidosis, (2) patients with MTC and Hirschsprung's disease, and (3) patients who develop only MTC (the old Familial MTC subtype of MEN2). MEN2B is defined by MTC (100% of patients), PCC (50% of patients), mucosal ganglioneuromas, and a marfanoid habitus.

Ninety-five percent of patients with MEN2 have type 2A and 5% have type 2B. There are strong phenotype/genotype correlations based on the mutated *RET* codon. For example, patients with codon 634 mutations have high risk for classical MEN2A, while patients with mutations at codon 918 almost exclusively have the MEN2B phenotype [54]. The 2015 guidelines suggest that patients with high-risk mutations (codons 918, 634, and 883) be screened annually for PCC starting at age 11 and those with moderate-risk mutations (all other codons) be screened annually for PCC starting at age 16 [53]. The mean age at diagnosis of PCC in patients with MEN2 is 28 years [55], and patients develop bilateral PCC more than 50% of the time [55, 56]. There are rare reports of extra-adrenal PGL [46]. MEN2-associated PCC usually have epinephrine predominance [57], and malignant PCC occurs in less than 5% of cases [56].

Hereditary Paraganglioma Syndromes

The hereditary paraganglioma syndromes are autosomal dominant disorders caused by loss of function mutations in one of the *Succinate Dehydrogenase Subunit (SDH)* genes (*SDHA, SDHB, SDHC, SDHD*) or the cofactor *SDHAF2* [58–62]. The SDH complex, complex II of the mitochondrial respiratory chain, is a highly conserved heterotetrameric protein and the only respiratory chain complex also involved in the Krebs cycle. The SDH complex couples the oxidation of succinate to fumarate with the electron transfer to the terminal acceptor ubiquinone in the electron transport chain. SDHA and SDHB are the catalytic subunits located in the mitochondrial matrix, and these two proteins are anchored to the inner mitochondrial membrane by subunits SDHC and SDHD. Loss of function mutations in the SDH complex subunits result in accumulation of succinate. Because succinate has structural similarity to α-ketoglutarate (also called 2-oxoglutarate), high levels of succinate competitively inhibit 2-oxoglutarate-dependant dioxygenases such as histone and DNA demethylases as well as HIF prolyl-hydroxylases [63, 64]. This inhibition leads to two main downstream consequences: (1) pseudohypoxia conditions because the inhibition of HIF prolyl-hydroxylase enzymes prevents the hydroxylation of the

proline residue on HIFα thereby preventing pVHL binding and subsequent proteasomal degradation and (2) global DNA hypermethylation and other epigenetic changes because of the inhibition of histone and DNA demethylases [65, 66]. Mutations in any of the SDH subunits or cofactor AF2 lead to increased risk of PCC/PGL formation as discussed below (Table 8.3).

The SDHB subunit is the most commonly mutated subunit of the complex associated with PCC/PGL. *SDHB* is located on chromosome 1p36.1 and encodes the 280 amino acid iron sulfur subunit of the SDH complex. Patients with *SDHB* mutations most often develop extra-adrenal PGL but also are at risk for HNPGL as well as adrenal PCC. The *SDHB*-associated tumors tend to secrete norepinephrine and/or dopamine, and the mean age at initial diagnosis ranges from 28.7 to 36.7 years [67–69]. Compared to all of the other PCC/PGL susceptibility genes, the risk of malignancy is highest in patients with *SDHB* associated PCC/PGL (~23%) [70]. Of those patients with metastatic disease, about half have germline *SDHB* mutations [18].

SDHD is the next most common subunit of the complex that is mutated in association with PCC/PGL. *SDHD* is located on chromosome 11q23.1 and encodes the 103 amino acids anchoring subunit with an ubiquinone binding site to which the electrons are transferred from the iron sulfur clusters within the SDHB subunit [71, 72]. *SDHD* mutations are expressed with a parent-of-origin effect, almost exclusively showing paternal inheritance with extremely rare exception [67, 73–76]. Patients with paternally inherited *SDHD* mutations develop HNPGL and are at risk to develop multiple primary tumors including adrenal PCC and extra-adrenal PGL as well [75, 77, 78]. The mean age at diagnosis is 35.7 years [67], and the rate of malignancy is less than 5% [70]. The *SDHD*-associated HNPGLs tend to be biochemically silent or produce only dopamine or methoxytyramine, whereas the PCCs can have norepinephrine secretion [57].

The *SDHC*, *SDHA*, and *SDHAF2* genes are mutated at a lower frequency in association with PCC/PGL. *SDHC* is located on chromosome 1q23.3, spans 35 kb, and encodes a large subunit with the cytochrome b in the SDH complex. Patients with *SDHC* mutations develop HNPGL and thoracic PGL most commonly, although PCC/PGL in other locations can be seen [79–81]. The mean age at initial diagnosis is 29–38 years [67, 79], and there is a negligible risk of malignant disease in *SDHC* mutation carriers [67, 79]. *SDHA* is located on chromosome 5q15 and encodes the flavoprotein catalytic subunit of SDH complex. Biallelic mutations in *SDHA* lead to Leigh's syndrome (an early onset neurodegenerative disorder) [82, 83]. It was subsequently discovered that *SDHA* mutations also are associated with PCC/PGL and account for about 3% of PCC/PGL cases with low penetrance [60, 84]. The phenotype associated with *SDHA*-related PCC/PGL is still being uncovered. *SDHAF2* is located on chromosome 11q12.2 and encodes the protein needed for flavanation of the SDHA catalytic subunit. Only a few families have been described with mutations in *SDHAF2,* and those affected mutation carriers tend to have multiple HNPGL [61, 85, 86]. Similar to *SDHD* mutations, *SDHAF2* mutations show a parent-of-origin effect associated with paternal transmission of disease. The average age of onset is 33 years and the rate of malignancy is negligible.

Inherited *SDHx* gene mutations are associated with increased risk of developing other cancers in addition to PCC/PGL [87] (Table 8.3). Renal cell carcinoma occurs in about 14% of *SDHB* and 8% of *SDHD* mutation carriers [75]. *SDHC* and *SDHA* mutation carriers also are at higher risk than the general population to develop RCC albeit at a low frequency [88]. In addition, all *SDHx* mutation carriers are at increased risk for developing gastrointestinal stromal tumors (GISTs) [89], and numerous case reports suggest an association between *SDHx* mutations and pituitary adenomas [90]. Thus far, no strong genotype/phenotype correlations have been discovered between the specific mutation types and the spectrum of disease.

There are no formal guidelines for screening asymptomatic or unaffected *SDHx* mutation carriers. PCC/PGL have been reported in children as young as 5 years old, yet the penetrance of PCC/PGL and the other *SDHx*-associated tumors is not well defined. Most experts suggest screening for *SDHx*-associated tumors in asymptomatic mutation carrier patients with whole body MRI every 2–5 years starting between ages 5 and 10 along with annual biochemical screening for PCC/PGL.

Familial Pheochromocytomas and Paragangliomas

Several families with inherited PCC/PGL had none of the above susceptibility gene mutations. Applying genomic approaches to DNA and RNA from tumors from these families, identified additional susceptibility genes as causing autosomal dominant transmission of PCC/PGL, including *TMEM127*, *MAX*, and *FH* (Table 8.3), discussed below. In addition, there are rare case reports of families with germline mutations in *MDH2*, *KIF1B*, and *EGLN1* [91–93].

The *TMEM127* gene is located on chromosome 2q11.2 and loss of function mutations lead to PCC/PGL [94]. *TMEM127* encodes transmembrane protein 127 believed to play a role in mTORC1 signaling pathway [94]. The patients with germline *TMEM127* mutations have a low penetrance of disease. These mutation carriers can develop PCC/PGL in any location, often having bilateral adrenal PCC [95, 96]. The average age of onset is similar to sporadic tumors at 45 years old, and the rate of malignancy is low. Rare cases of RCC have been found in *TMEM127* mutation carriers [97].

The *MAX* gene, located on chromosome 14q23, was found to be a PCC/PGL susceptibility gene through germline exome sequencing [98]. *MAX* encodes a basic helix-loop-helix leucine zipper protein called MYC-Associated Protein X (MAX), which heterodimerizes with MYC or MYC repressors to act as a transcription factor for numerous genes involved in cell proliferation, differentiation, and apoptosis [99, 100]. Patients with *MAX* mutations tend to have adrenal PCC which are often bilateral and can be multifocal within a single adrenal gland; furthermore, there may be an association with an increased risk of malignancy, although this has not been replicated in a second cohort study [98, 101]. The number of patients with *MAX* mutations identified thus far is quite small making the phenotype difficult to define.

Inactivating mutations in the *Fumarate Hydratase (FH)* gene cause Hereditary Leiomyomatosis and Renal Cell Cancer (HLRCC) syndrome which is an autosomal

dominant condition where patients develop smooth muscle tumors (leiomyomas) and type 2 papillary renal cell carcinoma [102]. Rare families with PCC/PGL have been found to carry germline *FH* mutations, and there may be an associated increased risk of metastatic disease [103, 104]. The *FH* gene is located on chromosome 1q42.1 and encodes the enzyme fumarate hydratase which converts fumarate to malate in the Krebs cycle. PCC/PGL with *FH* mutations have similar molecular consequences to PCC/PGL with *SDHx* mutations including pseudohypoxia and hypermethylation as fumarate shares structural similarities to succinate leading to similar inhibition of the 2-oxoglutarate-dependent dioxygenases [64, 103, 104].

Polycythemia Paraganglioma Syndrome

Germline gain-of-function mutations in *EPAS1* are known to be associated with hereditary polycythemia [105]. *EPAS1* is located on chromosome 2p21 and encodes the HIF2A (Hypoxia-Inducible Factor 2α) protein. Interestingly, somatic mosaic *EPAS1*-activating mutations located at the HIFα stabilizing prolyl sites or close by in the protein have been identified in patients with PCC/PGL, polycythemia, and somatostatinoma (Table 8.3) [106–108]. The molecular consequences of mutated HIF2A are similar to that for pVHL; the mutations prevent binding between pVHL and HIF2A, allowing HIF2A to avoid ubiquitination and degradation and instead move to the nucleus and activate downstream target genes. As expected, *EPAS1*-associated PCC/PGL have similar pseudohypoxia expression patterns as *VHL*-associated PCC [109].

Somatic Genetics of PCC/PGL

Several groups have performed large-scale somatic sequencing studies using targeted or exome sequencing which have provided additional insights into tumorigenesis. Most recurrent somatic mutations occur in sporadic PCC/PGL, those tumors without known germline susceptibility gene mutations. For example, somatic mutations in *NF1*, *VHL*, *RET*, and *HRAS (Harvey Rat Sarcoma Viral Oncogene Homolog)* each occur in 10–20% of sporadic PCC [110–114]. *NF1*, *VHL*, and *RET* are known susceptibility genes, so it is not surprising that they are commonly somatically mutated in PCC. Despite the relatively high number of somatic *HRAS* mutations in sporadic PCC, no germline mutations in *HRAS* have been reported to date. Remarkably, almost no somatic mutations have been reported in the *SDHx* genes.

Through somatic DNA sequencing, genes involved in chromatin remodeling have been shown to be mutated in up to 20% of PCC/PGL, although most individually mutated genes were not highly recurrent [115]. This suggests that epigenetic alterations may play a role in tumorigenesis. To date, there are two chromatin remodeling genes that are recurrently somatically mutated in PCC/PGL, *KMT2D*,

and *ATRX*. *KMT2D* mutations were seen in 14% of sporadic PCC, but not in PGL [116, 117]. *KMT2D*, also known as *MLL2* or *Lysine (K)-Specific Methyltransferase 2D*, encodes a histone methyltransferase responsible for methylating the lysine 4 position of histone H3. Heterozygous germline mutations in *KMT2D* are associated with Kabuki syndrome, a rare developmental disorder, while somatic mutations have been identified in several solid cancer types as well as lymphoma (reviewed in [118]). Another chromatin remodeling gene, *ATRX*, was found to be recurrently somatically mutated in almost 13% of PCC/PGL often in association with germline *SDHB* mutations and clinically aggressive disease [119]. ATRX heterodimerizes with the protein DAXX to recruit histone H3.3 for normal telomere maintenance [120, 121]. Germline *ATRX* mutations cause Alpha Thalassemia X-linked Intellectual Disability syndrome, and a high rate of somatic mutations in *ATRX* has been found in other neuroendocrine tumors, such as well-differentiated PNETS and neuroblastomas, as well as some gliomas [122–126]. Interestingly, in gliomas, the somatic *ATRX* mutations are associated with *IDH* mutations [122, 125], similar to the association in PCC/PGL with *SDHB* mutations [119]. This might suggest a link between somatic *ATRX* mutations and global hypermethylation seen in both *IDH*- and *SDHB*-mutated tumors [65, 122], but the true consequence of the association is unknown. In several cancers including PCC/PGL, somatic *ATRX* mutations also are associated with alternative lengthening of telomeres [119, 122, 123, 127, 128], used by cancer cells instead of, or in addition to, telomerase to maintain chromosome integrity during continued proliferation. Interestingly, recurrent *TERT (Telomerase Reverse Transcriptase)* promoter mutations have been found in a few *SDHx*-associated tumors, both extra-adrenal PGL and GIST [129].

Expression and Methylation Profiling in PCC/PGL

PCC/PGL are subdivided into well-described clusters based on expression and methylation profiling (Table 8.4). Based on RNA expression, PCC/PGL divide into two clusters, the pseudohypoxia cluster, containing tumors with *VHL*, *SDHx*, and *EPAS1* mutations, and the kinase cluster containing tumors with *NF1*, *RET*, *TMEM127*, and *MAX* mutations [48, 113, 130, 131]. Sporadic tumors are divided between both clusters. Not surprisingly based on the mutations which cluster together, a common feature of the pseudohypoxia cluster is activation of the HIF proteins and the downstream targets. By methylome analysis, PCC/PGL divide into hypermethylated and non-hypermethylated groups. The *SDHx*-associated tumors are hypermethylated compared to other PCC/PGL, with *SDHB*-associated tumors having the highest levels of methylation [65, 132]. Similar results were seen in *SDHx*-associated GIST which are hypermethylated compared to other GIST tumors [132]. The tumors with *RET* and *NF1* mutations have low global methylation, and *VHL*-associated tumors are in between [65]. Further work needs to be done to understand the full consequences of global methylation differences between PCC/PGL leading to tumorigenesis.

Table 8.4 PCC/PGL genomic clusters

Expression profile cluster	Pseudohypoxia		Kinase signaling
Germline mutations	*VHL*	*SDHx*, *FH*	*NF1*, *RET*, *TMEM127*, *MAX*
Somatic mutations	*VHL*, *EPAS1*	Possibly *ATRX*, *TERT*	*NF1*, *RET*, *HRAS*
Methylation profile	Low	High	Lowest
Molecular alteration	Activation of hypoxia inducible genes	Inhibition of histone and DNA demethylases and HIF prolyl-hydroxylases	Increased cell signaling for proliferation and decreased apoptosis
Malignancy risk	Low	High (*SDHB*, possibly *FH*)	Low
Biochemical secretion	Noradrenergic	Noradrenergic, dopaminergic, or silent	Adrenergic and noradrenergic

Despite what has been learned about PCC/PGL through germline and somatic genomics, many questions about tumorigenesis and malignant transformation remain. Why do only *SDHB*-associated tumors appear to increase risk of malignancy, especially compared with other *SDHx* mutations? Why is the penetrance of disease for mutation carriers not 100% for most of the susceptibility genes? What are the protective modifier genes or the additional hits needed for tumorigenesis and malignant transformation? These areas of research are ongoing.

Model Systems of PCC/PGL

The pathogenesis of PCC/PGL has been difficult to study largely due to the lack of relevant *in vivo* and *in vitro* model systems. Several mouse models of PCC have been developed, but none really recapitulate the human disease, especially metastatic disease. *Sdhb* and *Sdhd* knockout mice are embryonic lethal, and interestingly, both heterozygotes do not develop tumors [65, 133, 134]. Conditional *Sdhb* knockout mice are being developed and may offer a more reliable model system in the future for studying the disease. The *Nf1* heterozygous knockout mice do develop PCC in 10–20% of cases but late in the life cycle at 15–28 months [135]. The *Ret* M918T homozygous knockout mouse also develops PCC, and the heterozygotes have chromaffin cell hyperplasia [136]. Both the *Nf1* and *Ret* mouse models are useful for some studies, but neither develop clinically aggressive or metastatic disease, the area of true unmet need. Interestingly, mice with *Braf* V600E mutations develop PCC at 5 months [137]; however, *BRAF* mutations are seen only as extremely rare somatic (not germline) mutations in human PCC making this model less applicable to the human cancer.

PCC/PGL cell lines for *in vitro* models have largely been disappointing. PC12 cells were developed from rat PCC [138] and have been used extensively to study neuronal differentiation. These cells are not a great model system for PCC as that the cells experience phenotypic drift in response to different media conditions and additives [139]. MPC cells (mouse pheochromocytoma cells) from the irradiated *Nf1* knockout mice are another PCC cell line [140]. While MPC cells do capitulate the human *NF1*-associated PCC given the genetic background and the expression of PNMT enzyme [140], only a small percentage of human PCC are from patients with NF1 making this cell line less generalizable. Lastly, one group published a report of an immortalized cell line from a human PCC treated with lentivirus vector carrying the catalytic subunit of human telomerase reverse transcriptase (hTERT) [141]. This cell line does not express most enzymes in the catecholamine synthesis pathway including tyrosine hydroxylase and PNMT [141]. This abnormal enzyme expression pattern could be secondary to a change in differentiation in culture, or it could suggest the cultured cells are not the neuroendocrine tumor cells. The utility of this model is still to be determined.

Summary

PCC/PGL are unique tumors given the complications related to hormonal hypersecretion as well as the strong association with susceptibility gene mutations. There are now over 14 recognized susceptibility genes across a wide range of cellular pathways, and genomic studies have identified the driving mutation (germline or somatic) in at least 60% of tumors. The key mechanisms of tumorigenesis include alterations in the kinase signaling pathway and the pseudohypoxia pathway as well as epigenetic alterations. Unfortunately, thus far, the field lacks highly relevant *in vitro* and *in vivo* model systems to investigate the pathophysiology behind the malignant transformation and aggressive disease. Hopefully, new large-scale international collaborations and team science approaches will shed light on mechanisms of tumorigenesis and malignant transformation as well as identify novel targets for therapeutic intervention.

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Part III
Diagnosis/Treatment

Chapter 9
Diagnosis and Management of Adrenal Insufficiency

Xin He, James W. Findling, and Richard J. Auchus

Introduction and Background

Dr. Thomas Addison, who was interested in disorders of the skin, first described primary adrenal insufficiency (PAI), or Addison's disease, in 1855 [1]. At autopsy, he found that patients with a curious progressive hyperpigmentation of the skin had enlarged adrenal glands with tuberculosis granulomas. Until the second half of the twentieth century, tuberculosis had been the most common cause of PAI. Now, tuberculosis accounts for only <10% of cases, and autoimmune processes cause 80%; the remainder is due to other genetic, infections, vascular, and neoplastic etiologies (Table 9.1) [2]. PAI is a rare disease, with a prevalence of 90–140 cases per million and incidence of 4–6 cases per million per year [3].

Today, the prevalence of secondary and tertiary adrenal insufficiencies is 150–280 per million, which is much more common than PAI. Because it is often impossible and of little clinical utility to distinguish pituitary and hypothalamic sites of

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Table 9.1 Etiologies of primary and central adrenal insufficiency

Primary		
Genetic disorders		
	Congenital adrenal hyperplasia	
	Adrenoleukodystrophy	
	ACTH resistance syndromes	
	ACTH insensitivity: familial glucocorticoid deficiency, triple-A syndrome	
	Adrenal hypoplasia congenita	
	Other transcription factors: SF1 (NR5A1)	
Drug induced		
	Adrenostatic/adrenolytic: etomidate, ketoconazole, metyrapone, mitotane, osilodrostat, abiraterone	
	Glucocorticoid receptor antagonist: mifepristone	
Autoimmune		
Destructive		
	Hemorrhage	
Central		
Genetic disorders		
	Septo-optic dysplasia	
	Isolated ACTH deficiency	
Drug induced		
	Exogenous corticosteroids (any route of administration), medroxyprogesterone	
	Opioids	
	Immune-checkpoint inhibitor-induced hypophysitis: ipilimumab	
	Benzodiazepines: alprazolam	
	Atypical antipsychotics: olanzapine, quetiapine	
Acquired hypothalamic-pituitary disease		
	Pituitary tumors	
	Granulomatous diseases	
	Hypophysitis: granulomatous or lymphocytic	
	Surgery	
	Radiation	

dysfunction, the term central adrenal insufficiency (CAI) is often used to encompass secondary and tertiary adrenal insufficiencies. Etiologies include primarily pituitary pathologies: tumors, congenital or developmental causes, and hypophysitis (Table 9.1). Brain trauma, radiation damage, and granulomatous diseases primarily affect the neuroendocrine components of the hypothalamic-pituitary-adrenal (HPA) axis.

Of course, the most common cause of CAI is the administration of exogenous glucocorticoids. The biochemical findings are a low or normal plasma ACTH in the presence of a subnormal cortisol or impaired cortisol response to stimulation. Any route of glucocorticoid administration (oral, parenteral, inhaled, or topical) will suppress corticotropin-releasing hormone (CRH) and the HPA axis if the dose and

duration of use are sufficient. Insoluble suspensions, particularly when injected into a relatively avascular space, such as paraspinous injections of triamcinolone acetonide, can suppress the HPA axis for months. In addition, high doses of synthetic progestins with partial glucocorticoid agonist activity, such as megestrol acetate [4], will suppress the HPA axis, and many types of opiates also potently suppress the HPA axis [5–9]. Although the exogenous glucocorticoids compensate for the reduction in cortisol production, the atrophied zona fasciculata and zona reticularis of the adrenal glands are unable to produce cortisol when the glucocorticoids are withdrawn, which can precipitate clinical features of AI if abruptly discontinued.

As a general guide, HPA axis suppression from opiates is rapid in onset and, although variable among individuals, can occur with even small doses when taken regularly. There is some evidence that axis suppression reverses quickly upon discontinuation, even with chronic opiate use, and that acute stress such as insulin-induced hypoglycemia can overcome the suppression; however, data characterizing opiate-induced HPA axis suppression are limited. For exogenous glucocorticoids, HPA axis suppression persists starting about 3–4 weeks after a supraphysiologic dose and persists roughly for a period of time equal to the duration of treatment. Duration of use is more important than dose in determining the probable duration of axis suppression, and drugs with the longest the duration of action are the most suppressive. For example, HPA axis suppression from 4 mg dexamethasone daily for 6 months will typically last longer than from 40 mg prednisone daily for 3 months. In addition, the clinical state during treatment is important, as the HPA axis becomes resistant to suppression during critical illness.

Clinical Presentation

The presentation of chronic PAI encompasses vague and nonspecific symptoms, but some signs and symptoms are more specific than others, and the presence of certain symptoms can virtually rule out the diagnosis. For example, weight loss, hypotension, skin hyperpigmentation, and auricular cartilage calcification are more specific for AI than abdominal pain, nausea, vomiting, fatigue, general malaise, and myalgias. Of note, weight gain, in the absence of comorbidities such as heart failure, liver failure, or renal failure, essentially rules out PAI. On laboratory studies, hyponatremia, hyperkalemia, and eosinophilia are notable findings in PAI.

The presentations of PAI and CAI are similar, with a few notable differences. Patients with CAI do not experience skin hyperpigmentation, because elevated levels of pro-opiomelanocortin, a prohormone precursor of ACTH and melanocyte stimulation hormone, are absent. Due to intact mineralocorticoid production in CAI, hyperkalemia is absent, and hypotension is less prominent. However, hyponatremia may still be present due to the absence of glucocorticoid negative feedback on vasopressin secretion as well as the diminished glomerular filtration rate that may accompany cortisol deficiency. Consequently, the presence of unexplained hyponatremia should

always raise the possibility of hypocortisolemia. Gastrointestinal manifestations are less common in CAI than in PAI; it is possible that these symptoms are more prominent with mineralocorticoid deficiency. CAI may present with headaches, visual deficits, and symptoms of deficiencies in other pituitary hormones.

Acute AI, or adrenal crisis, presents predominantly with weakness, malaise, nausea, vomiting, and hypotension. Without appropriate therapy, shock and multisystem organ failure may ensue. Well-done longitudinal studies have shown that 6–8% of patients with a known diagnosis of either PAI or CAI will experience an episode of adrenal crisis annually, and the annual death rate may be as high as 0.5% [10]. Various situations can precipitate adrenal crisis: for example, patients with partial AI may be asymptomatic until they encounter a stressor, such as acute illness or surgery. Adrenal crisis is also seen in patients treated with glucocorticoids chronically, who do not take extra doses during acute stressors. Bilateral adrenal infarcts, adrenal hemorrhage, and pituitary infarcts can all precipitate adrenal crises. Table 9.2 lists the various signs and symptoms of PAI and CAI and their utility in supporting the diagnosis.

As alluded to above, in any patient suspected of having AI, it is important to take a thorough medication history. Glucocorticoids taken via various routes, including inhaled, intra-articular, transdermal, ocular, and rectal, can suppress the HPA axis. It should be emphasized that other commonly prescribed medications like opiates [5, 6] and benzodiazepines [11] are also associated with CAI. Because CAI of organic etiology is quite rare in the absence of other pituitary deficits, isolated CAI should be presumed due to exogenous substances until proven otherwise (Table 9.3).

Table 9.2 Clinical presentation of adrenal insufficiency

		Primary	Central
Sign and symptoms	Constitutional	Hypotension, weight loss, myalgias, arthralgias, malaise	Hypotension less prominent. Symptoms of other pituitary hormone deficiencies may be present
	Gastrointestinal	Abdominal pain, nausea, vomiting, constipation, diarrhea, splenomegaly	Less common
	Neuropsychiatric	Impaired memory, confusion, depression, psychosis	Other neurological deficits may also be present: headaches, visual field deficits
	Reproductive	Typically in women: decreased axillary and pubic hair, decreased libido, amenorrhea	Present
	Dermatologic	Hyperpigmentation, vitiligo	Absent
	Other	Auricular cartilage calcification	Present
Laboratory studies	Basic metabolic panel	Hyponatremia, hyperkalemia, hypoglycemia	Hyperkalemia absent, hypoglycemia more common
	Complete blood count	Leukocytosis, eosinophilia	Present

Table 9.3 Medical management of adrenal insufficiency

Setting	Recommended treatment	Comments
Chronic therapy		
Adult	Hydrocortisone 12 mg/m²/day in two or three divided doses, 15 to 25 mg total daily dose; prednisone 4–8 mg every morning; prednisolone 5–7.5 mg every morning; methylprednisolone 4–8 mg every morning	Titrate dosing every 3–6 months based on signs, symptoms, and serum sodium
	9α-fludrocortisone acetate 0.05–0.4 mg/day	Only for primary; titrate dosing every 6–12 weeks based on volume status, blood pressure, and serum potassium
Pregnancy	In third trimester, increase glucocorticoid by up to 50% Titrate fludrocortisone based on blood pressure and serum potassium	Avoid dexamethasone
Children	Hydrocortisone 12–17 mg/m²/day divided in two or three doses	Hydrocortisone preferred over other glucocorticoids
	9α-fludrocortisone acetate 0.05–0.4 mg/day	Titration is similar to that for adults
Acute stressors		
Acute illness with fluid loss and poor PO intake	Double or triple glucocorticoid dose and continue 3 days or until feeling well for 1 day	Monitor blood pressure and heart rate
Acute illness with inability to take PO glucocorticoid or maintain blood pressure	Hydrocortisone 100 mg intramuscular or subcutaneous (act-O-vial) once	Paramedics cannot administer glucocorticoids, so administer prior to transport to acute care facility
Minor surgery	25 mg hydrocortisone once prior to surgery and then resume normal therapy	Overtreatment can impair recovery
Major surgery, including childbirth	50 mg once, followed by 25–50 mg every 6 h for 2 days	Extend intensified therapy if complications
Acute adrenal crisis	Hydrocortisone 50 mg every 6 h Fluid resuscitation Electrolyte repletion	Monitor volume status and electrolytes; evaluate etiology of adrenal crisis

Approach to the Patient

There are basal and dynamic methods of testing for adrenal insufficiency. Basal testing measures hormone levels at specific times of the day, while dynamic testing involves measuring the hormonal response to a stimulus. As an initial evaluation in the outpatient setting in the absence of an acute illness, basal testing alone can be used to exclude all forms of adrenal insufficiency in the majority of patients. Because of the strong diurnal rhythm for these hormones, it is important to test

hormone levels when they are high; serum cortisol and plasma ACTH values are most useful when drawn before 0900. A serum cortisol of >14 µg/dL (400 nmol/L) obviates the need for dynamic testing [12]. Although ACTH values cannot be used to diagnose AI per se, the ACTH is used to distinguish primary (>100 pg/mL, >20 pmol/L) from secondary (<10 pg/mL, <2 pmol/L) AI if the cortisol is diagnostically low (<5 µg/dL, <140 nmol/L). Basal cortisol values 5–14 µg/dL, however, neither exclude nor establish the diagnosis and therefore prompt additional testing.

When performing basal testing, additional information can be obtained with the measurement of other ACTH-dependent products of the adrenal cortex, particularly dehydroepiandrosterone sulfate (DHEAS). Unlike cortisol and ACTH, serum DHEAS concentrations vary only slightly throughout the day due to its extensive protein binding; consequently, values need not be obtained in the early morning. Unless the patient is taking DHEA supplements, a serum DHEAS value >60 µg/dL (1500 nmol/L) at any time of day indicates normal adrenal function [13]. DHEAS measurements are generally used not as a primary test but as an ancillary criterion, typically when the basal serum cortisol is 10–14 µg/dL or when the cortisol is drawn after 0900 [14]. DHEAS production declines with age and is usually easily suppressed from any exogenous glucocorticoid use, even when cortisol secretion may remain normal; these properties limit its utility in the elderly (age > 65 years) and for patients who are already treated with glucocorticoids. Consequently, a low DHEAS level by itself is certainly not diagnostic of adrenal hypofunction. Serum aldosterone may also be useful in distinguishing PAI from CAI, since patients with PAI have a subnormal aldosterone and elevation of plasma renin activity.

When Is Dynamic Testing Necessary?

In an outpatient setting, when evaluating a patient with suspicion of chronic AI, basal morning testing often provides enough information to exclude the diagnosis, which obviates the need for dynamic testing. Difficulties in establishing or excluding a diagnosis of AI arise in a variety of settings, including patients who have already received treatment with glucocorticoids, patients seen in an afternoon clinic, patients who are taking certain medications, and patients with known pituitary disease. In general, the diagnosis of PAI is much easier than the diagnosis of CAI, particularly partial forms. Given the low prevalence of AI and the profound implications of the diagnosis, the primary objective should be to *exclude* the diagnosis in the *chronic* setting and to *establish* the diagnosis in the *acute* setting. As in all testing, the positive predictive value is based on the estimated pretest probability of disease, which is based on the aforementioned specific signs and symptoms. Considerable controversy has arisen for patients with critical illness, derived from the anesthesia and critical care literature. Criteria for critically ill patients are no different than for well outpatients, although hypoproteinemia should be taking into account as discussed below. Here we review the four most commonly used dynamic tests for AI.

To definitively exclude PAI and long-standing CAI, the standard test remains the conventional 250 µg cosyntropin stimulation test, called the short Synacthen test in Europe. The cosyntropin is administered as an intravenous bolus or intramuscular injection, and 1–3 serum cortisol samples are obtained after 30–60 min. The only criterion used for a normal test is a peak serum cortisol value of >18 µg/dL (500 nmol/L) anytime during the test [15]. The advantage to this test is that it can be performed any time of the day to elicit maximal cortisol production from the adrenals. The increase in serum cortisol is highly dependent on the basal value, which varies during the day, and the increase is no longer used as a diagnostic criterion. For example, a test is performed at 0900, and the cortisol rises from 16 to 19 µg/dL. This result should be interpreted as normal adrenal function. The normal change of serum aldosterone in response to cosyntropin is a doubling in value [16], and this change in aldosterone is also helpful for confirming normal adrenal function when cortisol testing is borderline normal and/or confounded.

The poorly named insulin tolerance test (ITT) is the gold standard test to diagnose any form of AI [15, 17]; however, only patients with suspected CAI and equivocal basal testing need to undergo an ITT. Furthermore, the logistical limitations render the study impractical in most circumstances, but the details of this test are nevertheless included for historical context. Insulin-induced hypoglycemia might not be a physiologically meaningful stimulus for most adults, but unlike other stimuli for the HPA axis, such as exercise [18, 19] or acute illness, the ITT follows a standardized protocol with normative criteria. The ITT should not be performed in patients who have seizure disorders or significant cardiovascular disease, in elderly and frail patients, or in patients who lack the ability to verbalize symptoms of hypoglycemia. The test is performed in the early morning after an overnight fast. Regular insulin or short-acting analog is given as an intravenous bolus of 0.1–0.2 unit/kg, and serum cortisol samples are drawn every 15 min for 75 min. In order for a negative test to be valid, the glucose should drop to <40 mg/dL (2.2 mmol/L), which typically occurs between 30 and 45 min after the dose. The normal response is a cortisol rise to >18 µg/dL at any time, including the basal value; the measurement of plasma ACTH during ITT provides no additional diagnostic information [20].

The low-dose 1 µg cosyntropin stimulation test has been proposed as a test to diagnose CAI of recent onset, to avoid false-positive results with the standard 250 µg test. This low-dose test, however, requires sampling precisely 30 min after bolus administration of diluted cosyntropin directly into an intravenous access without a significant length of tubing. To exclude CAI, the serum cortisol should rise to 18 µg/dL, although other criteria are found in the literature derived from results in specific patient cohorts. This test is most helpful when a diagnosis is urgently needed and/or appropriately timed basal samples cannot be obtained. In patients with partial CAI, the serum DHEA (not DHEAS) response to low-dose cosyntropin is lost before the serum cortisol response. Although strict cutoff values are not known, a cortisol/DHEA molar ratio >85 before or after low-dose cosyntropin is good evidence of impaired HPA axis integrity [21].

An alternative to the ITT for the diagnosis of CAI is the overnight metyrapone test, which also interrogates the entire HPA axis. Metyrapone inhibits steroid

11β-hydroxylase, the last step in cortisol synthesis. A dose of 30 mg/kg metyrapone (up to 3 g), administered with food at 2300, blocks the cortisol synthesis, increases morning ACTH, and increases precursors upstream of cortisol, particularly 11-deoxycortisol. A single serum sample is obtained at 0800 the following morning for measurement of cortisol and 11-deoxycortisol. A normal test result is an 11-deoxycortisol >7 µg/dL, and an abnormal test requires a serum cortisol of <5 µg/dL, which documents adequate inhibition of 11β-hydroxylase. In other words, the sum of cortisol and its immediate precursors should rise to >12 µg/dL (330 nmol/L) with a pharmacologic stimulus. An important caution is that 11-deoxycortisol cross-reacts significantly with cortisol for many commercial immunoassays [22]. Consequently, we recommend that tandem mass spectrometry assays are used to measure cortisol and 11-deoxycortisol with this test.

Abnormalities in Protein Binding

About 90% of cortisol ordinarily is tightly bound to corticosteroid-binding globulin (CBG), and much of the remaining 10% is weakly bound to albumin. The most common abnormality is found in patients receiving estrogen therapy, which significantly raises CBG [23]. Ordinarily, estrogen-induced increases in CBG do not obfuscate basal or dynamic testing of adrenal function in assessing cortisol, since its concentration should be high. We are aware of one well-documented case of partial CAI with a false-positive dynamic test, which was adjudicated with measurement of plasma-free cortisol [24]. Genetic deficiency of CBG [25] is very rare and is found in patients with very low serum cortisol of 0.5–2 µg/dL (1–5 nmol/L), who lack features of adrenal insufficiency and do not require treatment.

Hypoproteinemia reduces the fraction of plasma cortisol that is bound to proteins, which significantly lowers the total cortisol concentration needed to meet physiologic demands by elevating the corresponding free cortisol fraction. In patients with a serum albumin <2.5 mg/dL, total cortisol values as low as 7 µg/dL (200 nmol/L) can reflect normal adrenal function, which has been confirmed using plasma-free cortisol [26] or saliva cortisol [27, 28] values. The most common situation where hypoproteinemia may confound testing is in chronically ill patients with critical illness and hypotension, in whom the question of adrenal insufficiency often arises [29]. Furthermore, neutrophil elastase proteolytically cleaves CBG, which releases bound cortisol and thus enhances cortisol delivery to sites of inflammation.

When in doubt, plasma-free cortisol can be measured during dynamic testing. One study of 295 outpatients demonstrated excellent concordance of plasma-free cortisol with total serum cortisol during cosyntropin stimulation testing [24]. In that study, 3 of 43 women taking oral contraceptives showed discrepant results with the two types of cortisol assays. Because saliva is an ultrafiltrate of plasma, saliva cortisol values generally reflect plasma-free cortisol. Only a few studies, however, have tested the use of saliva cortisol measurements for the diagnosis of adrenal insufficiency [30].

Additional Testing

Most patients with a diagnosis of CAI have known pituitary and/or hypothalamic disease or damage with other pituitary hormone deficits, and testing is conducted to determine the integrity of the HPA axis. As stated previously, most cases of isolated ACTH deficiency are due to exogenous glucocorticoids or narcotics; however, a notable exception is lymphocytic hypophysitis, for which ACTH deficiency is common. Hypophysitis associated with immune checkpoint inhibitor therapy, particularly ipilimumab, has become increasingly common and often occurs in combination with central hypothyroidism. If an etiology is not known, a sella MRI is an essential diagnostic study. Given the dominant etiologies today, the first test following the diagnosis of PAI should be serum anti-21-hydroxylase antibodies [15], a very specific marker of autoimmune adrenal disease. If negative, young men should be tested for adrenoleukodystrophy with a measurement of plasma very-long-chain fatty acids [31] or genetic testing [32]. For all children with PAI, a genetic etiology should be considered and explored to the extent possible [33].

Treatment

Acute Adrenal Crisis

In the acute setting, the focus should be on circulatory support. Aggressive fluid resuscitation with normal saline or other isotonic crystalloid must be started immediately and continued until volume status is normalized. A bolus of intravenous hydrocortisone hemisuccinate should be given as soon as possible. A dose of 50 mg every 6 h affords trough serum cortisol concentrations roughly 50 μg/dL [29], which is well above the therapeutic range, so larger doses are never necessary. Instead, the goal is to continuously maintain serum cortisol in the therapeutic range with repeated moderate doses such as 25–50 mg every 6 h rather than large but infrequent doses. Besides organ damage from hypotension, hyperkalemia in PAI can cause life-threatening cardiac arrhythmias, so electrolytes, blood pressure, and heart rate should be monitored frequently.

Just as determining the etiology of adrenal insufficiency is an essential component of the outpatient evaluation, determining the reason for the adrenal crisis is likewise critical to the management of the patient. Typical causes of adrenal crisis include infections, from viral gastroenteritis to sepsis, cardiovascular events, and environmental stress such as extreme heat. Failure to recognize and to administer specific therapy for the precipitating illness can be catastrophic despite the administration of glucocorticoids.

The use of glucocorticoids in critically ill patients without bona fide adrenal insufficiency has been an area of controversy [29]. Cortisol values should be interpreted with caution in this population, because hypoproteinemia increases the

fraction of unbound cortisol, such that patients with an albumin <2.5 mg/dL can have a plasma-free cortisol well above normal despite at serum total cortisol of <10 μg/dL [26]. Cosyntropin stimulation testing has been used to identify patients with a poor cortisol rise as evidence of adrenal dysfunction, because mortality is high in this group. In reality, a poor response to cosyntropin identifies the sickest patients who already show maximal adrenal stimulation [34]. There is no strong evidence that these patients benefit from pharmacologic glucocorticoid dosing, which might suppress the inflammatory response. Large randomized trials such as CORTICUS failed to show an improvement in survival [35], but in a subanalysis, the sickest patients, identified by a poor response to cosyntropin, had slightly less septic shock when treated with hydrocortisone.

Chronic Therapy

The goals of replacement therapy are to provide sufficient glucocorticoid exposure to support normal physiologic function, maintain normal volume status, avoid supraphysiologic dosing and iatrogenic Cushing syndrome, and provide the education and preparation to prevent and manage adrenal crises. In PAI, physiologic doses of hydrocortisone do not provide sufficient mineralocorticoid activity to compensate for aldosterone deficiency, and aldosterone itself is not available for replacement therapy. Instead, 9α-fludrocortisone acetate is used at oral doses of 0.05–0.4 mg/day, sometimes higher in rare patients. The drug is generally started at 0.05–0.1 mg/d and titrated upward every 6–12 weeks if orthostatic hypotension or hyperkalemia persists. If hypertension and/or hypokalemia develops, the dose is reduced, and the drug can be given 2–4 times weekly for finer titration, since the effect on volume status is chronic and not acute. Some patients with PAI continue to salt crave despite normal blood pressure and potassium, but salt craving alone does not justify a dose increase, and measurement of plasma renin activity (or plasma direct renin) can aid in dose adjustment if other clinical and laboratory parameters are inconclusive. It is important to realize that chronic volume depletion is an under-recognized cause of persistent fatigue and exercise intolerance, which is often mistakenly attributed to glucocorticoid undertreatment. An important principle for PAI management is to first establish that plasma volume status is normalized before adjusting the glucocorticoid replacement regimen. By definition, patients with CAI do not have aldosterone deficiency and do not require 9α-fludrocortisone acetate therapy.

Glucocorticoid replacement aims to approximate a physiologic exposure to the normal diurnal rhythm of cortisol production. The available preparations of orally administered glucocorticoid tablets lack the proper pharmacokinetics to closely replicate this pattern, but divided doses of hydrocortisone provide a reasonable substitute. Hydrocortisone, which is another name for cortisol, is the preferred replacement

therapy because it is active as administered, achieves peak circulating concentrations in about an hour, and does not produce unnecessary lingering exposure throughout the night. The dose of hydrocortisone for PAI is about 12 mg/m^2/day, given in two or three divided doses with the highest dose in the morning, to distribute exposure similar to the normal diurnal rhythm. Typical regimens are 15 mg on arising and 5 mg after lunch (1300–1400) or 10 mg on arising and 5 mg with lunch (1200–1300) and supper (1700–1900). For CAI, the dose is slightly less, and evening or even afternoon doses might be omitted if the deficiency is partial. A disadvantage of hydrocortisone is that, in rare patients, very rapid metabolism might limit efficacy. Fortunately, drug exposure is easily assessed by measuring a serum cortisol 1 h after a dose (peak value) or prior to the second dose (trough value). As a rule of thumb, a 10 mg oral dose should yield a peak serum cortisol value of 15–20 μg/dL and a trough value 5 h later of 3–7 μg/dL [36].

In situations where rapid hydrocortisone metabolism is observed or multiple daily doses are impractical, longer-acting glucocorticoids might be effective as a single morning dose. Prednisone is widely available but is a prodrug, which requires hepatic conversion to prednisolone. Consequently, glucocorticoid exposure from an oral dose of prednisone is delayed and more variable than from prednisolone or methylprednisolone, particularly in patients with liver disease. Typical morning doses in PAI are 4–10 mg for prednisone, 5–7.5 mg for prednisolone, and 4–8 mg for methylprednisolone, or modestly lower doses for CAI. A liquid form of prednisolone is available for children and for finer dose titration. Dexamethasone is the most potent glucocorticoid available as tablets and elixir, and the dose is 0.2–0.75 mg/day. Dexamethasone gives relatively flat and prolonged exposure, which does not mimic the morning rise except at doses that are too high to provide a glucocorticoid-free window between doses. For this reason, dexamethasone might be useful in special situations for short-term therapy but tends to cause iatrogenic Cushing syndrome and should be avoided for chronic therapy.

There is no single laboratory test that can be used to titrate glucocorticoid dosing. Hyponatremia and recurrent fatigue that remits after a dose and recurs <8 h after a dose are reliable indicators of inadequate drug exposure. Symptoms and signs of AI such as weight loss, anorexia, and abdominal pain suggest under-replacement. Cushingoid features such as dermal atrophy, easy bruising, fat redistribution, insomnia, myopathy, and fragility fractures usually indicate over-replacement. Bronzing and easy tanning with elevated ACTH production will occur in PAI despite adequate cortisol replacement, possibly related to differences in cortisol transport in the brain and peripheral tissues [37]. As a general rule, a standard dose is administered and adjusted every 3–6 months based on laboratory findings, physical exam, and symptoms (Figs. 9.1 and 9.2).

Patient education and preparation for intercurrent illnesses are equally important as judicious medication titration. A simple plan is to advise doubling or tripling the glucocorticoid dosage for states of rapid fluid loss that the patient cannot replace with oral intake, including vomiting, diarrhea, and high fever or excessive
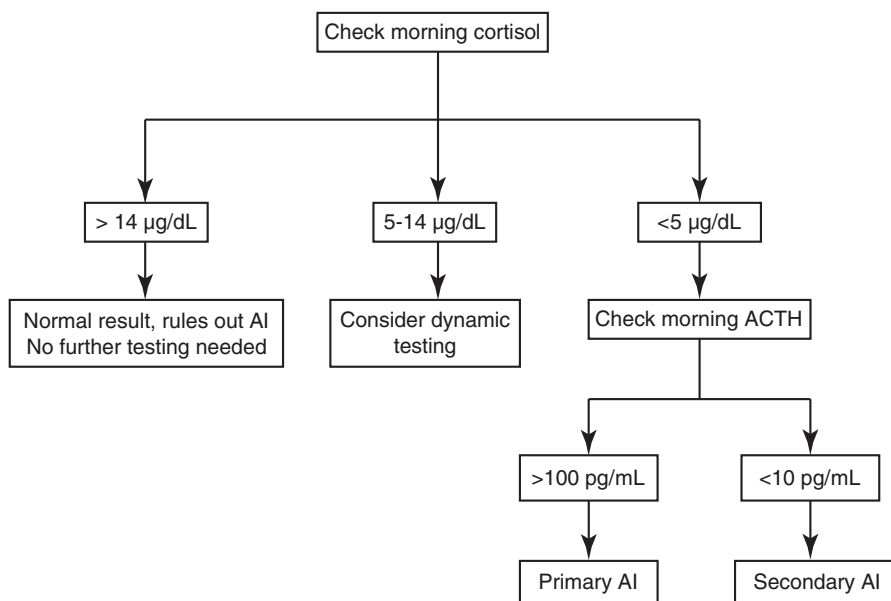
Fig. 9.1 Basal testing: appropriate for initial evaluation under most circumstances

sweating with strenuous exertion in a hot climate. The increased dose is continued for 3 days or until the patient is well for at least 1 day, and then the usual dose is resumed. Patients should also have injectable hydrocortisone hemisuccinate (Solu-Cortef), available as the Act-O-Vial for rapid mixing and emergency administration, and both the patient and at least one relative or close personal contact should be instructed in its use. If the patient is so sick that glucocorticoids cannot be taken orally or are ineffective in maintaining blood pressure, the entire contents of the Act-O-Vial (100 mg) is administered by intramuscular or subcutaneous injection [38], and the patient must be transported to the nearest emergency room. Patients should wear medical alert identification ("Addison's disease" or "adrenal insufficiency") and carry this medical information in their purse or wallet. It is important to recognize that in most states, ambulances and paramedical personnel neither carry hydrocortisone nor have authorization to administer the glucocorticoids in transit. Consequently, it is imperative that the emergency dose is administered prior to transport.

During planned surgeries, extra hydrocortisone is customarily administered for the impending stress. The most critical dose is a bolus prior to the induction of anesthesia. The doses are not higher than those suggested above for critical illness. Dosing should be commensurate with the relative to degree of surgical stress and potential blood loss. For example, minor surgeries, such as a carpal tunnel release under regional anesthesia, do not place a patient under extreme stress. A single bolus dose of 25 mg hydrocortisone prior to surgery will easily suffice for 4 h of minor surgery, and the usual outpatient regimen can be resumed afterward if there

a

```
┌─────────────────────────────┐
│  Administer 250 µg cosyntropin at
│        any time of day       │
└─────────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│   Measure cortisol 2-3 times
│      after 30-60 minutes     │
└─────────────────────────────┘
```

Any cortisol measurement >18 µg/dL	All cortisol measurements <18 µg/dL
Normal adrenal function Rules out AI	Adrenal insufficiency Determine etiology

b

```
┌──────────────────────────────────────────────┐
│  Administer 0.1-0.2 units/kg of regular or short-acting insulin
│       in the morning after overnight fast      │
└──────────────────────────────────────────────┘
                       │
                       ▼
┌──────────────────────────────────────────────┐
│       Measure cortisol and blood glucose
│         every 15 minutes for 75 minutes        │
└──────────────────────────────────────────────┘
```

Any cortisol measurement >18 µg/dL	All cortisol measurements <18 µg/dL with glucose nadir <40 mg/dL
Normal adrenal function Rules out AI	Adrenal insufficiency Determine etiology

Fig. 9.2 Dynamic testing: (**a**) standard cosyntropin stimulation test and (**b**) insulin tolerance test

are no complications. In contrast, cardiac bypass surgery is a major physical stress, and a bolus of 50 mg followed by 25–50 mg every 6 h will be continued for about 2 days until the patient is healing well and then gradually tapered to the usual outpatient dose. The same rules apply to childbearing and cesarean section.

During pregnancy, the doses of adrenal replacement therapies do not need to be increased for at least the first and second trimester. Although no studies have addressed the question directly, most experts advise increasing the dose of glucocorticoid by up to 50% sometime in the third trimester [15]. Dexamethasone crosses the placenta and should be avoided during pregnancy, whereas hydrocortisone, prednisolone, and its derivatives are inactivated via placental 11β-hydroxysteroid dehydrogenase type 2 [39] and are thus safe to use. Because the placenta synthesizes CRH, patients with incomplete CAI can have an increase in endogenous cortisol production during the third trimester, when the placental CRH production exceeds the production of its binding protein. Fludrocortisone acetate is pregnancy category C and should be used with caution in nursing mothers. The dose of fludrocortisone acetate is adjusted during pregnancy based on standing blood pressure and serum potassium. The physiology of normal pregnancy involves an increase in plasma volume, vasodilation, and risk of hypertension, particularly in the third trimester. These factors need to be taken into account and monitored in order to properly titrate adrenal replacement therapy.

Adrenal insufficiency is uncommon in children, but these children are at greater risk for adrenal crises than adults. In part, this tendency is due to the poor response of the kidney to mineralocorticoids in early life, as well as the low sodium content of breast milk (~7 meq/L). Hydrocortisone is preferable in children whenever possible, because prednisolone and dexamethasone are increasingly suppressive of growth and easily overdosed. The daily dose of hydrocortisone ranges from about 12 mg/m^2/day up to 17 mg/m^2/day divided in two or three doses. The high end of the dose range is used for children with congenital adrenal hyperplasia [40, 41], in whom suppression of adrenal androgen synthesis is a second goal of adrenal replacement therapy. The same parameters used to titrate fludrocortisone acetate in adults are also used in children. Because of their resistance to mineralocorticoid action, children receive fludrocortisone acetate doses as comparable to adults, despite the vast differences in body sizes.

Implications of the Adrenal Insufficiency Diagnosis

The labeling of a patient as adrenal insufficient carries considerable implications, not only for their medical condition and treatment but also for their social and economic status. Patients with adrenal insufficiency as a group still suffer from excess mortality due to adrenal crises, independent of education and despite adequate preparation for emergencies [10]. Adrenal insufficiency often occurs as a consequence of an underlying predisposing condition, such as polyglandular autoimmune syndromes, hypercoagulable states, or systemic infections (Table 9.1). Side effects of

chronic glucocorticoid treatment are common, including low bone mass, cognitive disturbances, and metabolic derangement [42]. In many countries, patients with adrenal insufficiency are ineligible for employment in fields with high risk for injury, such as police, firefighting, military service, and some types of construction. Life insurance policies can be difficult or impossible to obtain aside from group coverage through an employer. Consequently, the diagnosis of adrenal insufficiency should be absolutely certain before becoming a permanent fixture of the medical record. In the acute setting, when treatment takes priority over laboratory testing, presumptive treatment might be unavoidable; however, the treatment can be tapered in the outpatient setting once the patient is well to allow definitive testing shortly after the acute event has resolved.

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Chapter 10
Adrenal Cushing's Syndrome: Updates on Overt and Mild Hypercortisolism

Jose Sanchez Escobar, Aarti Ravikumar, and Alice C. Levine

Introduction

Although the typical description of adrenal hypercortisolism remains clinically unmistakable, the introduction of the concept of mild hypercortisolism has led to a shift in the approach to the disease's management. Similarly, the use of more frequent abdominal imaging has led to increased recognition of adrenal incidentalomas (AIs).

Many of these AIs exhibit a wide range of abnormalities in cortisol secretion. Retrospective and prospective data suggests that even small degrees of hypercortisolism, and indeed the mere presence of an adenoma, may be associated with an increased risk of death. It has been proposed that labeling mild hypercortisolism as "subclinical" might not accurately reflect the clinical risks to the patient, given the degree of substantial pathology associated with this condition. Recent studies demonstrate the negative impact of mild hypercortisolism particularly on cardiovascular disease. This knowledge has led to changes in the diagnostic and therapeutic recommendations, although significant controversy remains. These reports have exposed the pitfalls associated with the current tools used for the diagnosis of cortisol excess due to adrenal disease.

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When overt hypercortisolism is confirmed due to adrenal adenoma or carcinoma, the management is generally surgical removal. Advancements in surgical techniques have led to improvements in the prognosis for these patients. When appropriate, laparoscopic adrenalectomy has significantly reduced the morbidity that was typically associated with an open approach, reducing perioperative complications and mortality. In spite of this progress, research is still needed to tackle the issue of persistent morbidity and increased mortality, even after remission.

Current medical approaches for the treatment of hypercortisolism due to benign adrenal adenomas include the use of adrenal enzyme inhibitors or glucocorticoid receptor blockade. Most of the clinical studies of the available pharmacologic therapies have been carried out in patients with overt adrenal hypercortisolism; more data is presently needed in the subgroup of patients who exhibit mild hypercortisolism only. As our understanding of the pathophysiology, genetic determinants, and sequelae of mild and overt adrenal hypercortisolism advances, new therapeutic options may emerge and treatment may be better individualized for maximal patient benefit.

Definitions

Adrenal Cushing's syndrome is defined as a syndrome of glucocorticoid excess arising from an adrenal source, independent from adrenocorticotropic hormone (ACTH) secretion and control from the hypothalamic-pituitary axis.

Cushing's syndrome from adrenocortical pathology often presents in the setting of an *adrenal incidentaloma* (AI). These are defined as adrenal masses seen on radiological imaging that is ordered for other indications. AIs can be further classified into benign adrenal adenomas, metastatic lesions, adrenal carcinomas, and pheochromocytomas.

Benign adrenal adenomas are typically <4 cm in size with noncancerous imaging characteristics. On noncontrast CT and in-phase and out-of-phase MRI, benign adrenal adenomas are intracellularly lipid rich. On noncontrast CT, this correlates to a density of <10 Hounsfield units (HU). Contract CT studies can also be used to distinguish benign adrenal adenomas, by examining their contrast washout kinetics. A benign adenoma will have a washout of >50% after administration of contrast.

Overt hypercortisolism is defined as the presence of typical clinical features of Cushing's syndrome (e.g., moon facies, striae, and proximal muscle weakness), in addition to two abnormal results in tests designed to objectively identify glucocorticoid excess [1].

In contrast, *mild hypercortisolism* (also known as subclinical Cushing's syndrome) exhibits biochemical evidence of cortisol excess without the typical physical stigmata. Many of these patients may also have complications from excess cortisol such as the metabolic syndrome, diabetes, or hypertension. Unfortunately, there is no consensus on the biochemical definition of mild hypercortisolism, and there has been disagreement over what diagnostic thresholds to use.

Laboratory reference ranges for the following markers of adrenal function may vary between different laboratories and techniques:

   AM cortisol, 5–25 μg/dL (137.9–689.7 nmol/L)*
   Cortisol after dexamethasone suppression test, <1.8 μg/dL (<50 nmol/L)*
   Adrenocorticotropic hormone, 10–60 pg/mL (2.2–13.2 pmol/L)*
   24 h urinary free cortisol, 4–50 μg/24 h (11–138 nmol/L)*
   Midnight salivary cortisol, <0.09 μg/dL, <90 ng/dL (<3.6 nmol/L)*

*Conventional units are listed first, followed by SI units in parenthesis. μg/dL to nmol/L conversion factor = 27.8.

Epidemiology of Adrenal Hypercortisolism

Overt Hypercortisolism

Overt Cushing's syndrome from both ACTH-dependent and ACTH-independent causes remains a relatively rare malady, with a peak incidence of around two cases per million people/year [2].

Only 20–27% of cases of non-iatrogenic cortisol excess are thought to derive from adrenal etiologies. In a European registry of 481 patients with Cushing's syndrome from any etiology, the disease was more predominant in women (4:1 ratio). Patients with adrenal hypercortisolism tended to be older when compared to patients with other causes of hypercortisolism [3].

Articles focusing on florid Cushing's syndrome from adrenal causes give varying incidence rates according to the studied population and diagnostic method used (~0.2–0.6 new cases per million people/year) [4–6].

Case registry studies from large populations have estimated a total of around 40–80 cases of adrenal hypercortisolism per million people/year [7]. Of note, one of these cohorts followed in Spain showed linear increments in the prevalence of overt hypercortisolism during the study period of 1974–1992 [5]. This rise in the prevalence of the disease was not seen on a nationwide Danish registry that, aside from showing a higher prevalence of 79 cases per million people/year, benefitted from the fact that all citizens from Denmark hold a unique identification number, making the subject's information and medical history easier to retrieve. This same registry revealed 37 cases of overt hypercortisolism secondary to benign adrenal adenomas (75% from a total of 48 patients with an adrenal cause behind their cortisol excess); only 11 individuals were found to have adrenocortical carcinomas (ACC) [4].

Interestingly, both overt and mild hypercortisolism appear to be significantly more prevalent when populations with a high pretest probability of the disease were screened, such as individuals with diabetes or osteoporosis. Measurements as high as 5–10% were found when looking at these high-risk populations, although incidence and prevalence reports tended to be variable [8–10].

Mild Hypercortisolism

By far, the most common secretory syndrome stemming from AIs is subclinical Cushing's syndrome. Adrenal incidentalomas are estimated to be much more prevalent than adrenal hypercortisolism, with an increased probability of discovery seen with advancing age. The likelihood of detecting an AI in patients between the ages of 20–29 is approximately 0.2%, as opposed to 7% in a patient older than 70 years of age [11]. The majority of adrenal adenomas are nonfunctioning [12]. Benign adrenal adenomas comprise nearly 70% of AIs seen in the clinical setting. Previously, Cushing's syndrome was estimated to be present in 4–5% of patients with benign adrenal adenomas. However, in more recent studies that also include mild hypercortisolism, the prevalence is thought to be closer to 30% [13].

Morelli et al. studied an Italian cohort of 206 patients with mild adrenal hypercortisolism over a median of 6 years. They found that the size of the adenoma correlated with the cortisol after LDDST. The authors determined that the best cutoff size for an adenoma to predict the development of subclinical hypercortisolism was 2.4 cm [14]. A longitudinal cohort of 118 patients with AIs showed that the cumulative risk of mass enlargement was progressive for up to 8 years but globally low (only 25% of benign masses exhibited any growth) [15].

A recent retrospective analysis performed by Pasternak et al. [16] at UCSF compared unilateral versus bilateral adrenal adenomas. All adrenal incidentalomas were screened for primary aldosteronism, pheochromocytomas, and hypercortisolism. Mild hypercortisolism was defined as any of the following: an AM cortisol after LDDST >1.8 μg/dL and two 24 h UFC >50 μg/dL or multiple ACTH levels of <10 pg/mL. One hundred thirty-five AIs were identified, of which 23 were bilateral lesions. The authors found that 5/23 (21.7%) of bilateral adenomas exhibited mild adrenal hypercortisolism as compared to 7/112 (6.3%) of unilateral lesions. Another prospective long-term follow-up study performed by Vassilatou et al. in 77 patients with AIs found that hypercortisolism tended to be found more often in bilateral masses and adenomas with an annual growth rate of >0.5 cm. Of note, they defined subclinical disease as a post-dexamethasone suppression test cortisol level >50 nmol/L, in addition to one more abnormal test suggestive of cortisol excess (either ACTH <1.54 pmol/L, abnormal midnight serum cortisol, or 24 h urinary free cortisol >248 nmol/L) in the absence of clinical signs of Cushing's syndrome [17].

Although mild hypercortisolism remains the most frequently observed hormonal hypersecretion syndrome detected in patients with AIs, few of these patients go on to develop signs and symptoms of overt hypercortisolism. A large cohort of 284 individuals with AIs showed that for masses with subclinical glucocorticoid overproduction, the estimated risk of developing overt Cushing's syndrome was low at 12.5% after 1 year. All other nonfunctioning adenomas and AIs with mild hypercortisolism remained as such on follow-up (median 56 months, range 1–12 years) [18].

This phenomenon has been observed in other prospective longitudinal studies, where either no progression or low rates of conversion to overt hypercortisolism were shown. In the same cohort followed by Vassilatou et al., none of the 77 patients

with AIs progressed to overt hypercortisolism, including 20 subjects with preexisting mild disease [17]. In the study performed by Giordano et al. [15], only 2 out of 24 individuals from an Italian cohort of 118 AIs showed conversion to overt hypercortisolism when followed for about 5 years; no correlation between hormonal conversion and adenomatous growth was observed.

Pathophysiology

Historically, benign adrenal hypercortisolism has been subdivided as either arising from adrenocortical tumors or from bilateral micronodular/macronodular adrenal hyperplasia. However, recent discoveries into the molecular mechanisms behind certain somatic and germline mutations have led to a greater understanding of the pathophysiology behind these various diseases (see Table 10.1).

It is now understood that while some gene mutations may primarily lead to tumor growth, others may prominently lead to excessive steroidogenesis [19]. As epidemiological data has shown that the majority of cases of mild hypercortisolism do not progress to overt disease, it has been hypothesized that some mutations might preferentially lead to subclinical disease, while others lead to greater cortisol overproduction and the typical phenotype of overt Cushing's syndrome [15, 17].

In the case of adrenocortical tumors, most seem to arise in a sporadic manner, with a minority of cases manifesting in the setting of neoplasia-prone hereditary syndromes (e.g., multiple endocrine neoplasia 1, McCune-Albright syndrome, and

Table 10.1 Biochemical and genetic differences between mild and overt diseases

	Mild hypercortisolism	Overt hypercortisolism
Diagnostic tests	Sensitivity/specificity	
DST[a]	75–100%/67–72%	100%/91%
UFC[b]	36%	97%/91%
MSC[c]	82%/60%	92–100%/93–100%
	Frequent mutations[d]	
Genetic mutations	CTNNB1 (16%) ARMC5 (55% of BMAH)[e]	PRKACA (35–69%) GNAS (5–17%) PRKAR1A (66% of PPNAD)[f]

[a]1 mg dexamethasone suppression test (DST): positive results defined as a failure of suppression of the plasma cortisol level to <1.8 μg/dL
[b]24 hour urinary free cortisol (UFC) by liquid chromatography-tandem mass spectrometry. Positive results defined as a value >55 mcg/day
[c]Midnight salivary cortisol (MSC) by tandem mass spectrometry. Positive results defined as a value >1 ng/dL (which may not apply to the elderly population)
[d]Reported frequency of genetic mutations in adrenocortical tumors presented in parenthesis
[e]*BMAH* bilateral macronodular adrenal hyperplasia. Course may be insidious and overt hypercortisolism can be seen with very large nodules
[f]*PPNAD* primary pigmented nodular adrenocortical disease

Carney complex) [20]. Mutations at various levels of the cAMP/protein kinase A (PKA) signaling pathway have recently been singled out as major contributors to adrenal Cushing's syndrome. The most common somatic activating mutations in these adenomas are those found in the catalytic subunit of PKA (PRKACA gene) and the G-protein alpha subunit (GNAS gene). These mutations have been reported in up to 35–69% and 5–17% of all sporadic adrenocortical adenomas causing overt Cushing's syndrome, respectively [21–23].

Aside from mutations affecting the cAMP/PKA pathway, alterations in Wnt β-catenin signaling have also been reported frequently in cortisol-secreting adenomas. Sixteen percent of studied adenomas in a tissue repository were found to harbor mutations in the CTNBB1 gene, which is responsible for encoding β-catenin [24].

Tumors that contain the PRKACA and GNAS mutations tend to be smaller and linked to overt disease, while neoplasms exhibiting mutations in the CTNBB1/Wnt β-catenin have more ineffective steroidogenesis while favoring tumoral growth. This phenomenon was confirmed in an exome sequencing study of 25 tumor-normal pairs, as only adenomas with PRKACA/GNAS mutations were linked to overt disease and earlier diagnosis (13/16 with *PRKACA* or *GNAS* mutation with overt hypercortisolism versus 16/39 without, $p = 0.008$). Of note, PRKACA/GNAS and CTNBB1 mutations appeared to be mutually exclusive, supporting the hypothesis of a distinct genetic precedent between the different phenotypes of mild vs. overt adrenal hypercortisolism [25].

Diagnosis

According to the 2008 Endocrine Society guidelines for the diagnosis of Cushing's syndrome, a thorough medical history and physical exam should be performed on all subjects suspected of having endogenous hypercortisolism. Special attention should be paid to any possible exogenous steroid exposure, as glucocorticoid intake remains the most common cause of pathologic hypercortisolism. Additionally, reviewing the patient's medications is crucial, as drugs that either induce or inhibit CYP3A4 metabolism (e.g., certain antiepileptics or itraconazole, respectively) or increase cortisol-binding globulin (such as estrogen-containing contraceptives) may lead to false-negative or false-positive results [1].

Three tests may be used to make the diagnosis of hypercortisolism in general: the *low-dose dexamethasone suppression test*, a *24-hour urinary free cortisol*, and a *midnight salivary cortisol*. These tests aim to identify the evidence of failure to suppress cortisol production in spite of ACTH suppression, the presence of excess production of cortisol, and the loss normal diurnal variation in cortisol secretion, respectively (see Table 10.1).

The *low-dose dexamethasone suppression test* (LDDST) is the standard exam used for ruling out hypercortisolism from any cause. When using a LDDST, suppression of the plasma cortisol level to <1.8 µg/dL has the best negative predictive value and overall best sensitivity to rule out hypercortisolism (100%) [26].

Several dexamethasone suppression tests are available for confirming the presence of hypercortisolism (with either a low-dose 1 mg overnight test or the 2-day 2 mg test). The 2-day, 2 mg LDDST has been postulated as a superior screening tool for Cushing's syndrome. The test is performed by taking 0.5 mg of oral dexamethasone every 6 h (usually starting at 8 a.m.) and measuring serum cortisol and dexamethasone at 8 a.m. on day 3. A retrospective study of 245 patients with pituitary Cushing's syndrome found the test to correctly identify hypercortisolism in 98% of cases [27]. Although this test retains excellent sensitivity while exhibiting better specificity when compared to the standard LDDST, it requires greater effort from the patient, as the frequency of dosing may be too cumbersome for some. Being a synthetic corticosteroid, dexamethasone does not interact with the antibody immunoassays currently in use and is therefore the glucocorticoid of choice when trying to suppress endogenous cortisol production.

Infrequently, dexamethasone may be poorly absorbed or excessively metabolized, such as in the case of concomitant use of CYP3A4 altering drugs (inducers such as rifampicin and anticonvulsants, inhibitors such as protease inhibitors and azole antifungals) [28]. The simultaneous measurement of the dexamethasone level during LDDST is critical in order to verify adequate serum concentrations. An ideal serum dexamethasone level should be ~200–650 ng/dL [29]. Hormonal contraceptive use in women also requires special consideration, as estrogen has been found to increase cortisol-binding globulin (CBG) levels and therefore potentially raise total cortisol levels and render the dexamethasone suppression test uninterpretable [30]. It is highly recommended to postpone the biochemical evaluation of adrenal hyperfunction until after cessation of hormonal contraceptives, given the risk of false-positive results.

The excellent negative predictive value of the LDDST comes an increased risk of false-positive results; therefore, a positive test should be followed by additional confirmatory testing, including the collection of 24 h urine free cortisol and a midnight salivary cortisol assay. Given the variable nature of Cushing's syndrome, two measurements of each of these tests are recommended when confirming the diagnosis.

The use of *24-hour urine free cortisol* (UFC) measurement is a unique way of quantifying global glucocorticoid exposure in the tissues, primarily reflecting serum cortisol not bound to CBG or other proteins. This is ideal under conditions where CBG abnormalities may be suspected, such as during pregnancy where higher CBG levels are to be expected or during conditions with protein wasting such as the nephrotic syndrome, where lower CBG levels might lead to false-negative results. Structure-based analysis such as tandem mass spectrometry is preferred over immunoassays, as this eliminates the risk of interference from cortisol precursors and metabolites [31].

Care must be taken when interpreting mildly elevated 24 h UFC values (<2 times the upper limit of normalcy), as false-positive results may be seen in conditions such as pseudo-Cushing's or physiologic hypercortisolism [32]. Other disorders such as renal insufficiency, polydipsia (water intake >5 L daily), or polyuria (urine output >3 L over 24 h) may also decrease the accuracy of the test; when present,

other screening methods should be favored. A study by Mericq et al. on normal subjects showed that high fluid consumption caused the mean UFC values to increase (126 ± 33 (SD) vs. 77 ± 18 μg/day, $p < 0.005$). Additionally, more samples exceeded the upper limit of normalcy of 95 μg/day (23/30 vs. 6/30, $p < 0.005$) [33].

Midnight salivary cortisol testing is a convenient way of looking for the loss of diurnal variation in cortisol secretion, which can be easily performed in an outpatient setting. As with the other available tests for evaluating hypercortisolism, late-night salivary cortisol measurements may be influenced by factors such as age, sex, weight, and sleep habits of the individual as well as the presence of cyclical cortisol secretion in certain adenomas [34, 35].

Once endogenous hypercortisolism has been diagnosed biochemically, a morning ACTH should be measured. A suppressed level (<10 pg/mL) establishes independence from pituitary control and the absence of an ectopic ACTH source as the cause of hypercortisolism, confirming the diagnosis of an adrenal cause. However, in some patients with mild adrenal hypercortisolism, the degree of excess cortisol production may not be sufficient to suppress the hypothalamic-pituitary-adrenal (HPA) axis, and a low-normal morning ACTH level may be seen. In these cases, an ACTH can be checked after LDDST. If the ACTH level is not suppressed in the setting of a therapeutic dexamethasone level, other causes such as pituitary or ectopic ACTH overproduction should be considered.

Other labs may also be supportive of the diagnosis, including a low dehydroepiandrosterone (DHEA) sulfated (DHEA-S) level. A low DHEA-S (<40 μg/dL) has been found to have a sensitivity of 68%, specificity of 75%, PPV of 43%, and NPV of 90%. This decrease may be less clinically impactful in the elderly population, as serum DHEA and DHEA-S are known to decrease with aging [36].

Overt Hypercortisolism

Patients with overt Cushing's syndrome typically exhibit clinical features that best discriminate from other conditions. In this setting of high pretest probability of disease, tests that favor a high degree of specificity over sensitivity are preferred as "rule-in" tools to diagnose hypercortisolism. The 24 h urine free cortisol (when obtained appropriately and multiple times) arguably has the best accuracy with more severe forms of cortisol excess, particularly when the value is higher than three times the upper limit of normal [1]. A meta-analysis looking at 646 individuals with confirmed disease found that a patient with an abnormal 24 hour UFC result had a high likelihood of having endogenous hypercortisolism (LR 10.6, 95% CI 5.5–20.5) [37].

There is also data demonstrating that midnight salivary cortisol measurements are very sensitive and specific for establishing the diagnosis of overt Cushing's syndrome. In a study assessing the predictivity of the test in 13 patients with overt disease and 14 patients with mild hypercortisolism, late-night salivary measurements at a threshold of 12 nmol/L displayed a 100% sensitivity and specificity for the diagnosis of overt Cushing's syndrome [38]. Of note, the cohort came from a mixed setting (both outpatient- and hospital-based measurements).

Given its ease of use and widespread availability, a LDDST still remains an essential exam to rule out pathological cortisol secretion. As mentioned previously, the test retains an excellent degree of sensitivity in the setting of overt cortisol excess. While ideal as a screening tool, LDDST carries some pitfalls. There have been retrospective reports of cortisol suppression to <2 μg/dL with established Cushing's syndrome; a single center cohort examined by Findling et al. showed a minority (6 out of 80 patients) to exhibit this response to a LDDST [39].

Mild Hypercortisolism

As mentioned previously, controversies as to the biochemical definition and the diagnostic parameters needed to identify the presence of subclinical hypercortisolism remain. The LDDST has been shown to be the most sensitive test for the detection of mild adrenal hypercortisolism. The morning serum cortisol value after 1 mg dexamethasone the night before that is considered diagnostic of mild hypercortisolism has been debated; historically, literature suggested that >5 μg/dL (>138 nmol/L) after LDDST was diagnostic. However, more recently authors have proposed that the traditional threshold after LDDST, a morning cortisol of >1.8 μg/dL (>50 nmol/L), be employed to establish the diagnosis of mild hypercortisolism. There are others who suggest using a morning cortisol of >3 μg/dL (>80 nmol/L), a value to help balance between sensitivity and specificity. The discrepancy arises because of the difference in clinical index of suspicion between Cushing's syndrome and mild hypercortisolism. In patients being screened for symptomatic Cushing's syndrome, there is a high pretest probability of disease, so the likelihood of a false-positive result with a morning cortisol of >1.8 μg/dL is low. However, patients with mild hypercortisolism do not have the typical stigmata so the clinical index of suspicion is lower, and therefore a test with greater specificity may be preferable despite the loss of sensitivity.

As mentioned previously, a morning serum cortisol after LDDST of >5 μg/dL has a high specificity (83–100%) but a relatively low sensitivity (44–58%). In contrast, a morning serum cortisol >1.8 μg/dL after LDDST has a high sensitivity (75–100%) but a lower specificity (67–72%). Given the low specificity, there is a risk of false-positive values and overdiagnosis when using the cutoff of 1.8 μg/dL.

The use of midnight salivary cortisol levels has also been evaluated to diagnose adrenal mild hypercortisolism. However, its utility seems limited. One study that compared the MSC concentrations from patients with adrenal adenomas and known mild adrenal hypercortisolism to those with adrenal adenomas without hypercortisolism found that there was no difference. Other similar studies have replicated this outcome.

A 24 hour UFC level may also be elevated; mild increases of up to two times the upper limit of normal may support the diagnosis. However, many patients with mild hypercortisolism have normal UFC levels. As with the use of midnight salivary cortisol, mildly elevated 24 h UFC levels can help support the diagnosis of mild adrenal hypercortisolism, but normal levels do not rule out the disease.

Sequelae

Morbidity/Mortality in General

The presence of hypercortisolism, from any cause, induces a constellation of complications that ultimately impact life expectancy (see Fig. 10.1). The increase in mortality associated with hypercortisolism persists even after biochemical remission. A retrospective review of the entire population of Denmark from 1980 to 2010 found that of a cohort of 343 cases of mixed adrenal pituitary hypercortisolism, 74 patients died. The risk of death was double that of age-matched subjects with similar clinical characteristics. This effect was most pronounced 1 year after diagnosis, where the hazard ratio for death jumped to 5.2 (95% CI 2.7–9.7) when compared to the population without Cushing's syndrome. The rates were identical when adjusted for an adrenal or pituitary cause [40].

After treatment and induction of remission, death due to cardiovascular causes continued to be higher in patients with adrenal hypercortisolism. In a cohort from New Zealand, long-term follow-up showed a HR of 1.6 (95% CI 1.3–2.1). This is striking, given that their case series revealed high biochemical cure rates of >90% with surgery. In this same retrospective analysis, the most commonly reported causes of death after exclusion of ACC were 19% from ischemic heart disease,
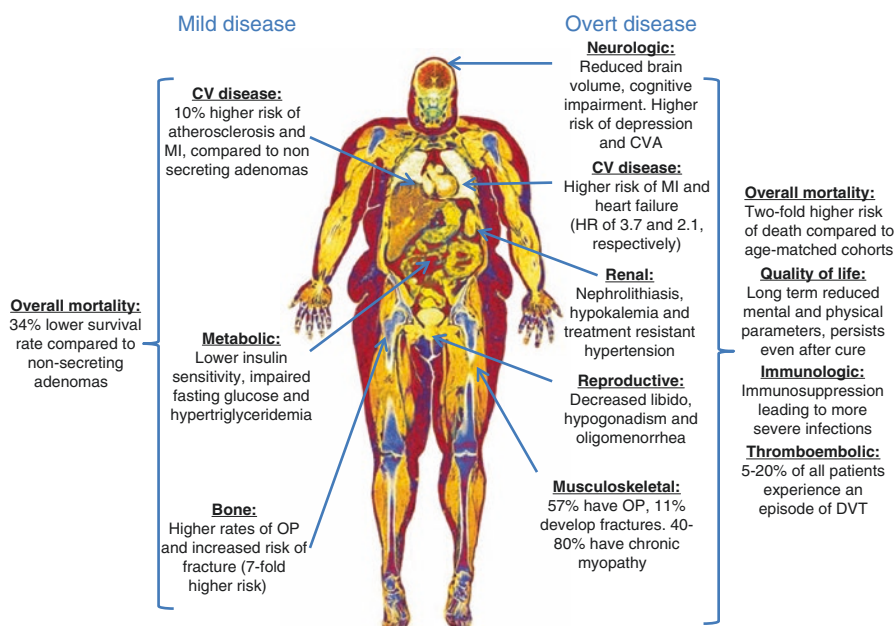
Fig. 10.1 Clinical consequences of mild and overt hypercortisolism. *MI* myocardial infarction, *CV* cardiovascular, *OP* osteoporosis, *CVA* cerebrovascular accident, *HR* hazard ratio, *DVT* deep vein thrombosis

17% from stroke, 17% from sepsis (including pneumonia), and 11% from pulmonary embolism [41].

The duration of cortisol exposure appears to correlate with the deleterious long-term effects on morbidity and mortality even after biochemical cure. An analysis performed by Lambert and Geer et al. of 346 patients after curative treatment for Cushing's disease showed a positive correlation between the duration of glucocorticoid exposure and death ($p = 0.038$) [42]. As it is difficult to ascertain the duration and degree of cortisol excess, particularly in patients with mild hypercortisolism, earlier intervention would be desirable.

The damages inflicted by adrenal Cushing's syndrome are widespread and exert a significant impact in quality of life (see Fig. 10.1). The most commonly reported comorbidities are cardiovascular disease (CVD), venous thromboembolism (VTE), cerebrovascular accidents (CVA), infections secondary to immunosuppression, neuropsychiatric disorders, and musculoskeletal diseases [43, 44]. Similar to the mortality outcomes, the risk for the development or deterioration of these conditions persists even after the resolution of hypercortisolism through surgical means [45, 46].

Taken together, it is no surprise that adrenal hypercortisolism leads to long-term impaired quality of life. Data from a 10-year registry of 123 patients with Cushing's syndrome showed that all mental and physical parameters of quality of life remain impaired, even after curative treatment, albeit with moderate improvements in the physical markers of disability [47].

Cardiovascular Disease

Under physiologic conditions, cortisol regulates metabolism and energy expenditure. Excessive cortisol secretion leads to a series of metabolic derangements, which ultimately cause significant impact on the cardiovascular system [48]. Glucocorticoids, by direct and indirect measures, promote changes in lipid and glucose utilization in myocytes and adipocytes. These changes lead to increased insulin resistance, adipocyte proliferation, and lipogenesis, which are prominent features of the metabolic syndrome [49, 50].

Hypertension, an almost universal feature of adrenal hypercortisolism, is an important contributor to the risk of cardiovascular disease. Initially, patients with CS may only have blunting of the physiologic dip in nocturnal blood pressure [51]; however, a recent study demonstrated that long-standing hypercortisolism may lead to treatment-resistant hypertension that may persist even after adrenalectomy [52]. The main mechanism underlying hypercortisolism-induced hypertension involves activation of the mineralocorticoid receptor by cortisol. Under physiologic conditions, cortisol is inactivated to cortisone in the kidney by 11-beta-hydroxysteroid-dehydrogenase 2 (11β-HSD 2). However, in the presence of very high cortisol levels, the ability of 11β-HSD 2 to inactivate to the metabolite is overwhelmed, leading to cortisol activation of mineralocorticoid receptors in the kidney, with resultant sodium reabsorption and potassium excretion.

The convergence of hypertension, impaired glucose metabolism, and dyslipidemia leads to pro-inflammatory changes in the endothelium, which results in widespread atherosclerotic buildup and cardiovascular disease [53]. Other cardiovascular effects from chronic hypertension and hypercortisolism include left ventricular hypertrophy and myocardial fibrosis, which in turn may lead to heart failure [54]. Most patients with adrenal hypercortisolism share a particular phenotype of the metabolic syndrome, featuring abdominal obesity and visceral fat accumulation. Visceral fat can be very metabolically active, overproducing adipokines (as well as other pro-inflammatory cytokines), which further increases the risk of the metabolic syndrome [55].

As discussed previously, cardiovascular disease is the main cause of the increased mortality seen with *overt adrenal hypercortisolism*. Acute myocardial infarction, cerebrovascular accidents, and heart failure are all much more common with overt Cushing's syndrome (HR 3.7 95% CI 2.4–5.5, HR 2.0 95% CI 1.3–3.2, and HR 6.0 95% CI 2.1–17.1, respectively) [40]. Retrospective data from iatrogenic hypercortisolism reveals that the occurrence of cardiovascular complications exhibits a dose-dependent association with the amount of glucocorticoids prescribed and the duration of exposure. Patients with continuous use >6 months and who received a total glucocorticoid dose ≥7.5 mg of prednisolone were found to have an absolute risk difference of 6.9 more cardiovascular events per 1000 person-years, when compared to unexposed individuals. The magnitude of effect was greater with increasing steroid doses [56].

This dose-response effect seen with exogenous glucocorticoids supports the concept that *mild adrenal hypercortisolism* produces cardiovascular disease, as even small amounts of endogenous excess cortisol over time will likely lead to similar effects on the endothelium.

Recent epidemiological data supports this argument. In a retrospective analysis of 198 patients with AIs followed over 15 years by Di Dalmazi et al., *mild hypercortisolism* was found to be associated with an increased risk of cardiovascular disease. They categorized the patients as stable nonsecreting (cortisol level <1.8 µg/dL after LDDST; $n = 114$), stable intermediate (cortisol 1.8–5 µg/dL after LDDST), or subclinical Cushing's (cortisol level >5 µg/dL after LDDST; $n = 61$). In addition, 23 patients had worsening of their hypercortisolism over the course of the study. The reported incidence of cardiovascular events was higher in the patients with stable intermediate or subclinical Cushing's (16.7%; $p = 0.04$) and also with worsened secreting patterns (29.4%; $p = 0.02$) compared to those with stable nonsecreting adenomas (6.7%) [13].

Androulakis and colleagues recently published a case-control study which also demonstrated an association between mild hypercortisolism and increased cardiovascular risk. There were three groups: cortisol-secreting AIs (CSAIs), nonfunctioning AIs (NFAIs), and healthy controls. All patients with AIs had no known cardiovascular risk factors (hypertension, hyperlipidemia, or diabetes). CSAIs were defined as a morning cortisol after LDDST +2 standard deviations greater than the control group, which translated to a cortisol level >1.09 µg/dL, a value lower than previously suggested cutoffs. The authors measured intimal media thickness (IMT)

and flow-mediated vasodilatation (FMD), two measures associated with atherosclerosis and cardiovascular risk. The IMT was greater and FMD less in the subjects with CSAIs compared with NFAIs [57].

DeBono and colleagues have also found an association between mild hypercortisolism and an increase in cardiovascular disease and mortality. In a retrospective study of patients with adrenal adenomas, patients were categorized by their cortisol levels after LDDST. Cortisol levels of >1.8 μg/dL after LDDST were associated with decreased survival, and 50% of the deaths in that group were from cardiovascular causes [58].

As mentioned previously, the most common definition of a normal cortisol suppression following a DST is <1.8 μg/dL. However, a ROC analysis performed by Morelli et al. in search of the ideal cortisol suppression level to predict a cardiovascular event found the ideal threshold to range between 1.5 and 2.0 μg/dL [14].

Musculoskeletal Disorders

Adrenal hypercortisolism affects both the muscle and bone. This not only leads to the typical phenotype of reduced muscle volume and strength but significantly impacts quality of life and balance and may ultimately lead to an increased risk of falls and osteoporotic fractures [41, 46].

Hypercortisolism may cause myocyte damage in both an acute and chronic presentation. Acute myopathy, although uncommon, can be life threatening, as it leads to widespread and rapid involvement of the proximal, distal, and even respiratory muscles [59]. Chronic myopathy involves proximal muscles primarily, causing predominant weakness in the muscles of the pelvic girdle. This presentation is quite prevalent among patients with Cushing's syndrome, with rates reported between 40 and 80% [60]. The main mechanisms of muscular damage are by impairment of protein synthesis through decreased amino acid uptake, inhibition of myogenesis by downregulation of myogenin, and stimulation of proteolysis through the ubiquitin-proteasome system. In the end, hypercortisolism leads to the transformation of muscle fibers from fast to slow type and muscle atrophy [46].

At the level of the bone, glucocorticoid excess leads to a multifactorial insult that can cause loss of bone density and fragility fractures [61]. Hypercortisolism leads to impairments in osteoblastogenesis as well as apoptosis of osteoblasts and osteocytes. This causes a reduction in osteoblast function (primarily through inhibition of the Wnt β-catenin pathway) and a major impairment of trabecular bone formation, particularly in the vertebra. At the level of the osteocyte, even small doses of exogenous glucocorticoids have been found to induce autophagy; at higher doses and more prolonged exposure, they lead to apoptosis and disruption of bone turnover that is irreversible [61–63]. Aside from the effect on bone formation, cortisol excess also causes increased bone resorption by promoting osteoclastogenesis and upregulating colony-stimulating factors and the receptor activator of nuclear factor kappa-B ligand (RANK-L) [61].

The cumulative lifetime prevalence of low impact skeletal fractures among patients with *overt hypercortisolism* ranges between 11 and 76% of all patients [64]. Bone impairment is more prevalent among patients with adrenal disease than with other causes of overt Cushing's syndrome, with up to 64–100% showing some degree of bone disease. Although the most common manifestations are asymptomatic osteopenia (40–78%) and osteoporosis (22–57%), vertebral fractures can frequently be the initial finding in cases of undiagnosed CS [65, 66]. The reason for this excess risk of bone injury in patients with adrenal hypercortisolism, as opposed to ACTH-mediated causes, may be from the suppression of ACTH production from the pituitary. In a study by Zaidi et al., ACTH was found to lead to VEGF-mediated increments in osteoblast survival. Additionally, necropsy evaluation of rabbits exposed to depot methylprednisolone found that concomitant treatment with a cosyntropin infusion leads to a reduction in femoral trabecular osteonecrosis, a crucial step behind glucocorticoid bone damage [67].

The risk of osteoporosis from *mild hypercortisolism* has been documented in various studies. In 2009, Chiodini et al. published a multicenter, retrospective study done in Italy evaluating the bone mineral density (BMD), prevalence of vertebral fractures, and bone quality in patients with AIs with and without subclinical hypercortisolism (SH). SH in this study was defined as two out of three of the following: UFC >70 μg/24 h, morning cortisol >3 μg/dL after LDDST, and ACTH <10 pg/mL. The primary endpoint measured was the spinal deformity index (SDI), in which the number and severity of vertebral fractures are integrated. The SDI has been associated with vertebral fracture risk over time. The study showed that the patients with AIs (SH+) had lower BMD at the lumbar spine (trabecular bone) and femoral neck (cortical bone), more metabolic syndrome, and increased spinal deformity index compared to those with AIs (SH-) and controls (no AI). The odds ratio for vertebral fractures was 7.27 ($p = 0.0001$) for patients who were SH+ regardless of age, MD, menopause status, and gender.

More recently in 2011, the same group conducted a multicenter, prospective longitudinal study evaluating SDI and vertebral fractures at baseline and after 12 and 24 months in patients who were SH+ and SH− with AIs. The results were similar to those of the retrospective study. The authors reported that the prevalence of fractures and SDI was higher in the SH+ group regardless of age, sex, BMI, MBD, and menopausal status. Those who were SH+ had a higher risk of vertebral fractures than those who were SH− with an odds ratio of 12.264 ($p = 0.001$) [68].

Immunosuppression

Adrenal hypercortisolism directly impacts the innate immune system, both by affecting its humoral and cellular responses. The humoral immune system is primarily affected through downregulation of pro-inflammatory cytokines and blunted lymphocyte proliferation. The adaptive component of the immune response is also impaired, as evidence has shown glucocorticoid excess also leads to inhibition of

maturation and proliferation of antigen-presenting cells and T-helper lymphocyte subclasses 1 and 2 (mediators of the cellular and humoral immune response, respectively). These effects lead to decreases in the production of IgG and IgE immunoglobulins, which decreases the ability of the immune system to recognize and ultimately opsonize and kill pathogens [69]. The cellular immune response is impaired both by the previously mentioned selective downregulation of Th1 lymphocytes and by directly impairing the action, maturation, and proliferation of neutrophils, monocytes, eosinophils, and macrophages [70]. The final result is an increase in susceptibility toward intracellular pathogens and opportunistic infections [71–73].

Although immunosuppression is the major consequence of glucocorticoid excess during the active phase of hypercortisolism, an increased susceptibility to autoimmunity has also been documented after curative treatment. This phenomenon is poorly understood, although it may involve a rebound in the immune response after immunosuppression, as well as a selective Th1/Th2 lymphocyte imbalance [43]. The degree of immunosuppression is determined both by the length and severity of hypercortisolism. A systematic review of eight cohorts of patients with Cushing's syndrome showed a HR of 2.4 (95% CI 1.0–5.9) for the risk of infection, 3 years before diagnosis; the risk peaked in the immediate postoperative period, with a HR of 38.2 (95% CI 16.9–86.1). Therefore, infections are important determinants of perioperative death [40].

The most commonly reported Gram-positive infections are those caused by *Staphylococcus*, *Streptococcus*, and *Listeria* pathogens. The most commonly reported Gram-negative infections are those from members of the *Enterobacteria* and *Legionella* family of organisms. Community-acquired and nosocomial infections in hypercortisolemic patients tend to present in more severe and invasive forms. Fungal (e.g., *Candida* and *Aspergillus*) and viral (e.g., herpes zoster, CMV, and influenza) infections also tend to be more severe and common [69, 71].

Thromboembolic Disease

The pro-thrombotic effect of hypercortisolism is twofold, both by inducing the activation of the coagulation pathway and by inhibition of fibrinolysis [74]. Prior research has shown that glucocorticoids mainly induce the activation of coagulation factors VIII and IX and vWF. Evidence of increased production of fibrinogen has also been found with hypercortisolism. Fibrinogen plays an important role both for the coagulation pathway and with primary hemostasis through mediation of platelet aggregation. Endogenous hypercortisolism may additionally influence other components of hemostasis, by acting through the upregulation of fibrinolysis and subsequent increased thrombin generation as well as by directly stimulating thrombocytosis and platelet function [75].

The effects from the many metabolic dysregulations with glucocorticoid excess also play a role in thrombosis. Factors such as hypertension, hyperhomocystein-

emia, and hyperglycemia with subsequent hyperinsulinemia and dyslipidemia indirectly influence hemostasis, by upregulating vWF and tissue plasminogen activatosr inhibitor 1/tPa concentrations and activating the extrinsic coagulation pathway [74]. A meta-analysis of 476 cases of pituitary and adrenal hypercortisolism showed a high rate of thrombotic events (1.9–2.5%) before surgery; postoperatively, the rate climbed to as high as 5.6% with a maximum incidence of 20% of subjects in one study [76].

Other Complications

Under physiologic conditions, cortisol exerts significant control in the regulation of many neuropsychiatric, reproductive, and dermatologic functions. Although epidemiologic data for *overt hypercortisolism* has clearly shown the impact of excess glucocorticoids in these systems, the influence of *mild hypercortisolism* on these organs has not yet been elucidated.

The effects of glucocorticoid excess on the nervous system are not completely understood. Deleterious events affecting neuroplasticity, the secretion of stimulatory neurotransmitters such as glutamate, and suppression of neurogenesis in the hippocampus have been postulated as causal factors [77]. The psychiatric disorders in patients with endogenous hypercortisolism can be quite debilitating and resistant to even curative treatment [40, 77]. The most common manifestation is a psychoactive/hypomanic form of major depression (50–81%), although anxiety (66%) and bipolar (30%) disorders are quite frequent as well [78]. Long-standing hypercortisolism, if left untreated, may ultimately lead to the loss of brain volume and neurocognitive impairment [79].

Adrenal hypercortisolism negatively affects both the reproductive and sexual health of men and women [2, 5]. Excess cortisol can block gonadotropin hormone release by reducing the amplitude and frequency of gonadotropin-releasing hormone production in the hypothalamus, while visceral adiposity may lead to abnormalities in the metabolism of sex steroids and sex hormone-binding globulin as well as androgen excess [80]. Hypercortisolism may also cause direct damage to the ovaries and testis, leading to reduced primordial follicles with cortical stromal fibrosis and Leydig cell impairment with tubular atrophy, respectively [80, 81]. These phenomena disrupt the hypothalamic-pituitary axis and ultimately may lead to hypogonadism and infertility [82]. Decreased libido, hypogonadism in men, and menstrual irregularities in women are common (24–90%, 50–70%, and 43–80%, respectively) [40]. Long-standing hypogonadism may also negatively impact bone health and increase the risk of osteoporosis, particularly in men [64].

Dermatologic manifestations are easily identifiable and quite prevalent among patients with overt Cushing's syndrome (~60–90%) [2, 5]. The main mechanism of skin damage is through glucocorticoid-mediated skin atrophy, mucopolysaccharide accumulation, and impairment of keratinocyte proliferation. Excess cortisol states

have been found to directly impair the cross-linking of collagen, thereby interfering with its synthesis and turnover in the dermis. The most commonly observed are facial plethora, easy bruising, acanthosis nigricans, and abnormally pigmented, purple striae. Hair manifestations including thinning and hirsutism are also common [83].

Treatment

The ultimate goal of any selected therapy is to reestablish normal cortisol secretion, with the hope of reducing or eliminating complications, improving quality of life, and normalizing the life expectancy of patients suffering from Cushing's syndrome (see Fig. 10.2). A multidisciplinary approach, including input from an experienced endocrinologist, surgeon, radiologist, and other medical subspecialties, is essential. Given the widespread sequelae from hypercortisolism, the management should include a thorough multi-system evaluation at diagnosis and throughout the course of the disease. This should include the assessment and treatment of cardiovascular, metabolic, musculoskeletal, psychiatric, and reproductive/sexual health. The patient and family members should be counseled on the disease process and, when possible, what to expect from treatment. Shared decision-making between the medical team and patient, with full knowledge of the risks and benefits of interventions, is highly recommended. Given the impact Cushing's syndrome can have on life expectancy and quality of life, the psychosocial needs of the patient and family should be addressed, and prompt referral to support groups, when available, is suggested.

Restablish eucortisolemia, reduce complications, improve survival and quality of life

- Patient and family counseling

- Referral to support groups and physical therapy

- Assesment and treatment of co-existing morbidities (cardiovascular, musculoskeletal, psychiatric)

Surgical adrenalectomy

- Standard of treatment when adenomatous lesion is present

- Laparoscopic adrenalectomy is favored

- High volume, experienced surgeons have better cure rates and lower surgical adverse events

- Improved survival and lowered consequences seen, however residual excess mortality remains

Medical treatment

- Effective in reducing effects of hypercortisolism in the short term, remains second line for long term management

- Limited use given concerns for side effects and drug-drug interactions

- Available modalities include steroidogenesis inhibitors and glucocorticoid receptor antagonists

Fig. 10.2 Overview of the goals of therapy and available treatment modalities in the management of adrenal hypercortisolism (Data adapted from Nieman LK, Biller BM, Findling JW, Murad MH, Newell-Price J, Savage MO, et al. Treatment of Cushing's syndrome: an endocrine society clinical practice guideline. J Clin Endocrinol Metab. 2015;100(8):2807–31)

Current clinical practice guidelines specify that management recommendations pertain to overt hypercortisolism. They defined "subclinical" disease as values <1.5 times the upper limit of normal [84]. Unfortunately, there is no consensus as to how best define mild hypercortisolism. This limits the applicability of present evidence in decision-making, as varying definitions are given in studies looking at both the diagnosis and treatment of mild disease. Given that the risk of death and complications appears to be positively correlated to the length of exposure to cortisol excess, this approach might lead to harm, particularly as the prevalence of mild hypercortisolism might be higher than expected in high cardiovascular risk groups [13, 42].

Widening the indications for treatment to include mild hypercortisolism might be preferred; however, this should be balanced by the risks of overtreatment. In certain scenarios, such as mild hypercortisolism without evidence of high-risk cardiometabolic features, biochemical monitoring might be an acceptable approach. Unfortunately, current evidence is lacking to provide a tailored approach to surveillance and treatment of mild hypercortisolism due to adrenal disease.

Surgery

Overt Hypercortisolism

When an adenomatous lesion is detected (in the presence of positive clinical and laboratory evidence of hypercortisolism), surgical resection of the mass is the gold standard of treatment (see Fig. 10.2). The introduction of minimally invasive laparoscopic adrenalectomy has dramatically reduced the rate of perioperative complications stemming from surgery. Mortality rates for overt disease have dramatically fallen over the last decades [40]. In retrospective surgical cohorts, the biochemical cure rate was significantly high as well, approaching 100% with experienced hands. As mentioned previously, unfortunately some residual increased risk of death due to prior hypercortisolism remains even after surgical correction [41, 42]. Features of the metabolic syndrome such as hypertension, obesity, and glucose intolerance typically improve or resolve, but up to 25% of cured patients may exhibit persistence of these high-risk conditions [41, 53]. Similar improvements in musculoskeletal and psychosomatic outcomes are seen, although a full return to baseline is usually blunted [63, 78].

The experience of the surgeon additionally plays a role in the rate of adverse events from adrenalectomy [85]. Surgeons with lower number of cases had more complications when compared to high-volume practitioners (18.2% vs. 11.3%, $p < 0.001$), and their patients had a longer postoperative length of stay (5.5 vs. 3.9 days, $p < 0.001$). Bilateral adrenalectomy is indicated in the presence of bilateral macronodular adrenal hyperplasia (BMAH) and primary pigmented nodular adrenocortical disease (PPNAD) when overt Cushing's syndrome is present, although permanent adrenal insufficiency is the inevitable outcome.

Mild Hypercortisolism

Accumulating retrospective and prospective data may point toward the effectiveness of prompt unilateral adrenalectomy in patients with mild hypercortisolism. These studies have shown improvements in a variety of cardiovascular and metabolic outcomes, although no definitive evidence toward a mortality benefit is currently known (see Table 10.2).

In a study performed by Chiodini et al., 108 patients with adrenal incidentalomas were evaluated for unilateral adrenalectomy. Fifty-five patients (23 with subclinical hypercortisolism and 22 without) exhibited statistically significant long-term improvements in weight loss, blood pressure, and fasting blood glucose. This benefit remained irrespective of baseline cortisol secretion. Of note, subclinical hypercortisolism was defined as more than two abnormalities of the following parameters: UFC >70 μg/24 h, cortisol after 1 mg DST >3.0 μg/dL, and ACTH <10 pg/mL [86].

A recent prospective longitudinal study also evaluated the effect of adrenalectomy on vertebral fractures in those with a unilateral adrenal adenoma and mild hypercortisolism. Fifty-five patients were offered surgery: 32 patients proceeded with surgery and 23 patients proceeded with conservative management. All patients had vitamin D repleted to >30 and calcium was given if intake was <1000 mg/day. DXA (lumbar spine and femoral neck) and an assessment for vertebral fractures were done at baseline and follow-up every 2 years. The authors found that >50% of the nonsurgical group had new vertebral fractures over the 2-year follow-up period as opposed to 9.4% of the surgical group. The surgical patients had a decreased risk of vertebral fractures regardless of age, sex, duration of follow-up, degree of hypercortisolism, lumbar DXA, and the presence of fractures at baseline [87].

Table 10.2 Recently performed studies highlighting the benefits of unilateral adrenalectomy in patients with mild hypercortisolism

	Mild hypercortisolism		
	Toniato et al.	Chiodini et al.	Salcuni et al.
Study type	RCT, 7-year follow-up	Retrospective, 29-month follow-up	Prospective, 40-month mean follow-up
Number of patients	23 surgeries; 22 controls	25 surgeries, 20 controls	32 surgeries, 23 controls
Criteria for surgery	DST >2.5 μg/dL + another HPA axis alteration + no signs of CS	2 out of 3 HPA axis alterations; DST >3 μg/dL	2 out of 3 HPA axis alterations + DST >3 μg/dL or DST >5 μg/dL
Reported A/E	No complications; no postoperative AI	None reported	None reported
Reported benefits	100% CR; DM2 improved in 62% of patients; HTN in 67%; obesity in 50%	Reductions in weight (32%), blood pressure (56%), and glucose levels (48%) improved	42.8% lower incidence of vertebral fractures

A/E adverse events, *RCT* randomized controlled trial, *DST* dexamethasone suppression test, *HPA* hypothalamic pituitary adrenal, *AI* adrenal insufficiency, *CR* curative rate, *DM2* diabetes mellitus type 2, *HTN* hypertension

Perioperative Management

In the immediate postoperative period, a high risk of thrombotic events may be seen. Incidence rates of having at least one major episode of deep vein thrombosis vary between 5 and 20%. Patients may require a tailored, extended antithrombotic regimen, even in the absence of acute thrombosis, particularly in cases with very high preoperative cortisol levels. The appropriate duration, type, and dose of thromboprophylaxis are not yet known. As most thrombotic events occur within the first 4 weeks of curative surgery, this period might be appropriate to offer anticoagulation coverage, although a minority of events may still occur up to 3 months after adrenalectomy [76].

Transient postoperative adrenal insufficiency is almost universal in overt hypercortisolism. A systematic review of 377 patients with overt Cushing's syndrome showed that, after adrenalectomy, 99.6% exhibited abnormal results in ACTH, serum cortisol, or 24 h UFC levels after surgery. Although less common in subclinical hypercortisolism, 65.3% of patients with mild disease exhibited long-term cortisol deficiency requiring replacement after surgical resection [88]. The possibility of contralateral adrenal atrophy and suppression of the HPA axis makes intra- and postoperative glucocorticoid replacement mandatory after adrenalectomy to avoid an adrenal crisis, although the ideal dose, duration, and velocity of titration are unknown. In the case of a unilateral adrenalectomy, since the remaining normal adrenal gland produces sufficient amounts of mineralocorticoids and catecholamines, lower and shorter regimens of glucocorticoid replacement therapy may be needed, typically on the order of 10 mg hydrocortisone in the early morning and 5 mg approximately 6 h later. Patients generally need replacement-dose glucocorticoids for at least 6 months after successful unilateral adrenalectomy for Cushing's syndrome.

Medical Treatment

When adrenal hypercortisolism is present, several medical therapies are currently available to reduce cortisol secretion and action (see Fig. 10.3). The available treatment modalities include inhibition of steroidogenesis and direct antagonism of the glucocorticoid receptor. In spite of the significant advances in our understanding of the pathophysiology of Cushing's syndrome due to various etiologies, this has not translated to novel treatment modalities specifically for adrenal hypercortisolism. All currently available agents share some common side effects. They can all potentially lead to symptomatic hypoadrenalism, even at therapeutic doses. Many interact with CYP3A4 metabolism; therefore, reviewing pharmacological interactions which may lower or increase serum drug concentrations is necessary throughout treatment. Some of these drugs may lead to electrocardiographic prolongation of the QT interval, although the ultimate significance of this effect is presently unknown.
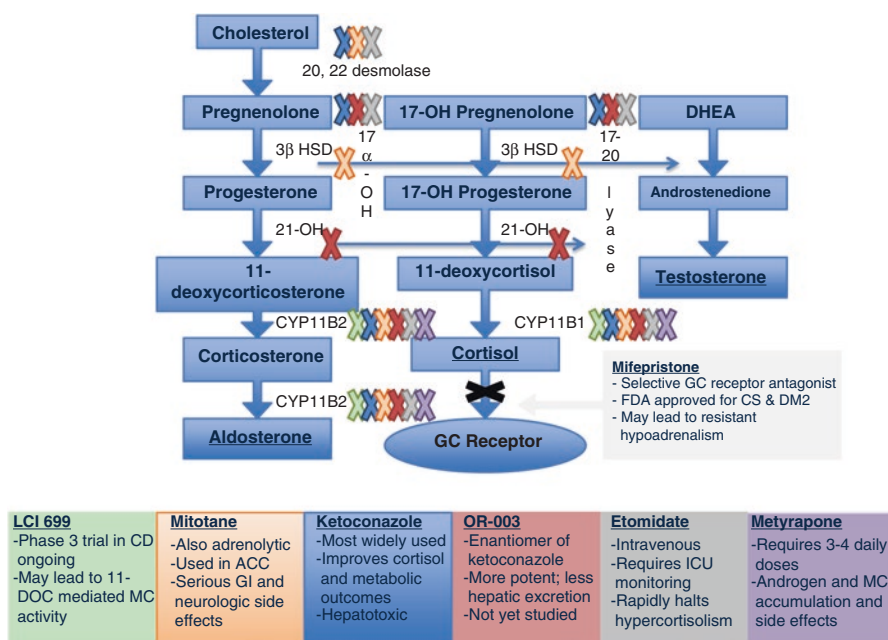
Fig. 10.3 Action of established and novel pharmacologic agents and their respective targets in the steroidogenesis pathway. *ACC* adrenocortical carcinoma, *CD* Cushing's disease, *DOC* deoxycorticosterone, *MC* mineralocorticoid, *DHEA* dehydroepiandrosterone, *GC* glucocorticoid, *ICU* intensive care unit, *FDA* Food and Drug Administration, *CS* Cushing's syndrome, *DM2* diabetes mellitus type 2

The most widely used steroidogenesis inhibitor remains *ketoconazole*. Typically used against systemic fungal infections, it inhibits glucocorticoid synthesis at two steps, both by blocking 17,20-lyase and 11β hydroxylase enzymes. In this fashion, it's an effective suppressor of adrenal and gonadal steroid synthesis and retrospectively has been found to lead to improvements in both cortisol secretion parameters and metabolic outcomes such as blood pressure, glucose tolerance, and hypokalemia. The usual doses required to block cortisol synthesis range from 200 to 1200 mg daily depending upon the degree of hypercortisolism. A multicenter retrospective analysis performed in France in patients with overt hypercortisolism found ketoconazole to be effective in reducing UFC levels in up to 75% of patients, with 50% having normalization of UFC preoperatively [89]. Its use in Cushing's syndrome is off-label. Unfortunately, ketoconazole is restricted as it carries a black box warning because of the possibility of severe liver injury. Monitoring of liver function tests is mandated while in use.

Metyrapone has a similar mechanism of action to ketoconazole. However, it only blocks the final 11β hydroxylase enzyme and therefore leads to increases in precursors with androgenic and mineralocorticoid activity that may produce side effects (e.g., increased acne, hirsutism, hypokalemia, hypertension, and edema). Retrospective data has also found short-term effectiveness with up to 80% of all

patients achieving reductions of at least 50% in UFC levels [90]. Typical doses vary between 500 and 6000 mg daily, although a short half-life means the medication should be taken every 6–8 h.

Perhaps the most rapid means of lowering serum cortisol levels is through the use of *etomidate*. This short-acting, intravenous anesthetic has been found to inhibit multiple enzymes in the conversion of cholesterol to cortisol and leads to a rapid halt in hypercortisolism, making this drug particularly useful during acute life-threatening conditions made worse by severe cortisol excess, such as sepsis [91]. As expected, given its hypnotic characteristics, its use is restricted to an intensive care unit with respiratory monitoring to avoid hypopnea. An intravenous drip at a starting dose of 0.3 mg/kg/h. is typically titrated at doses lower than dose used for anesthesia.

Mitotane is an adrenolytic agent primarily used in the adjuvant treatment of ACC. It has cytotoxic characteristics against adrenocortical tissue, in addition to inhibiting various enzymes in the steroid synthesis pathway. Serious gastrointestinal symptoms, a higher risk of profound hypoadrenalism, and neurologic side effects limit its use in benign adrenal disease. Mitotane has a slow onset of action, making it inadequate for the management of acute severe hypercortisolism.

Several experimental adrenal enzyme inhibitors such as *osilodrostat* (LCI699) and *levoketoconazole* (COR-003) are currently being studied for the treatment of Cushing's syndrome. A phase 3 trial of LCI699 use in the setting of persistent or recurrent Cushing's syndrome due to pituitary disease is ongoing. Levoketoconazole is the 2S,4R enantiomer of ketoconazole. In vitro, it has been found to have a more potent inhibition of steroidogenesis and a more preferential hepatic excretion, which may mean a lower risk of liver injury when compared to its 2R,4S counterpart.

Finally, *mifepristone* holds the distinction of being the only pharmacologic agent currently approved by the FDA for the treatment of Cushing's syndrome, in the setting of impaired fasting glucose or diabetes. The drug's mechanism of action is through selective glucocorticoid receptor antagonism (and to a lesser extent progesterone opposition as well). Its effect is quite potent and long acting [92]. Usual doses are between 300 and 1200 mg daily. As mifepristone does not inhibit glucocorticoid production, cortisol measurements are not useful as a biomarker of response to treatment. Serious side effects have been found with the use of mifepristone. Hypokalemia from excessive activity of cortisol on the mineralocorticoid receptor may be severe and symptomatic. Additionally, the dominant binding of the drug to the glucocorticoid receptor may lead to resistant hypoadrenalism. As this drug is also an anti-progestational agent, it is contraindicated in women of reproductive age and may be associated with endometrial hyperplasia and uterine bleeding. These adverse events pose a significant limitation in its use for long-term control of hypercortisolism.

Overt Hypercortisolism

Per the most recent treatment guidelines, medical therapy with steroidogenesis inhibitors is supported by moderate-quality evidence for the treatment of persistent/recurrent Cushing's disease, ectopic ACTH-producing neuroendocrine tumors, and

as an adjunct to ACC-related hypercortisolism. Glucocorticoid antagonists may also be considered in the presence of diabetes or impaired fasting glucose. When adrenal disease is present, both medical treatment modalities are acceptable as second-line options when surgery is not possible (either because of patient preference or if a poor surgical risk profile is present) [84].

Medical therapy additionally plays an important role in the management of severe hypercortisolism as a temporizing measure prior to definitive adrenalectomy and when acute complications of hypercortisolism are present (such as life-threatening psychosis or infection). The use of combined regimens, particularly with ketoconazole and metyrapone, is frequently used and has been described in the literature as effective until more definitive adrenal suppressions with mitotane or adrenalectomy can be performed [93]. Mifepristone, with its rapid and prolonged action at the glucocorticoid receptor, may also be considered in these scenarios.

Mild Hypercortisolism

As of the writing of this chapter, only one study of medical therapy in subclinical cortisol excess has been reported. DeBono et al. followed six patients with mild hypercortisolism (defined as serum cortisol >1.8 µg/dL after DST) and normal glycemic parameters. In this proof-of-concept study, HOMA-IR (a validated index of insulin sensitivity) levels significantly improved in all patients after 4 weeks of treatment with mifepristone [94].

Summary

With the advent of the worldwide pandemic of obesity, hypertension, and diabetes, physicians should be aware of the signs and symptoms of hypercortisolism, particularly as Cushing's syndrome may be a correctable cause behind these conditions. Although overt endogenous hypercortisolism remains a rare disease, mild hypercortisolism associated with adrenal incidentalomas are more commonly being detected in older individuals.

Present data points toward a deleterious effect of even mild forms of cortisol excess on multiple systems and overall mortality. Many therapeutic strategies are available and have significantly improved the prognosis of patients with adrenal cortisol excess. However, the fact that many sequelae and a higher risk of death persist even after biochemical cure may mean that delays in diagnosis may lead to irreversible damage. Future research needs to be performed to determine an ideal screening protocol, but before this is possible, a more standardized definition for mild hypercortisolism is essential. The paucity of data on treatment of patients with mild disease needs to be addressed. In the future, this may help tailor treatment in order to improve outcomes.

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Chapter 11
Diagnosis and Management of Primary Aldosteronism

William F. Young Jr.

Hypertension, increased aldosterone secretion, and suppressed plasma renin activity (PRA) characterize the syndrome of primary aldosteronism (PA), first fully described in 1955 [1]. Aldosterone-producing adenoma (APA) and bilateral idiopathic hyperaldosteronism (IHA) are the most common subtypes of PA (Table 11.1). Somatic mutations account for about half of APAs and include mutations in genes encoding components of the Kir 3.4 (GIRK4) potassium channel (*KCNJ5*), the sodium/potassium and calcium ATPases (*ATP1A1* and *ATP2B3*), and a voltage-dependent C-type calcium channel (*CACNA1D*) (see Chap. 6) [2]. A much less common form, unilateral hyperplasia or primary adrenal hyperplasia (PAH), is caused by micronodular or macronodular hyperplasia of the zona glomerulosa of predominantly one adrenal gland. Familial hyperaldosteronism (FH) is also rare, and three types have been described [2]. FH type I (FH-1), or glucocorticoid-remediable aldosteronism (GRA), results from a chimeric gene (5′-end of CYP11B1 fused to 3′-end of CYP11B2) that is autosomal dominant in inheritance and is associated with variable degrees of hyperaldosteronism. FH-2 refers to the familial occurrence of APA or IHA or both. FH-3 is caused by germline mutations in *KCNJ5* and usually results in severe hypertension in infancy and usually treated with bilateral adrenalectomy. FH-4 is caused by mutations in the *CACNA1H* gene, which encodes the alpha subunit of a L-type voltage-gated calcium channel (Ca$_v$3.2).

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Table 11.1 Types of primary aldosteronism

Aldosterone-producing adenoma (APA)—30% of cases
Bilateral idiopathic hyperplasia (IHA)—60% of cases
Primary (unilateral) adrenal hyperplasia—2% of cases
Aldosterone-producing adrenocortical carcinoma—<1% of cases
Familial hyperaldosteronism (FH)
Glucocorticoid-remediable aldosteronism (FH type 1)—<1% of cases
FH type 2 (APA or IHA)—<6% of cases
FH type 3 (germline *KCNJ5* mutations)—<1% of cases
FH type 4 (germline *CACNA1H* mutations)—<1% of cases
Ectopic aldosterone-producing adenoma or carcinoma—<0.1% of cases

Prevalence

In the past, clinicians would not consider the diagnosis of PA unless the patient presented with spontaneous hypokalemia, and then the diagnostic evaluation would require discontinuation of antihypertensive medications for at least 2 weeks. This diagnostic approach resulted in predicted prevalence rates of <0.5% of hypertensive patients [3–9]. However, it is now recognized that most patients with PA are not hypokalemic [10–13] and that case detection testing can be completed while the patient is taking antihypertensive drugs with a simple blood test that yields the ratio of plasma aldosterone concentration (PAC) to PRA [11]. The use of the PAC/PRA ratio as a case detection test, followed by aldosterone suppression for confirmatory testing, has resulted in much higher prevalence estimates for PA—5–10% of all patients with hypertension [11–14].

Clinical Presentation

The diagnosis of PA is usually made in patients who are in the third to sixth decade of life. Few symptoms are specific to the syndrome. Patients with marked hypokalemia may have muscle weakness and cramping, headaches, palpitations, polydipsia, polyuria, nocturia, or a combination of these [10]. Periodic paralysis is a very rare presentation in Caucasians, but it is not an infrequent presentation in patients of Asian descent [15]. Polyuria and nocturia are a result of hypokalemia-induced renal concentrating defect, and the presentation is frequently mistaken for prostatism in men. There are no specific physical findings. Edema is not a common finding because of the phenomenon of mineralocorticoid escape. The degree of hypertension is typically moderate to severe and may be resistant to usual pharmacologic treatments [10, 16]. In the first 262 cases of PA diagnosed at Mayo Clinic (1957–1986), the highest blood pressure was 260/155 mmHg; the mean (±SD) was 184/112 ± 28/16 mmHg [16]. Patients with APA tend to have higher blood pressures than those with IHA. Hypokalemia is frequently absent, so all patients with hypertension are candidates for this disorder. In other

patients, the hypokalemia becomes evident only with the addition of a potassium-wasting diuretic. Deep-seated renal cysts are found in up to 60% of patients with PA and chronic hypokalemia [17]. Because of a reset osmostat, the serum sodium concentration tends to be high-normal or slightly above the upper limit of normal. This clinical clue is very useful in the initial assessment for potential PA.

Several studies have shown that patients with PA are at higher risk than other patients with hypertension for target organ damage of the heart and kidney [18, 19]. Chronic kidney disease is common in patients with long-standing PA [20]. When matched for age, blood pressure, and duration of hypertension, patients with PA have greater left ventricular mass measurements than patients with other types of hypertension (e.g., pheochromocytoma, Cushing syndrome, essential hypertension) [21]. In patients with APA, the left ventricular wall thickness and mass were markedly decreased 1 year after adrenalectomy [22]. A case-control study of 124 patients with PA and 465 patients with essential hypertension (matched for age, sex, and systolic and diastolic blood pressure) found that patients presenting with either APA or IHA had a significantly higher rate of cardiovascular events (e.g., stroke, atrial fibrillation, myocardial infarction) than the matched patients with essential hypertension [19]. A negative effect of circulating aldosterone on cardiac function was found in young nonhypertensive subjects with GRA who had increased left ventricular wall thickness and reduced diastolic function compared with age- and sex-matched controls [18].

Diagnosis

The diagnostic approach to PA can be considered in three phases: case detection tests, confirmatory tests, and subtype evaluation tests.

Case Detection Tests Spontaneous hypokalemia is uncommon in patients with uncomplicated hypertension; when present, it strongly suggests associated mineralocorticoid excess. However, several studies have shown that most patients with PA have baseline serum levels of potassium in the normal range [11, 13]. Therefore, hypokalemia should not be the major criterion used to trigger case detection testing for PA. Patients with hypertension and hypokalemia (regardless of presumed cause), treatment-resistant hypertension (poor control on three antihypertensive drugs), severe hypertension ($\geq$150 mmHg systolic or $\geq$100 mmHg diastolic), hypertension and an incidental adrenal mass, or onset of hypertension at a young age should undergo case detection testing for PA (Fig. 11.1) [10, 11].

In patients with suspected PA, screening can be accomplished by paired measurements of PAC and PRA in a random morning ambulatory blood sample (preferably obtained between 8 and 10 a.m.). This test may be performed while the patient is taking antihypertensive medications (with some exceptions, discussed later) and without posture stimulation [10]. Hypokalemia reduces the secretion of aldosterone, and it is optimal to restore the serum level of potassium to near-normal before performing diagnostic studies.

When to Consider Testing for Primary Aldosteronism:
• Hypertension and hypokalemia
• Resistant hypertension (3 drugs and poor BP control)
• Adrenal incidentaloma and hypertension
• Onset of hypertension at a young age (eg, <30 y)
• Severe hypertension (≥150 mm Hg systolic or ≥100 mm Hg diastolic)
• Whenever considering secondary hypertension

Case Detection Testing:
Morning blood sample in seated ambulant patient
• Plasma aldosterone concentration (PAC)
• Plasma renin activity (PRA) or plasma renin concentration (PRC)

PAC (≥15 ng/dL; ≥416 pmol/L)
and
↓ PRA (<1.0 ng/mL/hr) or ↓PRC (< lower limit of detection for the assay)

Confirmatory Testing (needed if spontaneous hypokalemia absent):
• 24-h urine aldosterone, sodium, creatinine on a high sodium diet

Fig. 11.1 Algorithm provides guidance on when to consider testing for PA, use of the plasma aldosterone concentration (PAC) and plasma renin activity (PRA) as a case detection tool, and 24-hour urinary aldosterone excretion for confirmatory testing. *PRC* plasma renin concentration

It may be difficult to interpret data obtained from patients treated with a mineralocorticoid receptor antagonist (spironolactone and eplerenone). These drugs prevent aldosterone from activating the receptor, resulting sequentially in sodium loss, a decrease in plasma volume, and an elevation in PRA, which will reduce the utility of the PAC/PRA ratio. For this reason, spironolactone and eplerenone should not be initiated until the evaluation is completed and the final decisions about treatment are made. However, there are rare exceptions to this rule. For example, if the patient is hypokalemic despite treatment with spironolactone or eplerenone, then the mineralocorticoid receptors are not fully blocked, and PRA or PRC should be suppressed in such a patient with PA. In this unique circumstance, the evaluation for PA can proceed despite treatment with mineralocorticoid receptor antagonists. However, in most patients already receiving spironolactone, therapy should be discontinued for at least 6 weeks. Other potassium-sparing diuretics, such as amiloride and triamterene, usually do not interfere with testing unless the patient is on high doses.

Angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) have the potential to falsely elevate the PRA in a patient with PA. Therefore, the finding of a detectable PRA level or a low PAC/PRA ratio in a

patient taking one of these drugs does not exclude the diagnosis of PA. However, an undetectably low PRA level in a patient taking an ACE inhibitor or ARB makes PA likely.

The PAC/PRA ratio, first proposed as a case detection test for PA in 1981 [23], is based on the concept of paired hormone measurements. The PAC is measured in nanograms per deciliter (ng/dL) and the PRA in nanograms per milliliter per hour (ng/mL/h). In a hypertensive hypokalemic patient, secondary hyperaldosteronism should be considered if both PRA and PAC are increased and the PAC/PRA ratio is <10 (e.g., renovascular disease). An alternative source of mineralocorticoid receptor agonism should be considered if both PRA and PAC are suppressed (e.g., hypercortisolism). PA should be suspected if the PRA is suppressed (<1.0 ng/mL/h) and the PAC is increased (e.g., >15 ng/dL). Although there is some uncertainty about test characteristics and lack of standardization, the PAC/PRA ratio is widely accepted as the case detection test of choice for PA [11, 24].

It is important to understand that the lower limit of detection varies among different PRA assays and can have a dramatic effect on the PAC/PRA ratio. As an example, if the lower limit of detection for PRA is 0.6 ng/mL/h and the PAC is 16 ng/dL, then the PAC/PRA ratio with an "undetectable" PRA would be 27; however, if the lower limit of detection for PRA is 0.1 ng/mL/h, the same PAC level would yield a PAC/PRA ratio of 160. Thus, the cutoff for a "high" PAC/PRA ratio is laboratory dependent and, more specifically, PRA assay dependent. In a retrospective study, the combination of a PAC/PRA ratio >30 and a PAC level >20 ng/dL had a sensitivity of 90% and a specificity of 91% for APA [25]. At Mayo Clinic, the combination of a PAC/PRA ratio of 20 or higher and a PAC level of at least 15 ng/dL is found in more than 90% of patients with surgically confirmed APA [16]. In patients without PA, most of the variation occurs within the normal range [26]. A high PAC/PRA ratio is a positive case detection test, a finding that warrants further testing [11].

It is critical for the clinician to recognize that the PAC/PRA ratio is only a case detection tool, and, in the absence of spontaneous hypokalemia, all positive results should be followed by a confirmatory aldosterone suppression test to verify autonomous aldosterone production before treatment is initiated [11]. In a study of 118 subjects with essential hypertension, neither antihypertensive medications nor acute variation of dietary sodium affected the accuracy of the PAC/PRA ratio adversely; the sensitivities on and off therapy were 73% and 87%, respectively, and the specificities were 74% and 75%, respectively [27]. In a study of African American and Caucasian subjects with resistant hypertension, the PAC/PRA ratio was elevated (>20) in 45 of 58 subjects with PA and in 35 of 207 patients without PA (sensitivity, 78%; specificity, 83%) [28].

The measurement of PRA is time-consuming, shows high interlaboratory variability, and requires special preanalytic prerequisites. To overcome these disadvantages, a monoclonal antibody against active renin is being used by several reference laboratories to measure the plasma renin concentration (PRC) instead of PRA. However, few studies have compared the different methods of testing for PA, and these studies lack confirmatory testing. It is reasonable to consider a positive PAC/PRC test if the PAC is >15 ng/dL and the PRC is lower below the lower limit of detection for the assay.

Confirmatory Tests An increased PAC/PRA ratio is not diagnostic by itself, and PA must be confirmed by demonstration of inappropriate aldosterone secretion [11, 12]. The list of drugs and hormones capable of affecting the RAA axis is extensive, and a "medication-contaminated" evaluation is frequently unavoidable in patients with poorly controlled hypertension despite a three-drug program. Calcium channel blockers and α_1-adrenergic receptor blockers do not affect the diagnostic accuracy in most cases [11]. It is impossible to interpret data obtained from patients receiving treatment with mineralocorticoid receptor antagonists (e.g., spironolactone, eplerenone) when the PRA is not suppressed (see above). Therefore, treatment with a mineralocorticoid receptor antagonist should not be initiated until the evaluation has been completed and the final decisions about treatment have been made. The favored confirmatory test at Mayo Clinic is aldosterone suppression testing with orally administered sodium chloride and measurement of urinary aldosterone and sodium [10].

It is important to recognize that confirmatory testing is not needed in patients with hypertension and spontaneous hypokalemia when the PAC is >20 ng/dL and PRA is suppressed because there is no other disorder except PA that could be responsible for this presentation (Fig. 11.1) [11].

Oral Sodium Loading Test After hypertension and hypokalemia have been controlled, patients should receive a high-sodium diet (supplemented with sodium chloride tablets if needed) for 3 days, with a goal sodium intake of 5000 mg (equivalent to 218 mEq of sodium or 12.8 g sodium chloride) [16]. The risk of increasing dietary sodium in patients with severe hypertension must be assessed in each case [29]. Because the high-sodium diet can increase kaliuresis and hypokalemia, vigorous replacement of potassium chloride may be needed, and the serum level of potassium should be monitored daily. On the third day of the high-sodium diet, a 24-hour urine specimen is collected for measurement of aldosterone, sodium, and creatinine. To document adequate sodium repletion, the 24-hour urinary sodium excretion should exceed 200 mEq. Urinary aldosterone excretion of more than 12 μg/24 h in this setting is consistent with autonomous aldosterone secretion [16]. The sensitivity and specificity of the oral sodium loading test are 96% and 93%, respectively [30].

Intravenous Saline Infusion Test The intravenous saline infusion test has also been used widely for the diagnosis of PA [11, 12]. Normal subjects show suppression of PAC after volume expansion with isotonic saline; subjects with PA do not show this suppression. The test is done after an overnight fast. It is important to be sure that the patient is normokalemic before this test because the sodium load will increase the renal excretion of potassium. Two liters of 0.9% sodium chloride solution are infused intravenously with an infusion pump over 4 h with the patient recumbent or seated (see below). Blood pressure and heart rate are monitored during the infusion. At the completion of the infusion, blood is drawn for measurement of PAC. PAC levels in normal subjects decrease to <5 ng/dL, whereas most patients with PA do not suppress to <10 ng/dL. Post-infusion PAC values between 5 and 10 ng/dL are indeterminate and may be seen in patients with IHA. Historically the saline infusion

test has been performed in the supine position, and the false-negative rate has been excessive; preliminary data suggest that if the saline infusion test is performed in the seated position, the accuracy is improved [31].

Fludrocortisone Suppression Test and Captopril Stimulation Test The fludrocortisone suppression test [32] and the captopril stimulation test [33] are less commonly used confirmatory tests.

Subtype Evaluation Tests After case detection and confirmatory testing, the third management issue guides the therapeutic approach by distinguishing between unilateral adrenal disease (e.g., APA and PAH) from bilateral adrenal disease (e.g., IHA and GRA). Unilateral adrenalectomy in patients with APA or PAH results in normalization of hypokalemia in all cases; hypertension is improved in all cases and is cured in 30–60% [34–36]. In IHA and GRA, unilateral or bilateral adrenalectomy seldom corrects the hypertension [16]. IHA and GRA should be treated medically. APA is found in approximately 35% of cases and bilateral IHA in approximately 60% (Table 11.1). APAs are usually small hypodense adrenal nodules (<2 cm in diameter) on computed tomography (CT) and are golden yellow in color when resected (Fig. 11.2). IHA adrenal glands may be normal on CT or may show nodular changes. In general, patients with APAs have more severe hypertension, more frequent hypokalemia, and higher levels of plasma aldosterone (>25 ng/dL) and urinary aldosterone (>30 μg/24 h) and are younger (<50 years), compared with those who have IHA [16, 37]. Aldosterone-producing adrenal carcinomas are almost always larger than 4 cm in diameter and have an inhomogeneous imaging phenotype on CT.

Abdominal CT PA subtype evaluation may require one or more tests, the first of which is imaging of the adrenal glands with CT (Fig. 11.3). If a solitary unilateral hypodense (HU < 10) macroadenoma (>1 cm) and normal contralateral adrenal morphology are found on CT in a young patient (<35 years) with severe PA, unilateral adrenalectomy is a reasonable therapeutic option (Fig. 11.3) [11, 39]. However, in many cases, CT shows normal-appearing adrenals, minimal unilateral adrenal limb thickening, unilateral microadenomas (≤1 cm), or bilateral macroadenomas. In these cases, additional testing is required to determine the source of excess aldosterone secretion.

Small APAs may be labeled incorrectly as IHA on the basis of CT findings of bilateral nodularity or normal-appearing adrenals. Also, apparent adrenal microadenomas may actually represent areas of hyperplasia, and unilateral adrenalectomy would be inappropriate. In addition, nonfunctioning unilateral adrenal macroadenomas are not uncommon, especially in older patients (>40 years) [40]. Unilateral PAH may be visible on CT, or the PAH adrenal may appear normal on CT. Thus, adrenal CT is not accurate in distinguishing between APA and IHA [37, 39, 41]. In one study of 203 patients with PA who were evaluated with both CT and adrenal venous sampling, CT was accurate in only 53% of patients; based on the CT findings, 42 patients (22%) would have been incorrectly excluded as candidates for
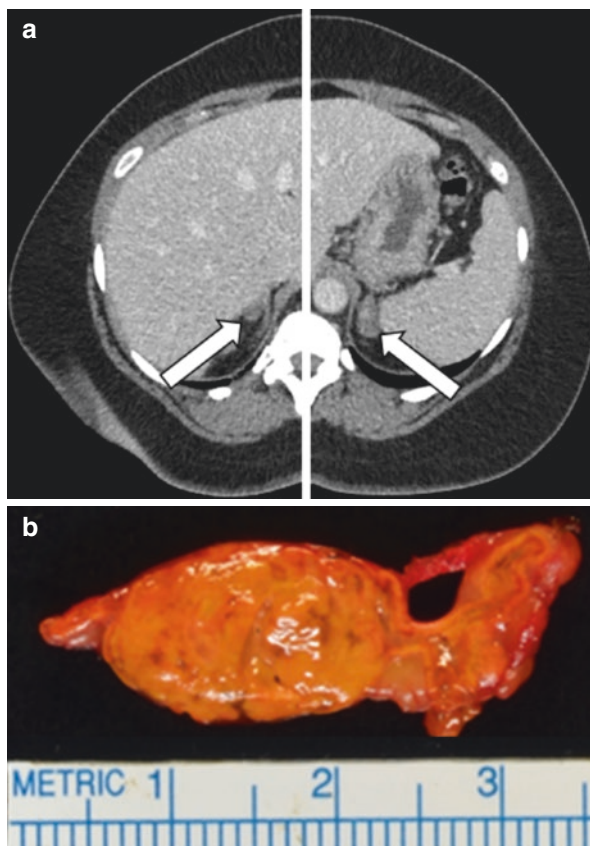
Fig. 11.2 A 49-year-old woman had a 22-year history of hypertension and 2-year history of hypokalemia. The case detection test for PA was positive, with a plasma aldosterone concentration (PAC) of 52 ng/dL and low-plasma renin activity (PRA) at <0.6 ng/mL/h. In view of the spontaneous hypokalemia, confirmatory testing was not needed. Panel (**a**), adrenal CT shows a 1.2 cm, low-density nodule (*arrow*) in the lateral limb of the right adrenal gland and a 2.2 cm low-density nodule (*arrow*) in the left adrenal gland. Adrenal venous sampling (Fig. 11.4) lateralized aldosterone secretion to the left adrenal gland and a yellow 2.1 cm cortical adenoma (shown in panel **b**) was found at laparoscopic left adrenalectomy. The postoperative plasma aldosterone concentration was <4.0 ng/dL. Hypokalemia was cured, and blood pressure was normal while taking two antihypertensive medications

adrenalectomy, and 48 (25%) might have had unnecessary or inappropriate surgery [37]. In a systematic review of 38 studies involving 950 patients with PA, adrenal CT/MRI results did not agree with the findings from adrenal venous sampling in 359 patients (38%); based on CT/MRI, 19% of the 950 patients would have undergone noncurative surgery, and 19% would have been offered medical therapy instead of curative adrenalectomy [41].

Adrenal Venous Sampling   Adrenal venous sampling (AVS) is the criterion standard test to distinguish between unilateral and bilateral disease in patients with PA [11, 39, 41]. AVS is an intricate procedure because the right adrenal vein is small and
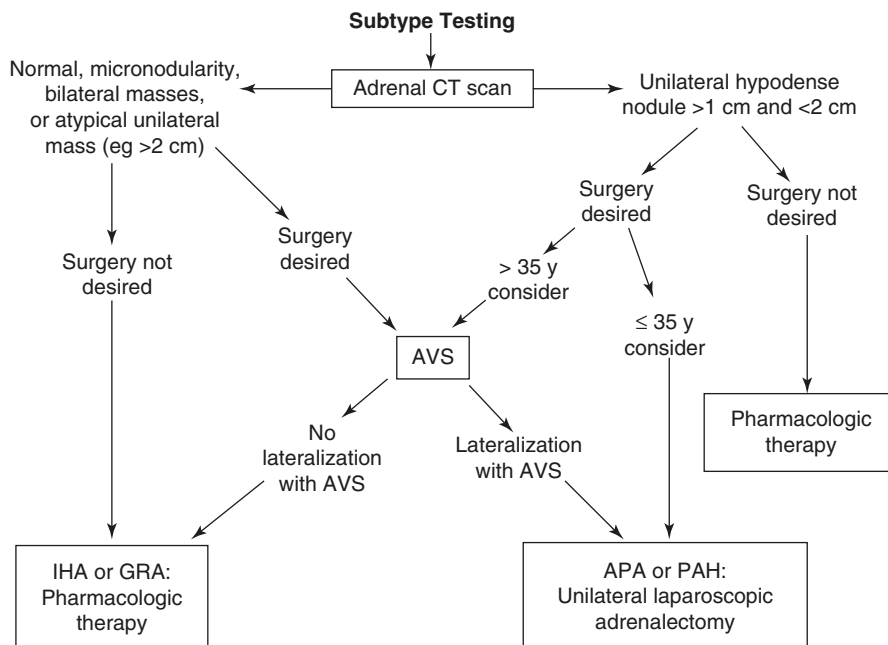
Fig. 11.3 Subtype evaluation of PA. For patients who want to pursue a surgical treatment for their hypertension, adrenal venous sampling is frequently a key diagnostic step (see text for details). *APA* aldosterone-producing adenoma, *AVS* adrenal venous sampling, *CT* computed tomography, *IHA* idiopathic hyperaldosteronism, *PA* primary aldosteronism, *PAH* primary adrenal hyperplasia (Modified from Young WF Jr., Hogan MJ: Renin-independent hypermineralocorticoidism. *Trends Endocrinol Metab.* 1994;5:97–106) [38]

may be difficult to locate and cannulate; the success rate depends on the proficiency of the angiographer [42]. A review of 47 reports found that the success rate for cannulation of the right adrenal vein in 384 patients was 74% [16]. With experience and focusing the expertise to one or two radiologists at a referral center, the AVS success rate can be as high as 96% [37, 43, 44].

The five keys to a successful adrenal venous sampling program are (1) appropriate patient selection, (2) careful patient preparation, (3) focused technical expertise, (4) defined protocol, and (5) accurate data interpretation [42]. A center-specific, written protocol is mandatory. The protocol should be developed by an interested group of endocrinologists, hypertension specialists, internists, radiologists, and laboratory personnel. Safeguards should be in place to prevent mislabeling of the blood tubes in the radiology suite and to prevent sample mix-up in the laboratory [42].

At Mayo Clinic, we use continuous cosyntropin infusion during AVS (50 μg/h starting 30 min before sampling and continuing throughout the procedure) for the following reasons: (1) to minimize stress-induced fluctuations in aldosterone secretion during nonsimultaneous AVS, (2) to maximize the gradient in cortisol from adrenal vein to inferior vena cava (IVC) and thus confirm successful sampling of the adrenal veins, and (3) to maximize the secretion of aldosterone from an APA [37, 42].

Results of Bilateral Adrenal Venous Sampling

Vein	Aldosterone (A), ng/dL	Cortisol (C), µg/dL	A/C ratio	Aldosterone lateralization ratio*
R adrenal vein	369	680	0.54	
L adrenal vein	6510	318	20.47	37.91
Inferior vena cava	74	31	2.39	

*L adrenal vein A/C ratio divided by R adrenal vein A/C ratio.

Fig. 11.4 Adrenal vein sampling results from patient in Fig. 11.2. Both adrenal veins were successfully sampled based on the cortisol gradient from adrenal vein to inferior vena cava (IVC) of 22:1 on the right and 10:1 on the left. The cortisol concentration from the left adrenal vein is usually lower than that found in the right adrenal vein because the blood sample on the left is obtained from the common phrenic trunk and adrenal vein effluent is diluted by the venous flow from the inferior phrenic vein. To correct this dilution, the aldosterone concentration from each adrenal vein is divided by the corresponding cortisol concentration, and then the aldosterone to cortisol ratio from each adrenal vein is compared. When corrected for venous dilution on the left, the aldosterone lateralization ratio was 37.9:1 (left/right). Surgery is indicated when the aldosterone lateralization ratio is more than 4:1. The aldosterone to cortisol ratio from the right adrenal gland was less than that in the IVC—this is termed "contralateral suppression" and is further supportive evidence for a left adrenal aldosterone-producing adenoma

The adrenal veins are catheterized through the percutaneous femoral vein approach, and the position of the catheter tip is verified by gentle injection of a small amount of nonionic contrast medium and radiographic documentation. Blood is obtained from both adrenal veins and from the IVC below the renal veins and assayed for aldosterone and cortisol concentrations. To be sure that there is no cross-contamination, the "IVC" sample should be obtained from the external iliac vein. The venous sample from the left side typically is obtained from the common phrenic vein immediately adjacent to the entrance of the adrenal vein. The cortisol concentrations from the adrenal veins and IVC are used to confirm successful catheterization; the adrenal vein/IVC cortisol ratio is typically >10:1, and we use a cutoff of >5:1 to define successful sampling of each adrenal vein.

Dividing the right and left adrenal vein PAC values by their respective cortisol concentrations corrects for the dilutional effect of the inferior phrenic vein flow into the left adrenal vein; these are termed *cortisol-corrected ratios* (Fig. 11.4). In patients with APA, the mean cortisol-corrected aldosterone ratio (i.e., the ratio of PAC/cortisol from the APA side to that from the normal side) is 18:1 [37]. A cutoff point of >4:1 for this ratio is used to indicate unilateral aldosterone excess. In patients with IHA, the mean cortisol-corrected aldosterone ratio is 1.8:1 (high side to low side), and a ratio of <3.0:1 suggests bilateral aldosterone hypersecretion [37]. Therefore, most patients with a unilateral source of aldosterone have cortisol-corrected aldosterone lateralization ratios >4.0 and ratios >3.0 but <4.0 represent a zone of overlap; aldosterone lateralization ratios no higher than 3.0 are consistent

with bilateral aldosterone secretion. The test characteristics of adrenal vein sampling for detection of unilateral aldosterone hypersecretion (APA or PAH) are $\leq$95% sensitivity and $\approx$100% specificity [37]. An additional and secondary metric that may be used to guide the assessment of lateralization is "contralateral suppression." When the aldosterone to cortisol ratio from the nondominant adrenal gland is less than that found in the IVC, there is contralateral suppression—a finding in approximately 93% of patients with APAs [37]. At centers with experience with AVS, the complication rate is 2.5% or less [37, 43]. Complications can include symptomatic groin hematoma, adrenal hemorrhage, and dissection of an adrenal vein.

Treatment

The treatment goal is to prevent the morbidity and mortality associated with hypertension, hypokalemia, nephrotoxicity, and cardiovascular damage. Knowing the cause of the PA helps to determine the appropriate treatment. Normalization of blood pressure should not be the only goal. In addition to the kidney and colon, mineralocorticoid receptors are present in the heart, brain, and blood vessels. Excessive secretion of aldosterone is associated with increased risk of cardiovascular disease and morbidity and chronic kidney disease. Therefore, normalization of circulating aldosterone or mineralocorticoid receptor blockade should be part of the management plan for all patients with PA. However, clinicians must understand that most patients with long-standing PA have some degree of chronic kidney disease that is masked by the glomerular hyperfiltration associated with aldosterone excess [45, 46]. The true degree of renal insufficiency may become evident only after effective pharmacologic or surgical therapy [45, 46].

Surgical Treatment of Aldosterone-Producing Adenoma and Unilateral Hyperplasia Unilateral laparoscopic adrenalectomy is an excellent treatment option for patients with APA or unilateral hyperplasia [47]. Although blood pressure control improves in almost 100% of patients postoperatively, average long-term cure rates of hypertension after unilateral adrenalectomy for APA range from 30 to 60% [34, 39, 48]. Persistent hypertension after adrenalectomy is correlated directly with having more than one first-degree relative with hypertension, use of more than two antihypertensive agents preoperatively, older age, increased serum creatinine level, and duration of hypertension and is most likely caused by coexistent primary hypertension [34, 48].

Laparoscopic adrenalectomy is the preferred surgical approach and is associated with shorter hospital stays and less long-term morbidity than the open approach. Because APAs are small and may be multiple, the entire adrenal gland should be removed [49]. To decrease the surgical risk, hypokalemia should be corrected with potassium supplements or a mineralocorticoid receptor antagonist, or both, preoperatively. These medications should be discontinued postoperatively. PAC should be measured 1–2 days after the operation to confirm a biochemical cure [39]. Serum potassium levels should be monitored weekly for 4 weeks after surgery, and a

generous sodium diet should be followed to avoid the hyperkalemia of hypoaldosteronism that may occur because of the chronic suppression of the renin angiotensin aldosterone axis [50]. Clinically significant hyperkalemia develops after surgery in approximately 5% of APA patients, and short-term fludrocortisone supplementation may be required. Typically, the hypertension that was associated with aldosterone excess resolves in 1–3 months after the surgery. It has been found that adrenalectomy for APA is significantly less expensive than long-term medical therapy [51].

Pharmacologic Treatment IHA and GRA should be treated medically [11]. In addition, APA may be treated medically if the medical treatment includes mineralocorticoid receptor blockade [52]. A sodium-restricted diet (<100 mEq of sodium per day), maintenance of ideal body weight, tobacco avoidance, and regular aerobic exercise contribute significantly to the success of pharmacologic treatment. No placebo-controlled, randomized trials have evaluated the relative efficacy of drugs in the treatment of PA [53].

Spironolactone has been the drug of choice to treat PA for more than five decades. It is available as 25, 50, and 100 mg tablets. The dosage is 12.5–25 mg/day initially and can be increased to 400 mg/day if necessary to achieve a high-normal serum potassium concentration without the aid of oral potassium chloride supplementation. Hypokalemia responds promptly, but hypertension can take as long as 4–8 weeks to be corrected. After several months of therapy, the dosage of spironolactone often can be decreased to as little as 25–50 mg/day; dosage titration is based on a goal serum potassium level in the high-normal range. Serum potassium and creatinine should be monitored frequently during the first 4–6 weeks of therapy (especially in patients with chronic kidney disease or diabetes mellitus). Spironolactone increases the half-life of digoxin, and the digoxin dosage may need to be adjusted when treatment with spironolactone is started. Concomitant therapy with salicylates should be avoided because they interfere with the tubular secretion of an active metabolite and decrease the effectiveness of spironolactone. Spironolactone is not selective for the mineralocorticoid receptor and that may lead to side effects. For example, antagonism at the testosterone receptor may result in painful gynecomastia, erectile dysfunction, and decreased libido in men, and agonist activity at the progesterone receptor results in menstrual irregularity in women [54].

Eplerenone is a steroid-based antimineralocorticoid that acts as a competitive and selective mineralocorticoid receptor antagonist and was approved by the US Food and Drug Administration (FDA) for the treatment of uncomplicated essential hypertension in 2003. The 9,11-epoxide group in eplerenone results in a marked reduction of the molecule's progestational and antiandrogenic actions; compared with spironolactone, eplerenone has 0.1% of the binding affinity to androgen receptors and <1% of the binding affinity to progesterone receptors. In a randomized, double-blinded trial comparing the efficacy, safety, and tolerability of eplerenone to that of spironolactone (100–300 mg vs. 75–225 mg, respectively) in patients with PA found spironolactone to be superior in terms of blood pressure lowering, but to be associated with higher rates of male gynecomastia (21% vs. 5% for eplerenone) and female mastodynia (21% vs 0%) [55]. Eplerenone is available as 25 and 50 mg

tablets. For PA, it is reasonable to start with a dose of 25 mg twice daily (twice daily because of the shorter half-life of eplerenone compared with spironolactone) and titrated upward; the target is a high-normal serum potassium concentration without the aid of potassium supplements. The maximum dose approved by the FDA for hypertension is 100 mg/day; however, much higher doses are frequently needed in patients with PA in order to achieve normokalemia. Potency studies with eplerenone show 25–50% less milligram-per-milligram potency compared with spironolactone. As with spironolactone, it is important to monitor blood pressure, serum potassium, and serum creatinine levels closely. Side effects include dizziness, headache, fatigue, diarrhea, hypertriglyceridemia, and elevated liver enzymes.

Patients with IHA frequently require a second antihypertensive agent to achieve good blood pressure control. Hypervolemia is a major reason for resistance to drug therapy, and low doses of a thiazide (e.g., 12.5–50 mg of hydrochlorothiazide daily) or a related sulfonamide diuretic are effective in combination with the mineralocorticoid receptor antagonist. Because these agents often lead to further hypokalemia, serum potassium levels should be monitored.

Before treatment for GRA is initiated, the diagnosis of GRA should be confirmed with genetic testing. In the GRA patient, chronic treatment with physiologic doses of a glucocorticoid normalizes blood pressure and corrects hypokalemia. The clinician should be cautious about iatrogenic Cushing syndrome with excessive doses of glucocorticoids, especially when dexamethasone is used in children. Shorter-acting agents such as prednisone or hydrocortisone should be prescribed, using the smallest effective dose in relation to body surface area (e.g., hydrocortisone, 10–12 mg/m^2/day). Target blood pressure in children should be guided by age-specific blood pressure percentiles. Children should be monitored by pediatricians with expertise in glucocorticoid therapy, with careful attention paid to preventing retardation of linear growth due to overtreatment. Treatment with mineralocorticoid receptor antagonists in these patients may be just as effective as glucocorticoids and avoids the potential disruption of the hypothalamic-pituitary-adrenal axis and risk of iatrogenic side effects. In addition, glucocorticoid therapy or mineralocorticoid receptor blockade may even have a role in normotensive GRA patients [18].

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Chapter 12
Pheochromocytoma/Paraganglioma: Update on Diagnosis and Management

Ivana Jochmanova and Karel Pacak

Abbreviations

^{18}F-DOPA	^{18}F-Dihydroxyphenylalanine
^{18}F-FDA	6-^{18}F-Fluorodopamine
^{18}F-FDG	^{18}F-Fluorodeoxyglucose
3PO	3-(3-Pyridinyl)-1-(4-pyridinyl)-2-propen-1-on
^{68}Ga-DOTATATE	^{68}Ga-DOTA(0)-Tyr(3)-octreotate
Acetyl-CoA	Acetyl coenzyme A
ACLY	ATP citrate lyase
AD	Autosomal dominant
AM	Morning
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
ATRX	Alpha thalassemia/mental retardation syndrome X-linked
BCH	2-Aminobicyclo(2,2,1)-heptane-2-carboxylic acid
BP	Blood pressure
BPTES	Bis-2-[5-phenylacetamido-1, 2, 4-thiadiazol-2-yl] ethyl sulfide

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CHC	α-Cyano-4-hydroxycinnamate
CT	Computed tomography
CVD	Cyclophosphamide, vincristine, dacarbazine
E	Epinephrine
EBRT	External beam radiation therapy
EGCG	Epigallocatechin gallate
EGLN1/2	Egl-9 family hypoxia-inducible factor 1/2 (see also *PHD2/1*)
FH	Fumarate hydratase
GDH1	Glutamate dehydrogenase 1
GLUT	Glucose transporter
GOT2	Glutamate oxaloacetate transaminase 2
GPNA	γ-L-Glutamyl-p-nitroanilide
GPT2	Glutamate pyruvate transaminase 2
HIF	Hypoxia-inducible factor
HIF2A	Hypoxia-inducible factor 2 alpha
HK	Hexokinase
HNPGL	Head and neck paraganglioma
HPLC	High-performance liquid chromatography
HR	Heart rate
HRAS	Harvey rat sarcoma viral oncogene homolog
IDH	Isocitrate dehydrogenase
KIF1Bβ	Kinesin family member 1B
LAT1	L-type amino acid transporter 1
LDHA	Lactate dehydrogenase A
MAX	myc-associated factor X gene
MCT	Monocarboxylase transporter
MDH2	Malate dehydrogenase 2
MEN2A/2B	Multiple endocrine neoplasia, type 2A/2B
MIBG	Metaiodobenzylguanidine
MN	Metanephrine
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MTY	Methoxytyramine
NE	Norepinephrine
NF1	Neurofibromatosis type 1
NF1	Neurofibromin 1
NMN	Normetanephrine
NS	Nonsecreting
PDH	Pyruvate dehydrogenase
PET	Positron emission tomography
PFK	Phosphofructokinase
PGC1α	Peroxisome proliferator-activated receptor-γ coactivator 1α
PGL	Paraganglioma
PHD1/2	HIF prolyl hydroxylase domain-containing protein 1/2 (see also *EGLN2/1*)

PHEO	Pheochromocytoma
PI	Paternal inheritance
PKM2	Pyruvate kinase, isoenzyme 2
RECIST	Response evaluation criteria in solid tumors
RET	Rearranged during transfection proto-oncogene
RFA	Radio-frequency ablation
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
SDHA, SDHB, SDHC, SDHD	Succinate dehydrogenase subunits A, B, C, and D
SDHAF2	SDH assembly factor 2
TAPGL	Thoracic and abdominal paraganglioma
TMEM127	Transmembrane protein 127
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau

Introduction

Pheochromocytomas (PHEOs) and paragangliomas (PGLs) are rare neuroendo-crine, catecholamine-producing tumors arising from adrenal medulla or extra-adrenal sympathetic and parasympathetic ganglia, respectively [1]. PHEOs/PGLs are mostly benign tumors, but metastatic disease is not rare, especially in patients with specific genetic backgrounds [2–4]. Although well known since the early twentieth century, PHEO/PGL diagnosis and treatment can still be a very challenging task. In up to 50% of patients, these tumors are not recognized [5]. Recently, research has brought new information about the pathophysiology of PHEO and PGL and enabled the development of new methods for diagnosis of these tumors as well as potential new treatment options.

Clinical Presentation of Pheochromocytoma and Paraganglioma

Deciphering the signs and symptoms of patients with a certain disease poses one of the most challenging parts of the diagnostic process. Comprehensive personal and family history and clinical examination are therefore the key components for the diagnostic process. Patients harboring PHEOs/PGLs exhibit a variety of nonspecific symptoms (Table 12.1) resulting from hemodynamic and metabolic actions of circulating catecholamines, or other amines and neuropeptides, secreted by the tumor [7, 10, 11]. Stimulation of α-adrenoceptors leads to an increase in systemic vasoconstriction and peripheral pressure and a decrease of perfusion in target organs (heart, brain, kidneys, gastrointestinal tract). Activation of β-adrenoceptors results in inotropic and chronotropic effects on myocardium and the release of renin [12].

Table 12.1 Clinical signs and symptoms exhibited by patients with PHEO/PGL

Symptoms	Frequency	Signs	Frequency
Headache	++++	Hypertension	++++
Palpitations	+++	– Sustained	++
Sweating	+++	– Paroxysmal	++
Anxiety/nervousness	++	Tachycardia or reflex bradycardia	+++
Abdominal/chest pain	++	Sweating/diaphoresis	+++
Nausea	++	Orthostatic hypotension	+++
Tremulousness	++	Pallor	++
Fatigue/weakness	++	Fever/hypermetabolism	++
Dyspnea	+	Hyperglycemia	++
Dizziness/faintness	+	Vomiting	++
Heat intolerance	+	Weight loss	++
Pain/paresthesias	+	Increased respiratory rate	++
Visual symptoms	+	Flushing	+
Constipation	+	Convulsions	+
Diarrhea	+	Psychosis (rare)	+

Frequency: up to 25%, +; 26–50%, ++; 51–75%, +++; 76–100%, ++++ [6–9]

Patients with primarily epinephrine-secreting PHEOs/PGLs more frequently display signs and symptoms compared to those with norepinephrine-producing tumors. Patients with dopamine-secreting PHEOs/PGLs usually present with less typical symptoms, such as hypotension, diarrhea, and weight loss. However, the severity of symptoms does not necessarily correlate with plasma catecholamine levels [7]. Clinical symptoms can mimic a number of different conditions (Table 12.2) and they vary from patient to patient. Moreover, approximately 8–13% of patients may be completely asymptomatic, usually due to a small (less than 5 mm) tumor or a dedifferentiated tumor without catecholamine-synthesizing enzymes [13, 14]. As a result, PHEOs/PGLs are often missed and are not discovered until autopsy [5, 10, 15]. Patients sometimes present with potentially life-threatening conditions due to excessive catecholamine release from a tumor (Table 12.3). PHEO/PGL-induced hemodynamic or metabolic attacks are variable in duration and frequency. They can occur daily or as infrequently as once a few months, lasting from seconds to several hours.

The classical PHEO/PGL symptoms include headaches, profuse sweating, and palpitations. A high number of patients suffer from sustained or paroxysmal hypertension [6, 10]. If these symptoms are present together, they are highly suggestive for PHEO/PGL [7, 10, 13, 18, 19].

Headaches are the most prevalent symptom (up to 90%) in patients with PHEO/PGL. Seriousness of headaches varies from mild to severe, and they can last up to several days [20]. Sweating and diaphoresis occurs in 60–70% of PHEO/PGL patients [7, 20]. Catecholamine effects, specifically epinephrine, on cardiac β-adrenoceptors can manifest as palpitations [7].

Sustained or paroxysmal hypertension, often resistant to treatment, is present in around 90% of PHEO/PGL patients. Those with sustained high blood pressure present disturbances in the diurnal blood pressure rhythm, reflected by the lack of nocturnal blood pressure dip [21, 22]. Hypertensive PHEO/PGL patients may also

Table 12.2 Differential diagnosis of PHEO/PGL

System	Diagnosis
Endocrine	Adrenal medullary hyperplasia
	Hyperthyreosis, thyrotoxicosis
	Carcinoid
	Hypoglycemia, insulin reaction
	Medullary thyroid carcinoma
	Hyperadrenergic essential hypertension
	Mastocytosis
	Menopausal syndrome
Cardiovascular	Heart failure
	Arrhythmias
	Ischemic heart disease, angina pectoris
	Myocardial infarction
	Mitral valve prolapse
	Abdominal catastrophe/aortic dissection
	Baroreflex failure
	Syncope
	Orthostatic hypotension
	Labile hypernoradrenergic essential hypertension
	Renovascular disease
Neurological	Migraine or cluster headaches
	Stroke
	Diencephalic autonomic epilepsy
	Meningioma
	Paroxysmal tachycardias including postural tachycardia syndrome
	Guillain-Barré syndrome
	Encephalitis
	Intracranial lesions
	Cerebral vasculitis and hemorrhage
Psychogenic	Anxiety or panic attacks
	Factitious use of drugs
	Somatization disorder
	Hyperventilation
Pharmacologic	Tricyclic antidepressant
	Cocaine
	Amphetamine
	Alcohol withdrawal
	Drugs stimulating adrenergic receptors
	Abrupt clonidine withdrawal
	Dopamine antagonists
	Ingestion of tyramine-containing foods or proprietary cold preparations while taking monoamine oxidase inhibitors
	Ephedrine-containing drugs
	Factitious use of various drugs including catecholamines
Other	Neuroblastoma, ganglioneuroma, ganglioneuroblastoma
	Acute intermittent porphyria
	Mastocytosis
	Unexplained flushing spells
	Recurrent idiopathic anaphylaxis
	Toxemia of pregnancy
	Unexplained shock
	Lead or mercury poisoning

Refs [7, 10, 179]

Table 12.3 Emergency situations associated with PHEO/PGL

Clinical setting	Symptoms
Pheochromocytoma multisystem crisis (PMC)	Hyper- and/or hypotension Multiple organ failure Body temperature $\geq$ 40 °C Encephalopathy
Cardiovascular	Collapse Hypertensive crisis Hypertensive crisis upon induction of anesthesia Hypertensive crisis induced by medication or other mechanisms Shock or profound hypotension Acute heart failure Myocardial infarction Arrhythmia Cardiomyopathy Myocarditis Dissecting aortic aneurysm Limb and/or organ ischemia, digital necrosis, or gangrene
Pulmonary	Acute pulmonary edema Adult respiratory distress syndrome Pulmonary hypertension
Abdominal	Abdominal bleeding Paralytic ileus Acute intestinal obstruction Severe enterocolitis and peritonitis Colon perforation Bowel ischemia and generalized peritonitis Mesenteric vascular occlusion Acute pancreatitis Cholecystitis Megacolon Watery diarrhea syndrome with hypokalemia
Neurological	Hemiplegia Limb weakness General muscle weakness Generalized seizures Stroke
Renal	Acute renal failure Acute pyelonephritis Severe hematuria Renal artery stenosis by compression of tumor
Metabolic	Diabetic ketoacidosis Lactic acidosis
Ocular	Acute blindness Retinopathy

Refs [12, 16, 17]

exhibit decreased cardiac output [23] or can present with an acute catecholamine cardiomyopathy/myocardial damage [24–26]. Severe hypertension may result in emergency situations requiring immediate medical attention and treatment [7]. Hypertensive crisis and symptoms associated with paroxysmal blood pressure elevations can occur due to excessive catecholamine release triggered by accidental tumor manipulation during diagnostic procedures (e.g., endoscopy), an increase in intra-abdominal pressure (e.g., palpation, defecation, urination, accident), and administration of anesthesia or certain drugs (Table 12.4) or through ingestion of food and beverages containing tyramine (certain cheeses, beers, wines, bananas, chocolate) or synephrine (citrus fruit juice) [10, 28, 30–34].

Although hypertension is the most common clinical sign, some patients (up to 10%) may have normal blood pressure or may present with hypotension, particularly postural hypotension, or alternating episodes of hyper- and hypotension [13, 35–37]. Orthostatic hypotension is usually accompanied by orthostatic tachycardia and is seen in epinephrine-secreting PHEO/PGL.

Other PHEO/PGL symptoms include flushing or pallor, nausea and vomiting (often exercise induced), anxiety or panic attacks, dyspnea, weight loss despite nor-

Table 12.4 Medications contraindicated in patients with known or suspected PHEO/PGL

Drug class	Examples	Relevant clinical uses
β-Adrenergic receptor blockers[a]	Propranolol, sotalol, timolol, nadolol, labetalol	But may be used to treat conditions that result from catecholamine excess (hypertension, cardiomyopathy, heart failure, panic attacks, migraine, tachycardia, cardiac arrhythmias)
Dopamine D2 receptor antagonists including antipsychotics	Metoclopramide, sulpiride, amisulpride, tiapride, chlorpromazine, prochlorperazine, droperidol	Control of nausea, vomiting, psychosis, hot flashes, tranquilizing effects
Tricyclic antidepressants and norepinephrine reuptake inhibitors	Amitriptyline, imipramine, nortriptyline, clomipramine	Treatment of insomnia, neuropathic pain, nocturnal enuresis in children, headaches, depression (rarely)
Other antidepressants (serotonin reuptake inhibitors)	Paroxetine, fluoxetine, duloxetine	Depression, anxiety, panic attacks, antiobesity agents
Monoamine oxidase inhibitors	Tranylcypromine, moclobemide, phenelzine	Nonselective agents rarely used as antidepressants (owing to "cheese effect")

(continued)

Table 12.4 (continued)

Drug class	Examples	Relevant clinical uses
Sympathomimetics[a]	Ephedrine, pseudoephedrine, fenfluramine, amfepramone, phendimetrazine, methylphenidate, phentermine, dexamfetamine	Control of low blood pressure during surgical anesthesia, as decongestants, antiobesity agents
Chemotherapeutic agents[a]		Antineoplastic actions and treatment of malignant pheochromocytoma
Oxazolidinone antibiotics	Linezolid	Treatment of infections caused by multiresistant gram-positive bacteria
Opioid analgesics[a] and naloxone	Morphine, pethidine, tramadol, oxycodone, heroin	Induction of surgical anesthesia
Neuromuscular blocking agents[a]	Succinylcholine, tubocurarine, atracurium	Induction of surgical anesthesia
Peptide and steroid hormones[a]	ACTH, glucagon, dexamethasone, prednisone, hydrocortisone, betamethasone	Diagnostic testing
Illegal recreational drugs	Ketamine, cocaine	
Chewing tobacco		

Partially adapted from [6, 27–29]

[a]These drugs have therapeutic or diagnostic use in PHEO/PGL, but usually only after pretreatment with appropriate antihypertensives (e.g., α-adrenoceptor blockers)

mal appetite, warmth with or without heat intolerance, or general weakness [6, 7, 35]. Less commonly, PHEO/PGL presents as fever of unknown origin, constipation due to catecholamine-induced decrease in intestinal motility, or cholesterol gallstones [38–40]. Due to the metabolic effects of epinephrine, hyperglycemia with low levels of plasma insulin associated with hypertensive episodes can occur. PHEOs/PGLs can also cause insulin resistance and diabetes mellitus manifestation [7, 41–47]. Rarely, PHEOs/PGLs can produce vasoactive intestinal peptide resulting in watery diarrhea, hypokalemia, and achlorhydria [48].

Patients may also complain of symptoms resulting from compression of tissues surrounding the tumor. For example, tumors located in the abdomen or chest can cause abdominal or chest pain. Patients with PGLs of the neck can present with dysphagia and dysphonia, and those with tumors growing in the head and neck area can display tinnitus, hearing loss, or cranial nerve palsy [49].

Since PHEO/PGL can have potentially life-threatening consequences, recognizing the signs and symptoms of these tumors leading to appropriate diagnostic sequence is critical. Evaluation for PHEO/PGL should be warranted in patients: (a) with a family history of PHEO/PGL or certain hereditary cancer syndromes (Table 12.5); (b) presenting with hypertension, tachycardia, sweating, and pallor; (c) presenting with resistant hypertension; (d) presenting with any paroxysmal symptoms; (e) presenting with hypertension and other symptoms in response to examination, anesthesia, surgery, certain medications, or foods and drinks; and (f) with adrenal incidentalomas [7, 11, 17, 51].

Table 12.5 Susceptibility genes and hereditary cancer syndromes associated with PHEO/PGL development and genotype-phenotype correlations

Molecular cluster	Gene	Locus	Mutation type	Inheritance	Syndrome	PHEO/PGL penetrance	Biochemical phenotype	Typical tumor localization	Malignancy rate	Associated clinical characteristics/other tumors
Krebs cycle (cluster 1a)	SDHA	5p15	Germline	AD	PGL5	Unknown	Unknown	HNPGL/TAPGL	0–14%	• Homozygots: Leigh's syndrome • Clear cell renal carcinoma • Gastrointestinal stromal tumors • Pituitary adenomas
	SDHB	1p36.13	Germline and somatic	AD	PGL4	30–100%	MN, NMN, MTY, NS	TAPGL; rarely HNPGL/adrenal	31–71%	• Clear cell renal carcinoma • Gastrointestinal stromal tumors • Pituitary adenomas • Possibly breast carcinoma • Possibly papillary thyroid carcinoma
	SDHC	1q23.3	Germline	AD	PGL3	Unknown	MN, NMN, MTY, NS	HNPGL; rarely TAPGL/adrenal	Low	• Clear cell renal carcinoma • Gastrointestinal stromal tumors • Pituitary adenomas
	SDHD	11q23	Germline and somatic	AD PI	PGL1	73–90%	MN, NMN, MTY, NS	HNPGL; rarely TAPGL/adrenal	Low (<5%)	• Clear cell renal carcinoma • Gastrointestinal stromal tumors • Pituitary adenomas
	SDHAF2	11q12	Germline	AD PI	PGL2	100%	Unknown	HNPGL	Unknown	
	FH	1q42.1	Germline	–		Unknown	NMN	Adrenal/TAPGL	Unknown (High?)	• Leiomyomatosis of skin and uterus • Clear cell renal carcinoma
	MDH2	7q11.23	Germline	–		Unknown	Unknown (NMN?)	Unknown (TAPGL?)	Unknown	

(continued)

Table 12.5 (continued)

Molecular cluster	Gene	Locus	Mutation type	Inheritance	Syndrome	PHEO/PGL penetrance	Biochemical phenotype	Typical tumor localization	Malignancy rate	Associated clinical characteristics/other tumors
Pseudohypoxic (cluster 1b)	VHL	3p25.3	Germline and somatic	AD	Von Hippel-Lindau	10–20%	NMN	Adrenal; rarely TAPGL/HNPGL	Low (<5%)	• Hemangioblastomas • Clear cell renal carcinoma • Tumors of pancreatic islets • Retinal angioma • Retinal, pancreatic, and testicular cysts
	HIF2A	2p21	Germline[a] and somatic	–	Pacak-Zhuang syndrome	Unknown	NMN	TAPGL/adrenal	Unknown	• Somatostatinoma, often multiple • Polycythemia • Eye changes • Organ cysts
	PHD1/EGLN2	19q13.2	Germline	–		Unknown	NMN	Unknown	Unknown	• Polycythemia
	PHD2/EGLN1	1q42.2	Germline	–		Unknown	NMN	Unknown (TAPGL?)	Unknown	• Polycythemia
Kinase signaling (cluster 2)	NF1	17q11.2	Germline and somatic	AD	Neurofibromatosis type 1	<6%	MN, NMN	Adrenal; rarely TAPGL	11%	• Café au lait spots • Neurofibromas • Freckles • Benign hamartomas of iris (Lisch nodules) • Gliomas and optical gliomas • Duodenal somatostatinomas • Sphenoid dysplasia/pseudoarthritis

Gene	Locus	Inheritance	AD	MEN2		Metabolites	Location	Malignancy	Associated tumors/conditions
RET	10q11.21	Germline and somatic			50%	MN, NMN	Adrenal	Low (<1–5%)	• Medullary thyroid carcinoma • Hirschsprung's disease • Hyperparathyreosis/hypercalcemia (MEN2A) • Marfanoid habitus, ganglioneuromas (MEN2B)
TMEM127	2q11.2	Germline	–		Unknown	MN and NMN	Adrenal/TAPGL/HNPGL	Low (4%)	• Possibly breast carcinoma • Possibly papillary thyroid carcinoma
MAX	14q23.3	Germline and somatic	–		Unknown	NMN and MN	Adrenal	10–25%	• Neuroblastoma
KIF1Bβ	1p36.22	Germline	–		Unknown	Unknown	Unknown (adrenal?)	Unknown	• Lung, colorectal adenocarcinoma • Neuroblastoma
H-RAS	11p15.5	Somatic	–		N/A	MN, NMN	Adrenal/TAPGL	Unknown	
K-RAS		Somatic	–		N/A	Unknown	Adrenal	Unknown	
ATRX	Xq21.1	Somatic	–		N/A	Unknown	Unknown (adrenal? TAPGL?)	Unknown	

AD autosomal dominant, *ATRX* alpha thalassemia/mental retardation syndrome X-linked, *EGLN1/2* Egl-9 family hypoxia-inducible factor 1/2, *FH* fumarate hydratase, *HIF2A* hypoxia-inducible factor 2α, *HRAS* Harvey rat sarcoma viral oncogene homolog, *HNPGL* head and neck paraganglioma, *IDH* isocitrate dehydrogenase, *KIF1Bβ* kinesin family member 1B, *MAX* myc-associated factor X, *MDH2* malate dehydrogenase 2, *MEN2A/2B* multiple endocrine neoplasia, type 2A/2B, *MN* metanephrine, *MTY* methoxytyramine, *NF1* neurofibromin 1, *NMN* normetanephrine, *NS* nonsecreting, *PGL* paraganglioma, *PHD1/2* prolyl hydroxylase domain-containing protein 1/2, *PHEO* pheochromocytoma, *PI* paternal inheritance, *RET* rearranged during transfection proto-oncogene, *SDHA, SDHB, SDHC, SDHD* succinate dehydrogenase subunits A, B, C, and D, *SDHAF2* SDH complex assembly factor 2, *TMEM127* transmembrane protein 127, *TAPGL* thoracic and abdominal paraganglioma, *VHL* von Hippel-Lindau [6, 50]

aPresent as PHEO/PGL with polycythemia only, most probably differ from presentation and characteristics of tumors associated with somatic *HIF2A* mutations

Biochemical Diagnosis of Pheochromocytoma

Initial Biochemical Testing

Most PHEOs and PGLs are characterized by excessive production of catecholamines, and thus, biochemical evidence of catecholamine production is an elementary step in diagnosis. Historically, biochemical diagnosis of PHEO/PGL relied on the measurement of urinary and plasma catecholamines (epinephrine and norepinephrine) together with measurement of urinary levels of catecholamine metabolites and vanillylmandelic acid. These tests can often lead to false negatives due to fluctuating levels of catecholamine release in many PHEOs/PGLs [10, 28]. Since PHEOs/PGLs often secrete catecholamines episodically, plasma or urinary levels of catecholamines may be normal. Approximately 30% of PHEOs/PGLs do not secrete catecholamines, even if they still synthesize them or they do not secrete catecholamines in amounts sufficient enough to produce the classical clinical presentation of a tumor with positive test results [52, 53]. Moreover, catecholamines are normally produced by the adrenal medulla and sympathetic nerves. Thus, high catecholamine levels are present in multiple different diseases and conditions and are not specific for these tumors [53].

On the other hand, metanephrines, the O-methylated metabolites of catecholamines, are produced continuously within PHEO/PGL cells, and their production is independent of catecholamine release [54, 55]. Therefore, diagnostic evaluation of plasma-free or urine-fractionated (i.e., normetanephrine and metanephrine measured separately) metanephrines is preferred and is currently the most sensitive diagnostic test (97% sensitivity and 93% specificity for measurement for plasma-free metanephrines) [8, 56–63]. Plasma metanephrines are usually measured in the free form. Metanephrines in urine are measured after deconjugation, although measurement of urine-free metanephrines is also possible [64, 65].

When interpreting the results, differentiating between a mild and high increase in catecholamine or metanephrine levels is very important. In patients with biochemically active tumors, an increase is usually two to four times higher than the upper reference limit. Mildly elevated levels of catecholamines are mostly due to interfering medications [10] (Table 12.6). If it is difficult to distinguish an increased catecholamine release due to sympathetic activation or from the presence of PHEO/PGL, a clonidine suppression test can be performed [66, 67]. Under physiologic conditions, clonidine suppresses release of neuronal norepinephrine (and so normetanephrine). A decrease in elevated plasma normetanephrine levels by ≥40% or within the reference limits after clonidine is administered indicates that sympathetic activation is the cause of elevation. Failure to depress plasma normetanephrine supports the presence of PHEO/PGL [67]. The clonidine suppression test has a high diagnostic sensitivity when combined with measurement of plasma normetanephrine responses to suppression. False positive elevations in plasma normetanephrine levels can be accurately identified with the combination of these tests. However, the reliability of the test can be compromised by tricyclic antidepressants

Table 12.6 Compounds that may cause false-positive elevations of plasma and urinary catecholamines or metanephrines

Compound group	Examples	Catecholamines		Metanephrines	
		NE	E	NMN	MN
Tricyclic antidepressants	Amitriptyline, imipramine, nortriptyline	+++	−	+++	−
α-Blockers (nonselective)	Phenoxybenzamine	+++	−	+++	−
α-Blockers (α₁-selective)	Doxazosin, terazosin, prazosin	+	−	−	−
β-Blockers	Atenolol, metoprolol, propranolol, labetalol	+	+	+	+
Calcium channel antagonists	Nifedipine, amlodipine, diltiazem, verapamil	+	+	−	−
Vasodilators	Hydralazine, isosorbide, minoxidil	+	−	?	?
Monoamine oxidase inhibitors	Phenelzin, tranylcypromine, selegiline	−	−	+++	+++
Sympathomimetics	Ephedrine, pseudoephedrine, amphetamines, albuterol	++	++	++	++
Stimulants	Caffeine, nicotine, theophylline	++	++	?	?
Miscellaneous	Levodopa, carbidopa	++	−	?	?
	Cocaine	++	++	?	?

Partially adapted from [8]

E epinephrine, *MN* metanephrine, *NE* norepinephrine, *NMN* normetanephrine, +++ substantial increase, ++ moderate increase, + mild increase, − little or no increase, ? unknown

and diuretics [42], as well as in patients with normal or only mildly elevated plasma catecholamine levels, despite the presence of PHEO/PGL [8].

In addition to measurement of metanephrines, recent evidence suggests that plasma 3-methoxytyramine is a biomarker for dopamine-producing tumors. Although it is currently only available in certain research centers, measurements of this biomarker are valuable for detecting very rare, exclusively dopamine-producing tumors (due to the lack of dopamine β-hydroxylase), which can be easily overlooked by solely measuring metanephrines [68, 69]. Moreover, methoxytyramine can also serve as an indicator of malignancy [4]. Exclusively dopamine-secreting tumors can also be detected by measuring serum levels of dopamine. Measurement of urinary dopamine levels is not useful, since it reflects dopamine production in the renal tubules, not in a potential tumor [27].

A nonspecific biomarker of neuroendocrine tumors, chromogranin A, is often measured in PHEO/PGL patients. Chromogranin A is commonly secreted by chromaffin cells, and its levels are elevated in 91% of patients with PHEO/PGL [70]. Despite its nonspecificity, chromogranin A, in combination with catecholamine measurement, can facilitate the diagnosis of PHEO/PGL, especially in tumors related to mutations in succinate dehydrogenase (SDH) subunit B gene [71, 72]. Chromogranin A is also a helpful diagnostic tool in patients with biochemically silent tumors and in disease monitoring [73, 74].

In very rare cases, PHEOs/PGLs can co-secrete other hormones, for example, ACTH or cortisol. These patients often present with the clinical picture of Cushing disease in addition to PHEO/PGL [75–77].

Determining the biochemical phenotype of PHEO/PGL can also be helpful in navigating further investigation and treatment, specifically localization of the tumor by imaging studies, genetic screening, determining the presence of metastatic disease, and an appropriate adrenergic blockade. Based on the type of catecholamines secreted, PHEOs/PGLs can be divided into three basic biochemical phenotypes: (a) adrenergic (epinephrine/metanephrine), (b) noradrenergic (norepinephrine/normetanephrine), and (c) dopaminergic (dopamine/methoxytyramine). Mixed phenotypes are common, and in such cases, PHEO/PGL-producing metanephrine and normetanephrine are considered adrenergic, and those secreting normetanephrine and methoxytyramine are considered dopaminergic. Since epinephrine/metanephrine are produced almost exclusively (99%) in the adrenal gland, adrenergic biochemical phenotype is typical for PHEO (Table 12.5).

Follow-Up Biochemical Testing

Although PHEOs/PGLs are rare tumors, a large number of patients are tested for these tumors in the process of differential diagnosis for secondary hypertension as well as other diseases. Because of this, false-positive results are expected, and they may outnumber true-positive results, even when tests with high specificities are used. This requires follow-up biochemical testing in patients with initially positive results, to confirm or rule out PHEO/PGL. However, when judging the likelihood of a PHEO/PGL from a single test, the degree of initial clinical suspicion or pretest probability of the tumor should be taken into account, which impacts the posttest probability of a tumor [7]. Moreover, patients with known hereditary syndromes associated with PHEO/PGL or with a history of tumors should periodically undergo screening. Biochemical testing is also used to confirm the success of surgical treatment and to evaluate activity of the disease in patients with metastases.

Sample Collection and Test Interferences

To ensure the reliability of biochemical test results, it is crucial to guarantee certain conditions during blood sample collection. Before collection of blood for measurements of plasma-free metanephrines, patients should be lying supine in a quiet room for at least 20–30 min with a previously inserted intravenous line (to minimize sympathoadrenal activation associated with venipuncture or upright posture) [53, 78]. Alternatively, with a higher risk of false-positive results, the sample may be collected from a seated patient, provided that upper reference limits obtained after supine rest are used [79]. If seated test returns back positive, it should be repeated after rest in supine position to rule out false positivity of initial test [8].

Although 24-h urine collection seems to solve the problem with the rigid conditions needed for blood sampling, it is not that simple. Twenty-four-hour urine results are not always accurate because of unreliable collection from the patient. Samples are also often influenced by diet and the activation of sympathoneuronal and adrenal medullary systems (e.g., during physical activity or changes of posture). To ensure more controlled conditions for urine collection, some investigators advocate spot or overnight urine collections with normalization of output catecholamines or metanephrines against urinary creatinine excretion [8, 80].

Blood samples should be stored on ice immediately after collection and separated plasma at −80 °C upon analysis. Urine samples should be refrigerated during the collection period while using HCl as a preservative. Urine sample aliquots should be stored frozen at −80 °C to minimize auto-oxidation and deconjugation [53].

When evaluating a patient for possible PHEO/PGL, it is necessary to ask the patient about their current medications. Certain compounds can increase catecholamine levels or interfere with the diagnostic analysis and, thus, result in catecholamine/metanephrine false positives [78]. The major source of interference is tricyclic antidepressants, which can lead to significant elevation of plasma or urinary metanephrines due to inhibition of catecholamine reuptake. Acetaminophen, a drug commonly used for pain and fever, as well as mesalamine and sulfasalazine, can interfere with high-performance liquid chromatographic (HPLC) assays used for measurement of catecholamines [67, 81]. An anxiolytic agent, buspirone, can cause falsely elevated levels of urinary metanephrine in some HPLC assays [67]. Other compounds that may distort catecholamine/metanephrine measurements are listed in Table 12.6.

Localization of Pheochromocytoma

PHEO/PGL localization should only be initiated if clinical evidence for the presence of a tumor is reasonably convincing and biochemical results are strongly positive [28, 59]. If biochemical evidence of a tumor is not compelling, imaging is only justified in patients with a higher probability of tumor development, such as those with hereditary predisposition, previous history of the tumor, or evidence of biochemically silent tumor in carriers with one of the PHEO/PGL susceptibility genes [8, 59]. Based on the tumor biochemical profile, imaging can initially be focused on certain areas of the body. In patients with elevated metanephrine/epinephrine levels, imaging should primarily center on the adrenal gland, as adrenergic phenotype is associated mostly with adrenal tumors. If the scan of adrenal glands is normal, imaging of additional areas of the body should be performed, specifically the abdomen, pelvis, chest, and neck ("eyes to thighs"). A detailed history and careful physical examination may also provide critical information about the possible location of a PHEO/PGL. For instance, postmicturition hypertension suggests urinary bladder PGL [8].

According to expert recommendations, optimal results for PHEO/PGL localization and confirmation are achieved by performing anatomical imaging studies (computed tomography (CT) and magnetic resonance imaging (MRI)) in combination

with functional (nuclear medicine) imaging studies [59]. Functional imaging is very useful in detecting primary or metastatic tumors, which could be missed with anatomical modalities [6]. Moreover, imaging plays an important role in the decision-making approach. For example, identification of multiple lesions or metastases before an initial surgery may completely change the treatment plan [8]. An algorithm for localization of PHEO/PGL is depicted in Fig. 12.1. Imaging is also an important part of screening patients with known genetic predisposition to PHEO/PGL development and for follow-up for patients with a history of PHEO/PGL. For
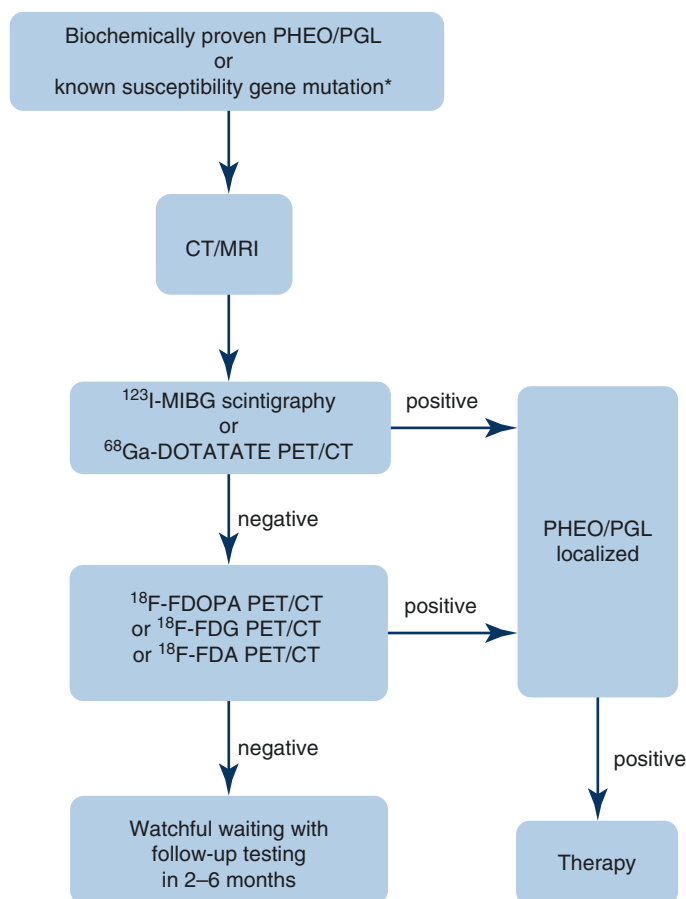
Fig. 12.1 Algorithm for localization of PHEO/PGL. In patients with a biochemically proven PHEO/PGL, as well as in patients with a susceptibility gene mutation known to be associated with a nonsecretory phenotype, anatomic imaging of adrenals/abdomen is suggested. If the result is negative, chest and neck CT or MRI scans should be performed. Afterward, the presence of PHEO/PGL should be confirmed or ruled out with functional imaging [8]. *18F-FDA* 18F-fluorodopamine, *18F-FDG* 18F-fluorodeoxyglucose, *18F-FDOPA* 18F-dihydroxyphenylalanine, *68Ga-DOTATATE* 68Ga-DOTA(0)-Tyr(3)-octreotate, *123I-MIBG* 123I-metaiodobenzylguanidine, *CT* computed tomography, *MRI* magnetic resonance imaging, *PET* positron emission tomography, *PGL* paraganglioma, *PHEO* pheochromocytoma

genetic mutation carriers, CT or MRI is recommended every few years along with biochemical evaluation. This is particularly important for carriers of the mutations in genes encoding succinate dehydrogenase subunits (*SDHx*: *SDHA*, *SDHB*, *SDHC*, *SDHD*) and patients with head and neck PGLs, as these patients often present with biochemically silent tumors [28, 82].

Anatomical Imaging

Anatomical imaging of PHEO/PGL should initially focus on the abdomen and pelvis, followed by the chest and neck if abdominal and pelvic scans are negative [59]. Computed tomography and MRI are widely used in the diagnostic workup for PHEO/PGL, and these modalities have been reported to have similar diagnostic sensitivities [10], although MRI may be superior to CT in detecting extra-adrenal tumors in certain locations (e.g., cardiac) [8]. Ultrasound is not recommended for initial PHEO/PGL localization. Exceptions include ruling out tumors in children and pregnant women, when MRI is not available.

Excellent spatial resolution, wider availability, and a relative low cost suggest a CT scan of the abdomen, with or without contrast, as an initial PHEO/PGL localization method [28]. Computed tomography can be used to localize tumors 1 cm or larger. The sensitivity of CT is approximately 95%, with specificity roughly 70% [83]. Use of intravenous contrast media is preferred to enhance the specificity of the method. However, the CT scan may fail to localize recurrent PHEOs/PGLs because of postoperative anatomical changes and the presence of surgical clips.

An MRI with or without gadolinium enhancement is a very dependable imaging method with sensitivity >95% and specificity similar to CT (70–80%) [10, 84, 85]. MRI has a high sensitivity in detecting adrenal lesions (93–100%) and is a good imaging modality for the detection of intracardiac, juxtacardiac, and juxtavascular PGLs. Moreover, MRI offers feasibility of multiplanar imaging and superior assessment of the relationships between tumor and surrounding vessels. This is important in the evaluation of patients with tumors in the adrenal and cardiac areas and for ruling out vessel invasion. The sensitivity of MRI for detection of extra-adrenal, metastatic, or recurrent PHEOs/PGLs is around 90%. Thus, MRI is preferred in patients with head and neck PGLs and metastatic disease and in patients with CT contrast allergies or in whom radiation exposure is contraindicated (pregnant women, children, patients with known germline mutations, patients with recent excessive radiation exposure) [10, 28].

Functional Imaging

Functional imaging plays an important role in PHEO/PGL workup. Specificity of anatomical imaging studies for PHEO/PGL is not sufficient. Thus, functional studies are needed to confirm the presence of a PHEO/PGL. Functional imaging may also help detect primary and/or metastatic tumors that could be missed on anatomical studies. It is also used to characterize the metabolic activity of the tumors in vivo and for

restaging aggressive tumors following treatment completion [86]. Functional imaging studies are enabled by the presence of the cell membrane and/or vesicular catecholamine transport systems in PHEO/PGL cells. Functional imaging modalities used to confirm PHEO/PGL and/or metastatic disease include ^{123}I-metaiodobenzylguanidine (^{123}I-MIBG)scintigraphy,6-^{18}F-fluorodopamine(^{18}F-FDA),[18]F-dihydroxyphenylalanine (^{18}F-DOPA), ^{11}C-hydroxyephedrine, and ^{11}C-epinephrine (not used anymore) positron emission tomography (PET) [87–93]. Currently, these methods are not widely available, and if needed, patients are referred to specialized centers.

In metastatic PHEO/PGL, tumor dedifferentiation may lead to loss of specific neurotransmitter transporters, resulting in difficulties with its localization. In such cases, ^{18}F-fluorodeoxyglucose (^{18}F-FDG) PET imaging or somatostatin receptor scintigraphy may be required. Metastatic PHEOs/PGLs often express somatostatin receptors, which enables somatostatin scintigraphy with the somatostatin analogue octreotide (octreoscan) or DOTA peptides analogues (^{68}Ga-DOTA(0)-Tyr(3)-octreotate (DOTATATE) PET/CT) [94–97].

Metaiodobenzylguanidine Scintigraphy

Historically, functional imaging has been performed using MIBG labeled with radioactive iodine (^{123}I and ^{131}I). MIBG is an aralkylguanidine similar to norepinephrine. ^{131}I-MIBG scintigraphy is not used for imaging because of its longer half-life and lower sensitivity (50%) compared to ^{123}I-MIBG (92–98% in nonmetastatic tumors, 57–79% for metastases) [90, 98–103]. Although the sensitivity of ^{123}I-MIBG for detection of metastases is low, it is very useful in identification of patients who can possibly benefit from palliative treatment with therapeutic doses of ^{131}I-MIBG. Besides PHEOs/PGLs, ^{123}I-MIBG uptake also occurs in other neuroendocrine tumors, such as glomus tumors, carcinoids, or in the sporadic and familial medullary carcinomas of the thyroid. [123/131]I-MIBG is physiologically accumulated in the myocardium, spleen, liver, urinary bladder, lungs, salivary glands, large intestine, and cerebellum. Furthermore, in 75% of patients, uptake is shown in normal adrenal glands.

Before MIBG scintigraphy, it is important to withhold the drugs interfering with accumulation of MIBG—it is suggested to do so for 2 weeks prior to exam. Interfering drugs include compounds that deplete catecholamine stores, compounds that inhibit cell catecholamine transporters, and other drugs such as calcium channel blockers or certain α- and β-blockers (Table 12.7) [104–106]. Appropriate blockade with potassium iodide, potassium iodate, 1% Lugol's solution, or potassium perchlorate is required to prevent an uptake and accumulation of free iodide ([123/131]I) in the thyroid gland [104].

Imaging scans are performed at 24 h and again at either 48 or 72 h after injection of the radioisotope, to decipher whether images from earlier scan are tumors or are physiological and fading out.

Table 12.7 Compounds interfering with MIBG uptake by tumors

Interfering agent group	Example	Mechanism of interference	Length of discontinuation before MIBG imaging/treatment
Combined α-/β-blocker	Labetalol	MIBG uptake inhibition	72 h
Adrenergic neurons blockers	Reserpine, bretylium	MIBG storage depletion	48 h
Calcium channel blockers	Amlodipine, diltiazem, nifedipine	MIBG uptake inhibition	48–72 h
Inotropic sympathomimetics	Dobutamine, dopamine	MIBG storage depletion	24 h
Vasoconstrictor sympathomimetics	Ephedrine, phenylephrine, norepinephrine	MIBG storage depletion	24–48 h
β2 adrenoceptor stimulants (sympathomimetics)	Salbutamol, terbutaline, fenoterol	MIBG storage depletion	24 h
Other adrenoceptor stimulants	Orciprenaline	MIBG storage depletion	24 h
Sympathomimetics for glaucoma	Brimonidine, dipivefrine	MIBG storage depletion	48 h
Tricyclic antidepressants	Amitriptyline, clomipramine, nortriptyline	MIBG uptake inhibition	24–48 h
Tricyclic-related antidepressants/atypical antidepressants	Maprotiline, trazodone, venlafaxine, mirtazapine	MIBG uptake inhibition	48 h–8 days
Antipsychotics (neuroleptics)	Chlorpromazine, haloperidol, perphenazine, risperidone	MIBG uptake inhibition	24 h–7 days, 1 month for depot forms
CNS stimulants	Amphetamines, cocaine, caffeine, phenylpropanolamine	MIBG uptake inhibition/MIBG storage depletion/unknown	24 h–5 days
Sedating antihistamines	Promethazine	MIBG uptake inhibition	24 h
Systemic and local nasal decongestants, compound cough and cold preparations	Pseudoephedrine, phenylephrine, phenylpropanolamine	MIBG storage depletion	48 h
Opioid analgesics	Tramadol	MIBG uptake inhibition	24 h

Refs [104, 105]

Positron Emission Tomography

PET has become a more widely available and valuable imaging method, offering high sensitivity, shorter acquisition times, low radiation exposure, and superior spatial resolution [86, 107]. Moreover, PET provides a quantifiable estimate of tumor metabolism using standard uptake values (SUV) [86].

Most of the tumors, including PHEO/PGL, exhibit increased glucose metabolism, which enables the use of glucose labeled with [18]F (fluoride) for imaging [91, 92]. ^{18}F-FDG PET is highly sensitive for the detection of metastatic PHEO/PGL (approx. 90%), especially in patients harboring SDH subunit B (*SDHB*) gene mutations. Sensitivity of ^{18}F-FDG PET for detection of primary, nonmetastatic PHEOs/PGLs is 88%, with a specificity similar to that of ^{123}I-MIBG. Thus, ^{18}F-FDG PET/CT is recommended for localization of metastatic disease [28, 108].

The majority of the radiopharmaceuticals used for PET detection of PHEO/PGL enter the tumor cell using the cell membrane norepinephrine transporter. A positron emitting analogue of dopamine, 6-^{18}F-FDA, is a very useful sympathoneuronal PET imaging agent for catecholamine-synthesizing cells [109]. ^{18}F-FDA PET has a high sensitivity for both primary PHEOs/PGLs and metastases (77–100% and 77–90%, respectively), with specificity more than 90% [100, 108]. Unfortunately, ^{18}F-FDA PET/CT is not yet widely available. Other PET imaging tracers, ^{11}C-hydroxyephedrine and ^{11}C-epinephrine, have been shown to only have limited application in diagnostic imaging because of the short half-life of [11]C (20 min) [110, 111].

^{18}F-DOPA is an amino acid analogue and catecholamine precursor that is taken up by the amino acid transporter [112]. Pretreatment with carbidopa enhances tumor uptake of tracer and improves sensitivity due to inhibition of DOPA decarboxylase [113]. ^{18}F-DOPA is extremely sensitive (81–100%) for the localization of nonmetastatic PHEO/PGL and head and neck PHEO/PGL [87, 108, 114, 115]. However, for detection of metastatic and *SDHB* mutation-related PHEOs/PGLs, sensitivity is not satisfactory (45% and 20%, respectively) [8].

Somatostatin Receptor-Based Imaging

Somatostatin receptors are expressed in up to 73% of PHEO/PGL cells in vitro [116], and scintigraphy using octreotide (^{111}In-pentreotide) has been used for PHEO/PGL localization, specifically for localization of head and neck PGLs. However, the sensitivity of this imaging modality is low, and thus, it is inferior to ^{123}I-MIBG scintigraphy [117]. Still, octreoscan can be useful in detection of tumors that express somatostatin receptors and are negative on other scans [118].

For somatostatin receptor-based PET/CT imaging, radiolabeled DOTA peptide analogues (DOTATATE, DOTATOC, and DOTANOC) were shown to be superior to all other imaging methods. For instance, ^{68}Ga-labeled DOTA peptides have been found to be highly sensitive for localization of neuroendocrine tumors, including PHEO/PGL [96, 97, 119–126]. In recent studies from Janssen et al. [94, 95], ^{68}Ga-DOTATATE PET/CT was shown to be clearly superior to all other functional imaging modalities, including ^{18}F-FDG PET/CT, ^{18}F-FDOPA PET/CT, and ^{18}F-FDA PET, for localization of both sporadic and *SDHB*-related metastatic PHEO/PGL.

The costs of imaging based on radiolabeled DOTA peptides are comparable to ^{18}F-FDG PET or ^{123}I-MIBG scintigraphy. These new modalities are expected to be more broadly available in few years.

Genetic Testing

From a clinical point of view, it is necessary to consider genetic testing in addition to diagnostics and appropriate therapy, especially in patients with a suspected hereditary form of the disease and in their first-step relatives. If the PHEO/PGL susceptibility germline mutation is present, patients need to be screened regularly, even if the disease is not obvious. Particular gene mutations present with a specific clinical and biochemical phenotype. Early identification of a mutation allows a physician to predict the course of disease, risk of malignancy, and heritability and helps to choose an appropriate treatment strategy. In order to diagnose PHEO/PGL and identify a specific mutation, it is necessary to take a comprehensive personal and family history, perform meticulous clinical and biochemical examinations, and use adequate imaging methods. In PHEO/PGL, it is important to particularly assess the location of a tumor, biochemical phenotype, age of a patient, and the presence of any tumors besides PHEO/PGL [6].

Hereditary disease should be suspected in patients from families with two or more cases of PHEO/PGL or with syndromes associated with PHEO/PGL. In syndromic forms of PHEO/PGL, underlying mutations can be predicted based on a combination of characteristic tumor types in the patient or their family members (e.g., renal cell carcinoma and hemangioblastoma or retinal angioma in von Hippel-Lindau (VHL) disease, medullary thyroid cancer in multiple endocrine neoplasia type 2) or based on characteristic clinical phenotype (e.g., café au lait spots and eye symptoms—Lisch nodules, optical gliomas—in neurofibromatosis type 1, polycythemia in mutations in hypoxia-inducible factor 2α (*HIF2A*) gene, Hirschsprung's disease in multiple endocrine neoplasia type 2) [127, 128]. Hereditary PHEO/PGL syndromes, associated tumors, and other characteristics associated with various mutations are in Table 12.5.

From a biochemical point of view, adrenergic mixed phenotype is observed in PHEOs/PGLs associated with *NF1* (neurofibromin 1), *RET* (rearranged during transfection), *KIF1Bβ*, and *MAX* (myc-associated factor X) mutations. *TMEM127* (transmembrane protein 127)-mutated tumors present with high metanephrine concentrations, which means they are solely adrenergic [50, 129]. PHEOs/PGLs associated with mutations in *VHL*, *SDHx*, *SDHAF2* (SDH assembly factor 2), *HIF2A*, *FH* (fumarate hydratase), *IDH* (isocitrate dehydrogenase), and *PHD1/2* (HIF prolyl hydroxylase domain-containing protein 1/2) genes are mostly extra-adrenal, except for *VHL*-mutated tumors [50, 130–132], and usually present with dopaminergic and/or noradrenergic biochemical phenotype [54, 133, 134]. *VHL*- and *HIF2A*-mutated PHEOs/PGLs typically exhibit a noradrenergic phenotype, and *SDHx* tumors are associated with a dopaminergic component. Rarely, they are biochemically silent [50, 129, 133, 134] (Fig. 12.2).

Presence of metastases in PHEO/PGL patient leads to suspicion of *SDHB* or *FH* mutations. Familial forms of PHEO/PGL usually show autosomal dominant inheritance, which means that children of mutation carriers have a 50% chance of inheriting the mutation from a parent. However, we cannot forget about the low penetrance

Fig. 12.2 Algorithm for genetic testing based on biochemical phenotype and clinical presentation of PHEO/PGL. In patients with a known family history of PHEO/PGL susceptibility mutation, this mutation should be tested first. Patients presenting with features of one of the syndromes associated with PHEO/PGL (NF1, VHL, MEN2, Pacak-Zhuang, etc.) should be tested for genetic mutations linked to those syndromes. In NF1, diagnosis is usually made on clinical presentation alone, and no genetic testing is needed. In patients without a known family history of PHEO/PGL, the direction of genetic testing is based upon the biochemical profile and localization of the tumor. For some of the known genetic mutations, biochemical profile and/or tumor location has not yet been elucidated (Table 12.5) [6, 135–137]. *FH* fumarate hydratase, *HIF2A* hypoxia-inducible factor 2α, *MAX* myc-associated factor X, *MEN2* multiple endocrine neoplasia, type 2, *NF1* neurofibromin 1, NF1 neurofibromatosis type 1, *PGL* paraganglioma, *PHEO* pheochromocytoma, *RET* rearranged during transfection proto-oncogene, *SDHA, SDHB, SDHC, SDHD* succinate dehydrogenase subunits A, B, C, and D, *SDHx* succinate dehydrogenase complex gene mutations, *TMEM127* transmembrane protein 127, *VHL* von Hippel-Lindau

of PHEO/PGL in some mutations (e.g., *SDHx*)—in these cases, a negative family history does not rule out the presence of a familial form of PHEO/PGL. In *SDHD*, *SDHAF2*, and *MAX* mutations, the risk of disease depends on which parent is the mutation carrier. The disease will only develop if the mutated gene is inherited from the father [138]. Germline mutations are present in 8–24% of apparently sporadic PHEOs/PGLs. These mutations often appear as *de novo* mutations or are associated with low penetrance [127, 128, 139]. In these patients, an underlying gene mutation should be considered if the tumor is extra-adrenal and malignant or if the diagnosis of PHEO/PGL is made at an early age [136].

Management of Patient with PHEO/PGL

Appropriate management of patients with PHEO/PGL requires a close collaboration of several specialists, including an endocrinologist, internist, radiologist, anesthesiologist, surgeon, and, if needed, oncologist [10, 59]. Currently, the only available curative treatment for PHEO/PGL is surgery. Thus, the optimal therapy for PHEO/PGL is a prompt, ideally complete, surgical removal of the tumor to prevent potentially life-threatening complications. Surgical debulking or extensive metastases removal may also allow for long-term remission in patients with locoregional or isolated resectable distant metastases, or it can palliate symptoms related to tumor mass or catecholamine excess [140]. Systemic chemotherapy and radiotherapy are possible treatment options for patients who are not surgical candidates, although these have only palliative character. However, recent progress in understanding the molecular mechanisms involved in PHEO/PGL development has driven introduction of new promising therapeutic options.

Medical Management and Preparation for Surgery

Immediately after diagnosis, all patients with biochemically active PHEO/PGL should be placed on sufficient adrenoceptor blockade to control symptoms and reduce the risk of hypertensive crises and organ damage mediated by the effects of released catecholamines [6]. There is no consensus regarding the drugs recommended for preoperative management because of wide-ranging practices, international differences in available or approved therapies, and a lack of studies comparing different medications. However, α-adrenoceptor antagonists, calcium-channel blockers, and angiotensin-receptor blockers are recommended [28, 141]. Drugs that can be used in symptom management and presurgical blockade, with suggested doses, are listed in Table 12.8.

If surgery is planned, medication should be introduced at least 7–14 days before the procedure to control blood pressure and heart rate, restore the catecholamine-induced volume depletion, and prevent a surgery-induced catecholamine storm and its consequences [28, 29, 59, 142]. Although there are some speculations regarding the need of preoperative adrenoceptor blockade, the potential benefit of preventing unpredictable

Table 12.8 Drugs used for symptom management and presurgical blockade in patients with PHEO/PGL

Drug	Classification	Suggested dose	Use	Common side effects
α-Adrenoceptor blockers				
Phenoxybenzamine	Long lasting, irreversible, noncompetitive	10 mg 1–3 times daily	First choice for α-adrenoceptor blockade	Orthostatic hypotension, nasal congestion, tachycardia, dizziness
Prazosin	Short acting, specific, competitive	2–5 mg 2–3 times daily	– When phenoxybenzamine is not available	
Terazosin	Short acting, specific, competitive	2–5 mg daily	– For patients who do not tolerate phenoxybenzamine	
Doxazosin	Short acting, specific, competitive	4–24 mg 2 times daily	– For patients with mild hypertension	
β-Adrenoceptor blockers				
Atenolol	Cardioselective	12.5–25 mg 2–3 times daily	To control tachyarrhythmia resulting from catecholamine excess or from α-adrenoceptor blockade	Fatigue, dizziness, exacerbation of asthma
Metoprolol	Cardioselective	25–50 mg 3–4 times daily		
Propranolol	Nonselective	20–80 mg 1–3 times daily		
Calcium channel blockers				
Amlodipine		10–20 mg daily	– To provide additional control of hypertension for patients on α-adrenoceptor blockers	Headache, edema, dizziness, nausea
Nicardipine		60–90 mg daily		
Nifedipine	Extended-release action	30–90 mg daily	– For patients who do not tolerate α-adrenoceptor blockers	
Verapamil	Extended-release action	180–540 mg daily	– For patients with intermittent hypertension	
Catecholamine synthesis inhibitors				
Metyrosine		250 mg every 8–12 h for a total dose of 1.5–2 g daily	To provide additional control of hypertension for patients on adrenoceptor blockade	Severe fatigue, sedation, depression, anxiety, galactorrhea, extrapyramidal side effects, nausea

Partially adapted from [6, 8, 141]

instability in blood pressure during surgery is much higher than a relatively low risk of medication-related adverse effects [28]. Preoperative preparation should also include a high-sodium diet and fluid intake to reverse catecholamine-induced blood volume contraction as a prevention of severe hypotension after tumor removal [28].

Alpha-adrenoceptor blockade is usually achieved with oral phenoxybenzamine, which exhibits long-lasting effects that diminishes only after *de novo* α-adrenoceptor synthesis. The initial dose of 10 mg twice daily is titrated upward until symptoms are controlled or side effects appear. Treatment goals are to normalize blood pressure, prevent paroxysmal hypertensive episodes, and eliminate tachyarrhythmias. Side effects of the treatment include orthostatic hypotension, tachycardia, nasal congestion, dry mouth, diplopia, and ejaculatory dysfunction. In patients who do not tolerate phenoxybenzamine, or if the drug is unavailable, other α-blocking agents can be used. These include prazosin, terazosin, or doxazosin. All three agents are short-acting, specific, competitive α$_1$-adrenergic antagonists, and they also have potential to cause severe postural hypotension [28, 59].

If adequate control of blood pressure and tachyarrhythmias is not achieved by α-blockers, β-blockade is initiated. β-Adrenoceptor blockers should be administered only after adequate pretreatment (at least for 3–4 days) with α-adrenoceptor antagonists to prevent unopposed epinephrine-induced vasoconstriction, which can lead to a hypertensive crisis [13, 141, 143]. Labetalol, which has both α- and β-adrenoceptor antagonistic activities, may also be used in PHEO/PGL patients in doses 200–600 mg twice daily [144]. However, the fixed ratio of α- and β-adrenoceptor blocker (1:4) is often not suitable for patients with PHEO/PGL, and thus, the use of individual α- and β-adrenoceptor antagonists is a better option.

A tyrosine hydroxylase inhibitor, metyrosine, is another valuable medication in management of patients with PHEO/PGL. Metyrosine competitively inhibits tyrosine hydroxylase, resulting in significant depletion of catecholamine production. Thus, metyrosine facilitates control of blood pressure both before and during surgery, especially during the induction of anesthesia and manipulation of the tumor, which can cause both extensive sympathetic activation and catecholamine release. Metyrosine crosses the blood-brain barrier. Therefore, it inhibits catecholamine synthesis not only in the periphery but also in brain and often causes sedation, depression, anxiety, galactorrhea, and, very rarely, extrapyramidal signs. Due to the severity of side effects, the use of metyrosine is reserved for patients with very high levels of catecholamines and those with extensive metastatic disease.

Calcium channel blockers control hypertension and tachyarrhythmias by blocking norepinephrine-mediated calcium influx into vascular smooth muscles. Calcium channel blockers can be used alongside α-adrenoceptor blockers if the blockade is not sufficient. They can replace α-adrenoceptor blockers in patients who are unable to tolerate them [29, 145].

For acute management of hypertensive crisis, either intravenous phentolamine (5 mg every 2 min until adequate control of hypertension or a continuous infusion of 100 mL of phentolamine in 500 mL of 5% dextrose) or sodium nitroprusside (continuous intravenous infusion) is effective. Sometimes, nifedipine (10 mg) administered orally or sublingually can be used to control hypertension. If tachycardia

is present, β-adrenoceptor blockers may be administered. However, they should never be used before α-adrenoceptor blockers, as previously discussed. A list of medications that can precipitate hypertensive episodes in patients with PHEO/PGL is in Table 12.4.

A proposed algorithm for presurgical management of PHEO/PGL patients is shown in Fig. 12.3.
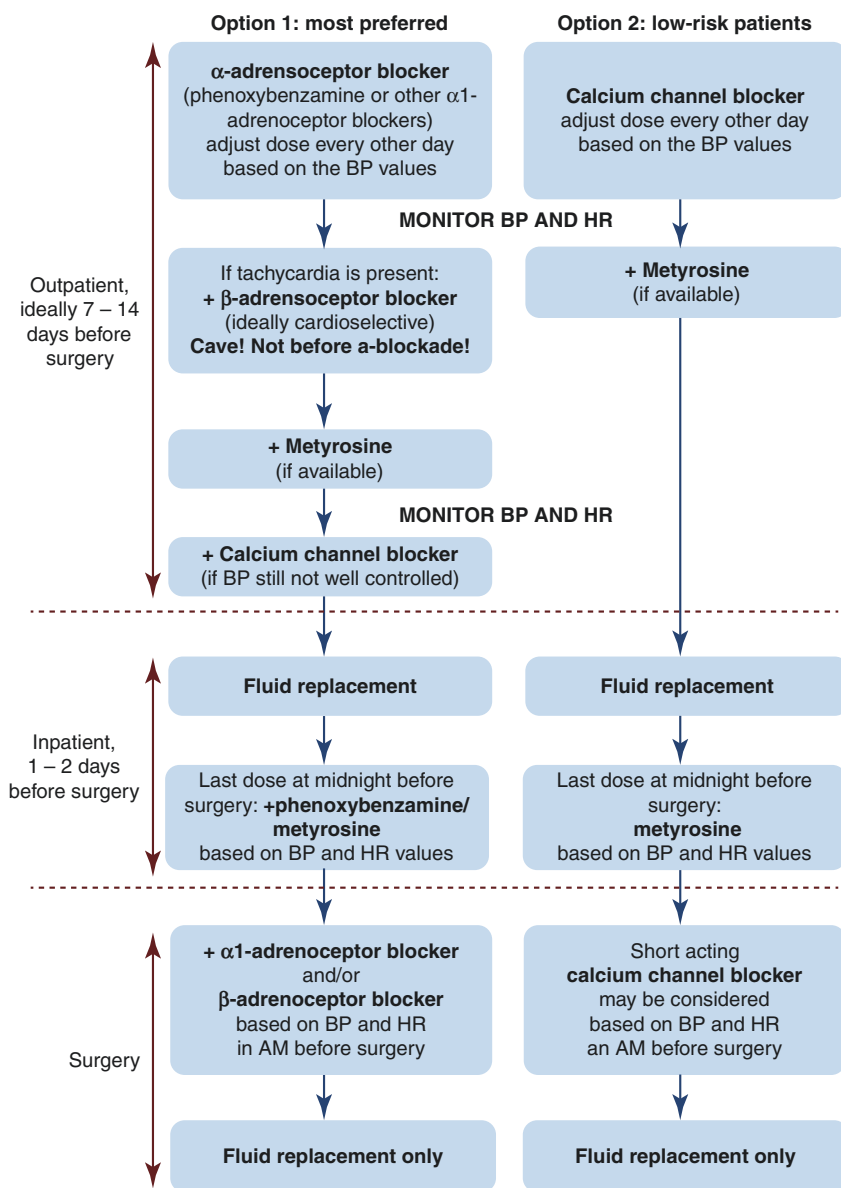
Fig. 12.3 Algorithm for presurgical management of patients with PHEO/PGL [29]. *AM* morning, *BP* blood pressure, *HR* heart rate, *PGL* paraganglioma, *PHEO* pheochromocytoma

Surgical and Perioperative Management

PHEO/PGL surgery requires a multidisciplinary approach and should preferably be performed in centers experienced with PHEO/PGL management and treatment. The current standard for surgical management of adrenal PHEOs is minimally invasive surgery, which can be performed as transperitoneal laparoscopic adrenalectomy or retroperitoneoscopic adrenalectomy [146–148]. For PGLs, open resection is suggested, since they are more likely to be malignant and often arise in areas not accessible for laparoscopy. However, laparoscopic surgery can be used to remove intra-abdominal PGLs smaller than 6 cm [28, 149]. Advantages of laparoscopic approach over an open procedure include lower morbidity, less postoperative pain, and a faster recovery [150]. However, caution should be exercised as the insufflation of carbon dioxide to produce a pneumoperitoneum is associated with iatrogenic acidosis and mechanical compression, which can cause catecholamine release from the tumor [150]. The operation should be converted to an open resection if laparoscopic approach is difficult [28]. For large tumors, laparoscopic surgery may be used, but it requires precise and gentle dissection in order to prevent profuse bleeding, the disruption of the tumor capsule leading to intraoperative dissemination, peritoneal seeding, and recurrence of the tumor [146, 147]. Another method, a single-port robotic adrenalectomy, has been reported as a safe and effective method for both complete and partial resection of PHEO with similar success rates, decreased postoperative pain, and shorter hospitalization, but with higher costs compared to laparoscopic and open surgery [151–153].

In patients with sporadic and hereditary bilateral adrenal PHEOs and in those with small tumors who have undergone a complete resection of a contralateral adrenal gland, a cortical sparing adrenalectomy is recommended [28]. This approach is safe, and if a sufficient amount of adrenal cortex is preserved, it could prevent postoperative adrenal insufficiency and requirements for glucocorticoid and mineralocorticoid replacement [154–156]. Retention of normal glucocorticoid function after cortical sparing partial adrenalectomy is achieved in >50% of patients [157–160]. The risk of recurrence due to residual medullary tissue is reported to be between 0 and 21% [157–159, 161].

In patients with metastatic disease, surgery can be performed for either noncurative debulking or for aggressive resection with the goal of complete remission. Palliative debulking surgery can be performed in some cases for immediate risk reduction and resection of the lesions affecting crucial structures [142, 162]. Surgical debulking can also improve the efficacy of adjuvant therapies by reducing tumor mass and burden [142]. Extensive surgery for metastatic disease can result in successful outcomes and remission in patients with metastases limited to the abdomen [163, 164]. Intraoperative use of gamma probes labeled with radiopharmaceuticals (e.g., ^{123}I-MIBG) can facilitate identification of metastatic sites missed on anatomical imaging [165, 166].

A critical component of intraoperative patient management is avid communication between the surgical and anesthesia teams to anticipate and reduce the likelihood of major hemodynamic events and other complications. The most frequent PHEO/PGL surgery complications include significant changes in blood pressure,

arrhythmias, and hyper- or hypoglycemia. Hypertension surges may occur during induction of anesthesia, intubation, tumor palpation and manipulation, or ligation of tumor vessels. Intraoperative hypertensive crises are managed by administration of intravenous sodium nitroprusside in conjunction with nitroglycerine (to reduce pre-load), phentolamine, nicardipine, and fenoldopam [29, 167]. Intravenous magnesium sulfate is also used for management of hypertension during PHEO/PGL resection, as it can inhibit catecholamine release from the adrenal medulla, enhance vasodilatation, block catecholamine receptors, and prevent arrhythmias [27, 168]. Severe hypotension may develop following tumor ligation. Hypotension results from a combination of several factors: the loss of catecholamine secretion from tumor, continued α-adrenoceptor blockade, increased systemic capacitance from antihypertensive drugs, contracted plasma volume, surgical bleeding, and anesthetic-induced vasodilation. It may become profound and persistent [27, 167]. In such situations, massive volume resuscitation with saline infusion should be initialized with discontinuation of vasodilators. Vasopressors should be administered only if hypotension persists after plasma expansion to euvolemia. In refractory cases, vasopressin or methylene blue can be utilized. In surgical settings it is important to exclude hemorrhage as a cause of persistent hypotension.

Arrhythmias usually occur during induction or maintenance of general anesthesia. Arrhythmias can be managed with esmolol, propranolol, or lidocaine, but caution is needed when administering these drugs to patients with severe ventricular dysfunction [27].

Hyperglycemia due to increased catecholamine-stimulated glycogenolysis and lipolysis is present in approximately 60% of patients with PHEO/PGL [143]. It is important not to overtreat hyperglycemia as insulin administered during surgical procedure may worsen hypoglycemia after tumor removal or during postoperative period [27]. To prevent hypoglycemia, which often occurs after tumor removal, intravenous glucose replacement (5% dextrose) can be administered.

Postoperative Management

In the postoperative period, patients are at risk for development of complications including hypertension, hypotension, arrhythmias, rebound hypoglycemia, renal dysfunction, or prolonged intubation. Therefore, blood pressure, heart rate, and plasma glucose levels should be closely monitored for 24–48 h after surgery [143]. Moreover, attention has to be paid to patients who underwent a bilateral adrenalectomy, bilateral cortical-sparing adrenalectomy, or unilateral cortical-sparing adrenalectomy of a sole remaining adrenal gland, because of the potential adrenal insufficiency. This requires cautious observation of plasma glucose, electrolytes levels, and endocrine functions.

For postsurgical hypotension, volume replacement is the first-choice treatment. Use of vasopressors may not be effective because of long-acting α-adrenoceptor blockers and catecholamine synthesis inhibitors (metyrosine) used in preoperative

treatment. Often, a high fluid volume is required during the first 24–48 h after surgery (until sympathetic nervous system resumes autoregulation). If hypotension persists regardless of adequate volume replacement, vasopressor agents should be administered [8].

Hypertension after surgery for PHEO/PGL may be caused by volume overload, autonomic instability, or pain. These are treated symptomatically. However, it may also indicate that not all tumor tissue has been resected or that there is a coexistence of essential hypertension.

Biochemical confirmation of remaining tumor should not be done earlier than 5–7 days after surgery to ensure that increases in plasma and urinary catecholamines produced during surgery have dissipated. Ideally, postoperative measurements of metanephrines and 3-methoxytyramine should be obtained 2–6 weeks after surgery. Measurements of 3-methoxytyramine should become commercially available in the near future. Follow-up biochemical screening for recurrent or metastatic disease should be done annually for lifetime or immediately if symptoms reappear [28]. In patients with biochemically silent tumors, annual or biannual imaging studies are performed instead of biochemical testing [169, 170].

Management of Metastatic Pheochromocytoma and Paraganglioma

In PHEO/PGL, there are no known cellular or molecular markers for determining malignancy. Metastatic PHEOs/PGLs are only defined by the presence of metastases at sites where chromaffin cells are not normally present [171]. Metastases can spread via hematogenous or lymphatic pathways, and the most common metastatic sites include lymph nodes, bones, lungs, and liver. In PHEO/PGL patients, metastasis may be present at time of diagnosis or appear months or years later [140]. Frequency of metastases in patients with PHEO/PGL ranges from 1% to 34%. Higher incidence of metastatic disease is associated with certain genetic backgrounds (e.g., mutations in *SDHB* and *FH* genes). Moreover, metastases are more prevalent in PGLs than in PHEOs, ~25% vs. ~10%, respectively [2, 142, 172]. Regardless of the genetic background, patients with PHEO/PGL need to be followed up on a long-term basis, as metastases may occur even 20 years after presentation of a primary tumor.

Clinical manifestation of metastatic PHEO/PGL is similar to benign tumors. Additionally, a patient may present with symptoms related to local invasion of tumors. Some patients may exhibit minimal symptoms or do not have any symptoms at all, despite significantly elevated catecholamine levels. This most likely occurs due to desensitization of adrenoceptors by constantly high catecholamine concentrations.

Biochemical diagnosis and localization of metastatic disease follow the same algorithm as benign PHEOs/PGLs. Metastatic tumors secrete mostly norepinephrine [4, 173, 174], and patients usually have higher levels of plasma and urinary

normetanephrine, which reflects a larger tumor size [175, 176]. Moreover, increased excretion of dopamine and its metabolite, 3-methoxytyramine, is associated with metastatic disease [4, 173, 177]. For localization of metastatic tumors, ^{18}F-FDG PET is more useful than specific positron-emitting compounds or ^{123}I-MIBG scintigraphy, because of dedifferentiation of the tumor and loss of expression of cell membrane and vesicular transporter systems [8, 101], as discussed in the tumor localization section.

Management of metastatic PHEO/PGL is a challenging task and requires a multidisciplinary approach. Currently, there is no effective treatment for metastatic PHEO/PGL. The aim of planned treatment interventions is an attempt for definitive cure of limited disease and palliation for advanced disease. In patients with slowly progressive disease, a wait-and-watch strategy can be applied with regular biochemical and radiological follow-ups. Each step in treatment should be individualized to the patient's needs and intended therapeutic goals. An active therapeutic intervention is needed in the presence of uncontrolled hormone- or tumor-related symptoms or high tumor burden as defined by RECIST criteria (seven or more bone metastases, replacement of >50% of the liver parenchyma, multiple pulmonary nodules >2 cm, or significant radiologic progression) [178, 179]. Besides pharmacotherapy of hypertension and other symptoms and surgical debulking, current treatment options for metastatic PHEO/PGL include local ablative therapies, radiotherapy, and chemotherapy (Fig. 12.4). Novel molecular-targeted therapies are under development and may be introduced in the near future.

Targeted Radiotherapy

^{131}I-MIBG therapy is a systemic targeted radiotherapy used in patients with inoperable metastatic disease and positive uptake on ^{123}I-MIBG scintigraphy. Treatment can be administered as a single dose (50–900 mCi) or as multiple doses (100–300 mCi) at intervals of 3–6 months, with a total dose limit of 1000 mCi [181, 182]. Higher doses are associated with serious hematologic and non-hematologic toxicity [182]. However, when multiple doses of low to intermediate ^{131}I-MIBG activities (50–350 mCi) are administered, treatment is well tolerated, with minimal toxicity, which includes mild bone marrow suppression, mildly elevated liver enzymes, some renal toxicity, and hypothyroidism. Around 30% of patients show partial response (<50% reduction of tumor mass). Complete response is low, 0–18%. Nevertheless, a significant number of patients report symptomatic and biochemical responses [182, 183].

Before treatment administration, several precautions must be taken: (a) patients should be on antihypertensive drugs that do not interfere with ^{131}I-MIBG uptake (phenoxybenzamine, nifedipine, atenolol), and all interfering drugs (Table 12.7) have to be discontinued before therapy; (b) 24–48 h prior to therapy and 10–15 days after therapy, patients have to take potassium iodine, saturated solution of potassium iodide, Lugol's solution, or potassium perchlorate to protect the thyroid from accumulation of radioiodine; (c) because of the hematologic, liver, and renal toxicity of ^{131}I-MIBG, the patient's hematopoietic parameters, liver, and renal function should be adequate [8, 182].
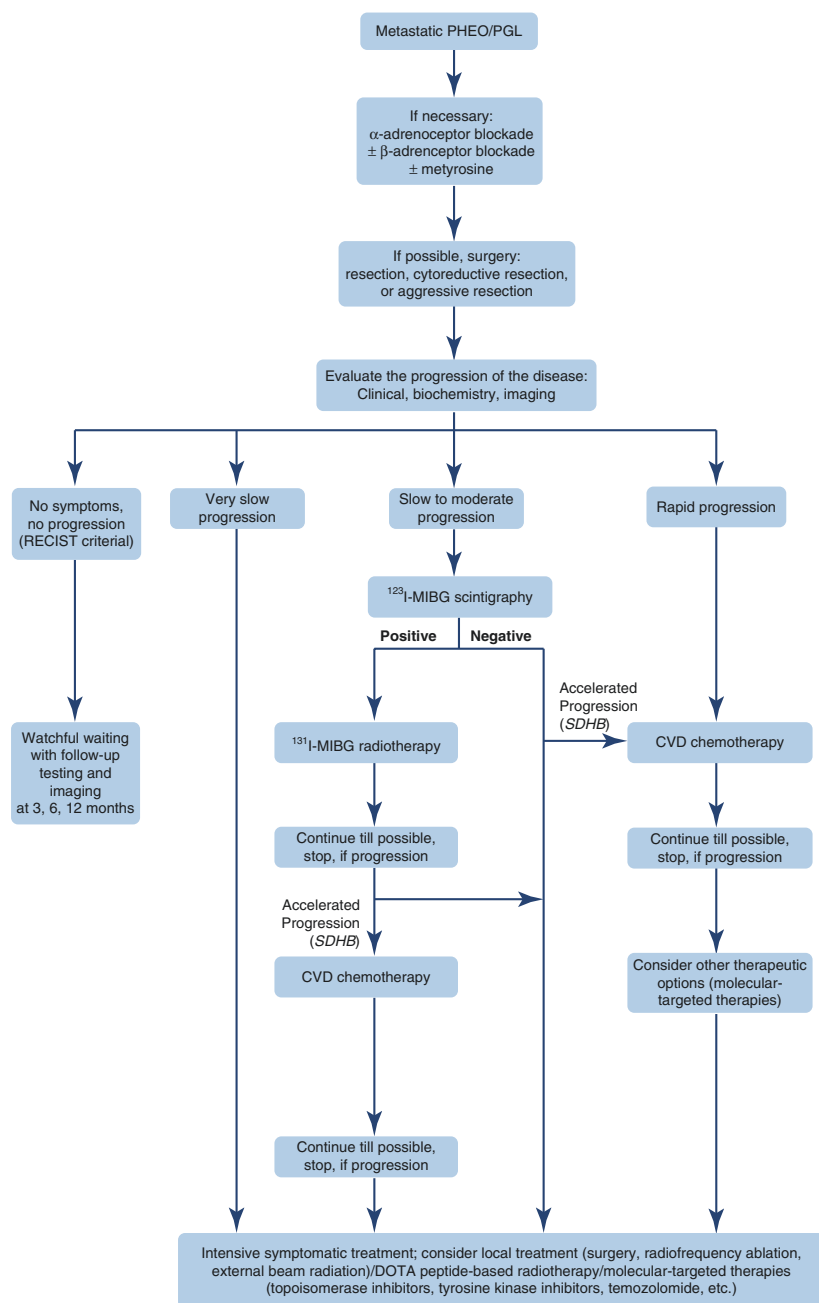
Fig. 12.4 Algorithm for management of metastatic PHEO/PGL [8, 180]. *CVD* cyclophosphamide, vincristine, and dacarbazine, *MIBG* metaiodobenzylguanidine, *PGL* paraganglioma, *PHEO* pheochromocytoma, *RECIST* response evaluation criteria in solid tumors, *SDHB* succinate dehydrogenase subunit B

Another promising targeted radiotherapy treatment is represented by radionuclide-labeled DOTA peptide-based therapy (DOTATATE, DOTATOC, DOTANOC), which targets somatostatin receptors and uses the long-acting, somatostatin analogue octreotide (TATE, TOC, NOC) conjugated with radionuclide-labeled DOTA (lutetium, ^{177}Lu; yttrium, ^{90}Y; or indium, ^{111}In). Since many PHEOs/PGLs express somatostatin receptors, treatment can be used in patients with positive octreoscan or ^{68}Ga-DOTATATE imaging [184–191].

Radiofrequency Ablation and External Beam Radiation

Percutaneous radiofrequency ablation (RFA) is a safe and minimally invasive treatment modality aimed at growth inhibition and control. It is used for management of painful metastasis and symptoms related to catecholamine excess. RFA has been shown to be particularly effective in the treatment of osseous and liver lesions, which are successfully ablated without recurrence [192–195].

In the past, use of external beam radiation therapy (EBRT) in PHEO/PGL treatment has been controversial, since metastatic PHEOs/PGLs were considered to be resistant to radiation. However, recent data suggests that EBRT can be used as adjuvant treatment to chemotherapy and systemic radiotherapy for local control of bulky metastases, especially with osseous lesions. EBRT seems to be effective in symptomatic control of disease in patients with a limited burden of metastases. Moreover, it is also becoming accepted as a first-line therapy for head and neck PGLs [196–198]. Head and neck PGLs are often localized in inaccessible areas or are extremely adherent to adjacent structures. Therefore, surgical resection cannot be performed, or it would result in devastating postoperative morbidity related to dysfunction of cranial nerves [199]. Radiotherapeutic options for the treatment of non-resectable head and neck tumors include the traditional fractionated EBRT or radiosurgery using Gamma Knife, linear accelerator (LINAC), or CyberKnife. Development of stereotactic radiosurgery allows for delivery of high doses of radiation with more precise targeting and a decreased toxicity in the surrounding tissue [200]. Several studies have shown successful tumor control (stabilization or regression of tumor volumes) along with symptomatic improvement in more than 95% of patients with glomus jugulare tumors [201–203].

Systemic Chemotherapy

Systemic chemotherapy is recommended for rapidly progressive inoperable metastatic PHEOs/PGLs and for patients with a high tumor burden or a large number of bone metastases. In such cases, it primarily serves as a palliative treatment option, improving patient's quality of life. In addition, it may also serve as a neoadjuvant therapy to improve chances of successful surgical treatment of large tumors.

Although there is not a chemotherapy combination or single treatment that would have long-term efficacy in metastatic PHEO/PGL, some of the used regimens can stabilize disease for several years. The combination of cyclophosphamide, vincristine, and dacarbazine (CVD), administered intravenously in 21-day cycles, is the

most extensively used for PHEO/PGL. This approach yields complete or partial response rates of 57% [180, 204]. Moreover, 79% of treated patients had a complete or partial biochemical response and showed objective improvement in performance status and clinical findings [204]. Effectiveness of CVD chemotherapy might be evident within 1–3 months after initiation of treatment. In responders, chemotherapy should be continued, although the evidence of increased overall survival is conflicting [205–208]. CVD chemotherapy has been found to be particularly beneficial in patients with mutations in the *SDHB* gene [209].

There is anecdotal evidence of successful treatment of individual cases using alternative chemotherapeutic agents alone or in combination, including temozolomide; cyclophosphamide and methotrexate, thalidomide, ifosfamide, etoposide, carboplatin, vincristine, cyclophosphamide, and doxorubicin; and cisplatin and 5-fluorouracil [206, 210–213].

Molecular-Targeted Therapies

Recently, progress in the understanding of pathophysiological mechanisms involved in development of PHEO/PGL was made, especially due to the identification of disease susceptibility genes. These discoveries allowed for identification of dysfunction of several critical signaling pathways and, thus, potential novel treatment targets.

For instance, the hypoxia-inducible factor (HIF) signaling pathway has been found to be activated in certain PHEOs/PGLs due to mutations in genes encoding Krebs cycle enzymes (*SDHx*, *FH*, *IDH*, *MDH2*), mutations in *HIF2A* gene, or upstream regulators of HIF signaling, reviewed in [214, 215]. Moreover, gene expression studies revealed an increased expression of hypoxia-angiogenic pathway components, e.g., vascular endothelial growth factors (VEGFs) and other growth factors. Therefore, inhibiting or modifying metabolic processes and enzymes participating in metabolic reprogramming poses a promising therapeutic strategy. Potential molecular/metabolic therapeutic targets are listed in Table 12.9.

Table 12.9 Potential molecular/metabolic therapeutic targets in PHEO/PGL

Therapeutic target	Treatment effects	Examples of agents
HIF signaling (reviewed in [216–218])		
HIF-α mRNA/protein expression	Inhibition of HIF-α mRNA or protein expression resulting in decreased HIF-α accumulation and activation	Wortmannin, LY94002, GFC-0941, PI-103, rapamycin, PP242, aminoflavone, glyceollins, topotecan, EZN-2968, 2ME2, ENMD-1198, geldanamycin and analogues, vorinostat, YC-1, PX-478, PX-12, cardiac glycosides, FM19G11, HIF-2α translational inhibitors
HIF-α dimerization	Inhibition of HIF-α/HIF-1β dimerization	Acriflavine, PT2385

(continued)

Table 12.9 (continued)

Therapeutic target	Treatment effects	Examples of agents
HIF binding to DNA	Inhibition of HIF dimers binding to DNA	Echinomycin, polyamides
HIF transcriptional activity	Inhibition of transcription of HIF target genes	Chetomin, bortezomib, amphotericin B, triptolide, AJM290, AW464
Hypoxia	Apoptosis of hypoxic cell	Hypoxia-activated prodrugs: TH-302, EO9, AQ4N, PR-104, tirapazamine, SN30000, TH-4000
Angiogenesis	VEGF, VEGFR inhibition	Sunitinib, sorafenib, pazopanib, bevacizumab
Glycolysis (reviewed in [217, 219])		
Glucose uptake	Inhibition of glucose transport	GLUTs inhibitors: flavonoids (phloretin, silybin), STF-31, WZB117, ritonavir
HK 1/2	Inhibition	3-Bromopyruvate 2-deoxyglucose, lonidamine
PDK1	Inhibition (to allow activity of PDH)	Dichloroacetate
PFKB3	Inhibition	3PO
LDHA	Inhibition	shRNA, gossypol/AT-101 and derivatives, galloflavin
PKM2	Induction of apoptosis	Somatostatin and its derivatives, TLN-232/CAP-232
MCTs	Inhibition of lactate transport	AZD3965 MCT1/2 specific inhibitors, CHC, phenylpyruvate, bioflavonoids
Glutaminolysis (reviewed in [220])		
Glutamine uptake	Glutamine transporters inhibition	BCH, GPNA, benzylserine
Glutaminase	Inhibition	BPTES/CB-839, compound 968
GOT2/GPT2	Inhibition	Aminooxyacetate
GDH1	Inhibition	Purpurin/R162, EGCG
Fatty acid and lipid synthesis (reviewed in [221–223])		
ACLY	Inhibition	SB-204990
Acyl-CoA synthetase	Inhibition	
Acetyl-CoA carboxylase	Inhibition, induction of apoptosis/autophagy	Soraphen A
Fatty acid synthase	Inhibition, induction of apoptosis	Cerulenin, C75, flavonoids
Choline kinase	Inhibition	MN58b
Phospholipid metabolism	Inhibition	Metformin
Dysfunctional Krebs cycle enzymes and metabolites (reviewed in [217, 224])		
IDH1/2	Inhibition of IDH mutants, inhibition of 2HG production	Mutant IDH inhibitors

Table 12.9 (continued)

Therapeutic target	Treatment effects	Examples of agents
α-Ketoglutarate-dependent dioxygenases	Restoring the function	α-Ketoglutarate analogues
Low citrate	Increase in citrate levels, inhibition of PFK, arrest of glycolysis, induction of apoptosis	Citrate [225]
Proton extrusion (reviewed in [217, 221])		
Na+/H+ exchanger	Inhibition	Cariporide, amiloride
Bicarbonate/Cl- exchanger	Inhibition	S3705
MCT1 lactate/H+ symporter	Inhibition	α-Cyano-4-OH-cinnamate
Carbonic anhydrases 9 and 12	Inhibition	Sulfonamide indisulam
F1F0 ATP synthase	Inhibition	Angiostatin
V-ATPase	Inhibition	Bafilomycin A1
Other (reviewed in [221])		
DNA methylation	Inhibition of methylation	DNA demethylating agents: decitabine [216]
AMPK	Activation	Biguanides, thiazolidinediones, bevacizumab, erlotinib
LAT1	Inhibition of amino acid transport	2-Aminocyclo (2.2.1)-heptane 2-carboxylic acid
SIRT1	Stimulation of SIRT1-dependent deacetylation PGC1α	Resveratrol
ROS	Neutralizing ROS by antioxidants to reduce HIF-α activation	N-Acetylcysteine, vitamin C
ROS	Induction of ROS overproduction	Menadione, gadolinium texaphyrin, β-lapachone
Antioxidant systems (GSH)	Inhibition to achieve ROS accumulation	Buthionine sulfoximine, isothiocyanates, mangafodipir

Adapted from [226]

3PO 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-on; *Acetyl-CoA* acetyl coenzyme A, *ACLY* ATP citrate lyase, *AMPK* AMP-activated protein kinase, *ATP* adenosine triphosphate, *BCH* 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid, *BPTES* bis-2-[5-phenylacetamido-1,2,4-thiadiazol-2-yl]ethyl sulfide, *CHC* α-cyano-4-hydroxycinnamate, *EGCG* epigallocatechin gallate, *GDH1* glutamate dehydrogenase 1, *GLUT* glucose transporter, *GOT2* glutamate oxaloacetate transaminase 2, *GPNA* γ-L-glutamyl-p-nitroanilide, *GPT2* glutamate pyruvate transaminase 2, *HIF* hypoxia-inducible factor, *HK* hexokinase, *IDH* isocitrate dehydrogenase, *LAT1* L-type amino acid transporter 1, *LDHA* lactate dehydrogenase A, *MCT* monocarboxylate transporter, *PDH* pyruvate dehydrogenase, *PFK* phosphofructokinase, *mRNA* messenger RNA, *PGC1α* peroxisome proliferator-activated receptor-γ co-activator 1α, *PHD* prolyl hydroxylase domain-containing protein, *PKM2* pyruvate kinase, isoenzyme 2, *ROS* reactive oxygen species, *SDH* succinate dehydrogenase, *SIRT1* sirtuin 1

One of the approaches is to modify/interrupt the HIF signaling pathway, and several possible strategies are being tested. For example, drugs inhibiting HIF mRNA or protein expression (such as antiangiogenic agents, drugs targeting PI3K/Akt/mTOR pathway, or heat shock protein 90 activity inhibitors). A few years ago, sunitinib, a tyrosine kinase inhibitor preventing angiogenesis through targeting the VEGF receptors, was introduced. In vitro studies have suggested that sunitinib induces apoptosis in rat PHEO cells and directly inhibits catecholamine synthesis by reducing the activity of tyrosine hydroxylase [227, 228]. However, when used in clinical settings, the results were conflicting [229–232]. Another compound, everolimus, targeting the mTOR pathway, was evaluated in a small number of patients with PHEO/PGL with disappointing results [233, 234]. However, there are current clinical trials evaluating the effects of combination therapy with mTOR and PI3K inhibitors in other cancers, which may show more significant results [235, 236].

Inhibition of HIF dimerization, which is one of the steps leading to HIF-α activation, and inhibition of HIF binding to DNA are other possible therapeutic targets [237], reviewed in [215, 218].

Cancer cell metabolism depends on glucose and glutamine. Therefore, altering the uptake of these nutrients by glucose transporters inhibitors, inhibitors of glycolytic enzymes, or inhibitors of glutaminolysis may be another promising treatment approach, reviewed in [219, 220, 238, 239].

Other options for targeted PHEO/PGL therapy include restoration of the enzymatic activity of nonfunctioning Krebs cycle enzymes, replenishment of depleted substrates for the cell, and inhibition of overexpressed enzymes. Therapeutic agents are under development, which include small molecule inhibitors of certain proteins or agents restoring functionality of Krebs cycle enzymes, reviewed in [215]. In *SDHB*-deficient cells, the use of proteostasis regulators (e.g., histone deacetylase inhibitors) leads to an increase in the total amount of SDHB protein in cell [240].

Tumors driven by *VHL* mutations display promoter hypermethylation of a few target genes and a prevalent hypomethylation outside CpG islands. *SDH*- and *FH*-mutated PHEOs/PGLs are characterized by the hypermethylator phenotype [241], suggesting the use of demethylating agents in the treatment of PHEO/PGL [216].

Although molecular therapies for PHEO/PGL are still under development, a deeper understanding of the molecular mechanisms associated with tumorigenesis facilitates identification of novel treatment targets and, in the near future, may help to improve outcomes of patients with metastatic PHEO/PGL.

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Chapter 13
Adrenal Cortical Carcinoma: Mitotane and Beyond

**Silvia De Francia, Paola Perotti, Vittoria Basile,
Antonina Germano, and Massimo Terzolo**

Introduction

Adrenocortical carcinoma (ACC) is a rare endocrine tumor characterized by a poor prognosis as the 5-year survival rate after diagnosis is less than 40% [1–3]. A limited range of therapeutic options is available for ACC: its rarity and aggressiveness have concurred to hamper progress in the development of treatment beyond surgery. In this grim scenario, mitotane remains a cornerstone in the management of patients with ACC. More than 50 years have passed since the introduction of mitotane in clinical practice; however, we still have many uncertainties on how to use this old drug and what we may expect in terms of activity [4]. Mitotane is currently used both in a postoperative adjuvant setting and in advanced disease. However, no data from randomized prospective trials are available to guide management.

Mechanism of Action of Mitotane

Mitotane, [1,1-dichlorodiphenildichloroethane (o,p'-DDD)], a parent compound of the insecticide dichlorodiphenyltrichloroethane (DDT), has been widely employed to treat ACC [1–3]. Mitotane has a profound effect on steroidogenesis [5, 6], but the specific mechanisms are not fully understood. The effect on adrenal steroidogenesis has been associated with the inhibition of a number of

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mitochondrial cytochrome P450-dependent enzymes: cholesterol side chain cleavage (CYP11A1), 11β-hydroxylase (CYP11B1), and 18β-hydroxylase (CYP11B2) [7, 8], as well as P450-independent enzymes, such as 3 β-hydroxysteroid-dehydrogenase [9]. Lin et al. [10] explored the effect of non-cytotoxic concentrations of mitotane on cortisol production by an immortalized clone of human ACC cell line (National Cancer Institute-Human 295 [NCI-H295] cells) and found that mitotane interferes with gene transcription of a number of steroidogenic enzymes. Steroidogenic acute regulatory (protein) (StAR) and CYP11A1, which are involved in the rate-limiting step of steroidogenesis, are most sensitive to mitotane, although at drug concentrations close to the therapeutic range (20–40 μM, i.e., 6.4–12.8 mg/L) (Fig. 13.1). Mitotane effect on 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) was biphasic, more stimulatory than inhibitory, contradicting early reports of a strong suppression of CYP11B1 activity [11]. These data are conflicting but may concur to explain why aldosterone synthesis is less affected than other steroid pathways. The anti-steroidogenetic effect of mitotane was also recently evaluated by van Koetsveld et al. [12], who investigated the effect of mitotane and interferon β in primary cultures of ACC and found that both drugs strongly inhibited mRNA expression of StAR, CYP11A1, 17α-hydroxylase (CYP17A1), and CYP11B1. Combination of mitotane and interferon β induced an additive inhibitory effect on cellular DNA number and cortisol secretion, suggesting that treatment with interferon β may increase sensitivity of ACC cells to mitotane. Lehmann et al. [13] studied the effect of a 24-h mitotane treatment on NCI-H295R cell viability and expression of genes involved in adrenal steroidosynthesis. It was found that mitotane markedly inhibited expression of genes coding for enzymes involved in generation of cortisol and dehydroepiandrosterone sulfate (CYP11A1 and CYP17A1). Moreover, mitotane reduced viability of NCI-H295R cells inducing cell apoptosis triggered by increased caspase 3 and caspase 7 activities. The mitotane-induced repression of genes of the steroidogenetic pathway has been confirmed by another study in the same cell line [14]. Chortis et al. [15] studied the steroid inhibitory effect of mitotane in vivo, using a novel steroidobolomic approach, to analyze 24-h urine samples from ACC patients receiving mitotane for adjuvant treatment or metastatic disease. It was found that mitotane downregulated the initial steps of steroidogenesis but did not influence CYP11B1 activity. As previously discussed, in vitro data are controversial about the mitotane effect on this enzymatic step. Moreover, mitotane was found to be a strong inducer of CYP3A4 activity leading to glucocorticoid inactivation and a consequent sharp rise in 6β-hydroxycortisol urinary excretion. It was calculated that mitotane is able to inactivate 50% of administered hydrocortisone, and this explains why patients on mitotane have an increased dose requirement of steroid replacement. Finally, mitotane proved to be a strong inhibitor of 5α-reductase activity, and this effect prompts to use 5α-dihydrotestosterone as androgen substitution in mitotane-treated men. An important mitotane-induced derangement of cortisol and testosterone metabolism has been also shown in a similar study [16]. To evaluate which are the intracellular targets of mitotane, Poli et al. [17] performed electron microscopy on human ACC H295R and SW13 cell lines. Increasing concentrations of mitotane
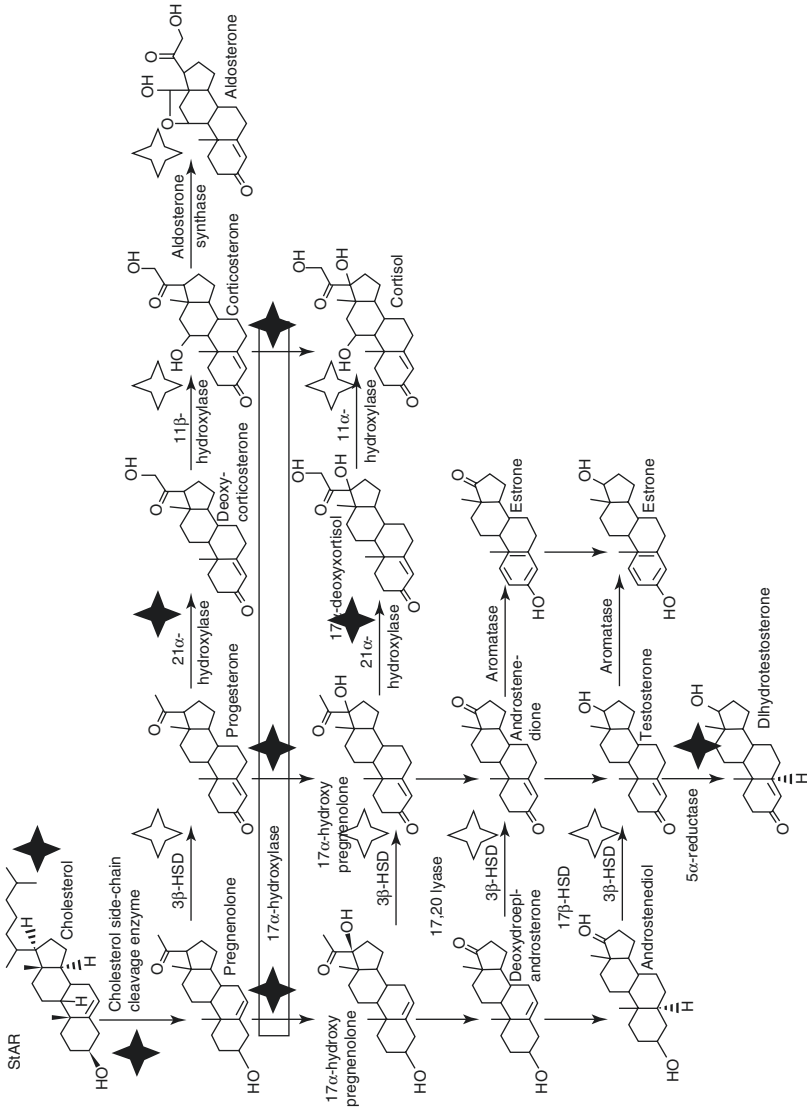
Fig. 13.1 Effects of mitotane on mRNA expression of several steroidogenic enzymes. Black diamonds identify a definitive inhibitory effect, whereas diamonds identify mixed effects

caused marked alterations in the morphology of mitochondria in a dose- and time-dependent manner. Mitochondria were finally disrupted leading to a drastic reduction of cell oxygen consumption. Mitotane was converted by the mitotane-sensitive H295R cells in its active metabolites and exerted cytostatic and cytotoxic effects at doses corresponding to the therapeutic window (30–50 μM, i.e., 9.6–16 mg/L). This study showed that mitotane effects seem to be mainly mediated by the mitochondria damage that activates an apoptotic process involving caspase 3 and caspase 7. Further data showing that mitotane affects mitochondrial function have been reported by Hescot et al. [18]. In H295R and SW13 cell lines, mitotane inhibited cell proliferation in a dose- and a time-dependent manner and suppressed cortisol and 17-hydroxyprogesterone through inhibition of a number of genes involved in steroidogenesis (StAR, CYP11A1, HSD3B2, CYP11B1, and CYP11B2). Mitotane hampered the mitochondrial respiratory chain function complex IV (cytochrome c oxidase), and this was accompanied by enhanced mitochondrial mass, as a compensatory mechanism in response to the respiratory chain defect. Furthermore, mitotane induced morphologic fragmentation of the mitochondrial membranes that are required for respiratory chain activity and presumably steroidogenesis.

More recently, Sbiera et al. [19] demonstrated that mitotane is an inhibitor of sterol-O-acyl-transferase 1 (SOAT1) leading to accumulation of free cholesterol at toxic levels for the cell. The fact that SOAT1 is predominantly expressed by the adrenals confers the specificity of action to mitotane. By inhibiting SOAT1, mitotane downregulates steroidogenesis and exerts its cytotoxic effect due to lipid-induced endoplasmic reticulum stress. In a small number of ACC tissues, SOAT1 expression correlated with the response to mitotane treatment, i.e., low SOAT1 expression was associated with poor response. Targeting cancer-specific lipid metabolism can then open new avenues for treatment of ACC. We should pay attention to potential drug binding, since mitotane is a lipophilic drug that accumulates in lipoproteins and induces dyslipidemia (hypercholesterolemia and/or hypertriglyceridemia). Previous studies suggested that the lipoprotein profile may influence mitotane drug distribution [20]. Moreover, high plasma mitotane levels have been described in dyslipidemic patients who did not exhibit any side effect, suggesting either methodological issues, or that plasma mitotane distribution in lipoprotein subtypes is a major determinant of its distribution in tissues [21]. Indeed, Hescot et al. recently reported that plasma mitotane levels were correlated with o,p′-DDD measured in HDL and LDL fractions [22], and in a subsequent case report, they showed the case of an ACC patient with severe dyslipidemia and very high levels of plasma mitotane but without any neurological side effects [23]. They demonstrated that dyslipidemia causes an overestimation of plasma mitotane levels explained by a so-called matrix effect. On this basis, only lipoprotein-free mitotane should be considered the therapeutically active fraction. This concept has been confirmed in vitro by Kroiss et al. [24] by means of demonstration of activity of mitotane inhibited by lipoprotein binding. However, measurement of lipoprotein-free mitotane levels has still to enter clinical practice even if the methodology is not technically demanding.

Mitotane in the Adjuvant Setting

The main predictor of outcome for ACC patients is the possibility of a radical surgery; still, fully half of the tumors that have been completely extirpated are doomed to relapse [25–30]. Since even stages I–II tumors recur frequently, surgical failure cannot be the only reason. Several potential predictive factors of recurrence in radically resected ACC have been identified [31, 32], but the issue of defining prognostic factors is complicated by the great variability of clinical presentation and biological heterogeneity of ACC. A so high recurrence rate has prompted to consider the use of systemic adjuvant therapy following ACC removal. However, the literature is conflicting for a variety of reasons (Table 13.1). First, most studies [26, 33, 38, 45] had limited statistical power. Second, many studies [26, 29, 37–39, 45] did not include a concomitant matched control group of untreated patients, whereas in some series a number of patients underwent multiple adjuvant treatments [28]. In addition, the definition of recurrence-free survival (RFS) has not been uniform, and the duration of response has been sometimes unclear. Finally, all studies but one [39] were retrospective and employed different formulations of mitotane at doses ranging from 3 to 20 g daily, which were given for different times.

Table 13.1 Outcome of adjuvant mitotane treatment

References	Patients treated with mitotane	Outcome
Schteingart [29]	4	Mean survival of 74 ± 33 months in patients who received adjuvant MIT. No control group
Venkatesh et al. [30]	7	After 1–4 years from surgery, 6/7 patients treated with adjuvant MIT are still alive. No control group
Bodie et al. [33]	21	No difference in survival between patients with or without ($n = 25$) adjuvant MIT. No information on DFS is given
Pommier and Brennan [28]	7	Mean DFS was 2.4 years for 10 patients treated adjuvantly (MIT in 7 and radiotherapy in 3 patients) and 2.5 years for 43 untreated patients (NS)
Vassilopoulou-Sellin et al. [34]	8	Median DFS was 10 months for the patients treated with adjuvant MIT vs 23 months for 6 untreated patients ($P < 0.01$). MIT was discontinued early in 5 patients for toxicity
Haak et al. [35]	11	Median survival of the patients treated with adjuvant MIT was 51 vs 61 months for untreated patients ($n = 15$) (NS). Six patients had MIT levels >14 mg/L
Barzon et al. [36]	7	Median DFS of 8 months in the patients treated with adjuvant MIT vs 13 months for untreated patients ($n = 11$) (NS). Nevertheless, 5/7 patients in MIT group are disease-free at the last follow-up (range 5–54 months), in contrast to 3/11 in the control group

Table 13.1 (continued)

References	Patients treated with mitotane	Outcome
Dickstein et al. [37]	4	DFS ranged 18–68 months. No control group
Kasperlik-Zaluska et al. [38][a]	55	At the last follow-up, 18/32 (56%) patients treated immediately after surgery are alive vs 6/27 (22%) patients treated with delay. Only 1/8 (12%) untreated patient is surviving. Adjuvant MIT was given irrespective of staging and completeness of surgery
Icard et al. [26][b]	83	Adjuvant MIT did not have an independent effect on survival. It is not reported whether the patients in MIT group had comparable prognostic factors with the untreated patients. No information on DFS is given
Baudin et al. [39]	11	Recurrence developed in 8 patients within 1 year; 6 of them had MIT levels >14 mg/L. No control group
Terzolo et al. [40]	47	Increased risk of recurrence in two concomitant control groups of untreated patients (group 2, $n = 55$ and group 3, $n = 75$) compared to the MIT group (group 1): group 2 vs group 1, HR 3.79 (2.77–6.32); group 3 vs group 1, HR 2.93 (1.74–4.94); $P < 0.001$ at multivariable analysis. Increased risk of death in group 2 vs group 1 (HR 2.47, 1.26–4.85) and group 3 vs group 1 (HR 1.96, 1.00–3.87); $P = 0.03$ at multivariable analysis
Grubbs et al. [41]	22	Increased risk of recurrence in the control group of untreated patients ($n = 196$) than in the MIT group: HR 1.95 (1.06–3.59); $P = 0.03$ at multivariable analysis
Fassnacht et al. [42]	35	Reduced risk of death in the MIT group than in the control group of untreated patients ($n = 114$): HR 0.38 (0.12–1.28); $P = 0.11$ at multivariable analysis
Wangberg et al. [43]	37	Reduced risk of death in the high-level MIT group ($n = 24$) than in the low-level MIT group ($n = 13$): HR 0.25 (0.06–1.00); $P = 0.049$ at Poisson regression
Else et al. [44]	105	Reduced risk of recurrence in the MIT group than in the control group ($n = 159$): HR 0.72 (0.53–0.98); $P = 0.037$ at multivariable analysis

DFS disease-free survival, *MIT* mitotane, *NS* not significant, *HR* hazard ratio
[a]The study includes the patients reported previously by Kasperlik-Zaluska et al. [45]
[b]The study includes the patients reported previously by Icard et al. [46]

Mitotane has a narrow therapeutic index [3, 35, 39] and can cause significant toxicity; thus, it is not an ideal drug to treat patients free of disease. This concept coupled with a limited evidence of efficacy in the literature [26, 28, 33–36, 39] made adjunctive treatment with mitotane less appealing until the last 10 years. As a matter of fact, no recommendation in favor or against adjuvant treatment was formulated at a consensus conference on ACC held at Ann Arbor, Michigan, USA, in

2003 [47]. In 2007, however, we published a retrospective analysis involving a large cohort of ACC patients, followed for up to 10 years at different institutions in Italy and Germany which challenged this view [40]. In that study, adjuvant mitotane was given to 47 Italian patients after radical surgery, and RFS in these patients was compared with that of two concomitant, independent groups of 55 Italian and 75 German patients who were left without any postsurgical treatment. RFS (the primary outcome of the study) was significantly prolonged in the mitotane group (42 months), as compared with the two groups of untreated patients (10 and 25 months, respectively) who had a significantly higher recurrence rate than those receiving mitotane. The mitotane group and the Italian control group were highly comparable for the clinical characteristics known to affect outcome, whereas the control group from Germany had better prognostic factors making mitotane effects even more impressive. Indeed, multivariate analysis confirmed that mitotane treatment gave a significant advantage for RFS. The benefit on OS was less evident, although being significant after adjusting for the difference in prognostic factors [40]. An important finding of the study is that a favorable effect was achieved with low doses of mitotane (1–5 g per day), which were associated with an acceptable toxicity [40]. Conversely, severe and disabling toxicity was observed in the previous series employing high doses of mitotane [28, 34].

Following publication of our study, Bertherat et al. [48] reported that in a cohort of 166 patients, mitotane use following complete tumor removal was not associated with any improvement in DFS. Since mitotane was given to only half of the patients referred to the authors' institution, a selection bias may be anticipated, implying that patients with unfavorable prognostic factors were selected for adjuvant mitotane treatment. This is a major difference with our study [40], in which the choice to recommend mitotane was made according to a predefined center policy irrespective of patient or tumor characteristics. The predefined treatment assignment and the inclusion of well-matched control groups were considered to be the major advantages of our study as compared with other studies that had less clear treatment assignments and often used historical controls or no controls at all [4]. Bertherat et al. [48] raised also the question whether the efficacy of mitotane may change as a function of the secretory activity of ACC since in a previous report by the same group a beneficial effect of mitotane in patients with Cushing's syndrome was reported [32]. It is biologically plausible that hypercortisolism may contribute to an unfavorable outcome in patients with advanced ACC and complicates management. By instance, susceptibility to infections poses a great challenge to application of chemotherapeutic protocols in patients with severe Cushing. However, a recent multicentric retrospective study showed that cortisol excess portends a worse prognosis also when tumors can be completely removed and Cushing be cured [49]. This implies that the negative prognostic effect of cortisol excess persists after its resolution; it is likely that secreting tumors have some still unknown biological characteristics that confer higher aggressiveness. At present, there is no firm evidence that controlling cortisol excess by employing steroid-inhibiting drugs (i.e., ketoconazole, metyrapone) improves prognosis of affected patients, although this is pursued in clinics.

The retrospective nature of our study, however, does not allow concluding definitively that adjuvant mitotane treatment is beneficial [50]. Arguments against are based on the methodological flaws of the available evidence, toxicity and complexity of mitotane treatment, and lack of factors predicting response to treatment [51]. Following our study, new evidence on the value of adjuvant mitotane has been published [41–43]. A study from the M.D. Anderson Cancer Center claimed that a state-of-the-art surgical approach may provide a similar survival to surgery plus adjuvant mitotane, but the lack of adjuvant mitotane treatment was a factor predicting a higher risk of recurrence [42]. Moreover, patients treated with adjuvant mitotane showed significantly better RFS even if they were mostly treated by less experienced surgeons in the community [41]. Fassnacht and colleagues [42] found that survival was improved in patients with stage II ACC who were managed by a specialized center early after surgery compared to patients who were referred at a larger stage, usually after tumor recurrence. Adjuvant mitotane was more frequently used in the first group and was associated with a survival advantage [42]. Wangberg and colleagues [43], reviewing their experience with ACC, showed that an aggressive surgical approach was associated with a satisfactory disease-specific survival. The benefit of mitotane was evident for patients with high-stage ACC and circulating drug levels >14 mg/L [43].

The availability of mitotane measurement across Europe, as a free service offered by the company distributing mitotane (info@lysodren-europe.com), gives the possibility to guide dose adjustments and to prevent severe toxicity. Mitotane monitoring is key for an appropriate management of adjuvant treatment giving the possibility to guide dose adjustments and target mitotane concentrations that have been associated with therapeutic effect. Results of our group demonstrated that plasma mitotane concentrations matter also for patient outcome in adjuvant setting [52]. We did a retrospective analysis of 122 ACC patients who were radically operated on and then treated adjuvantly with a monitored mitotane treatment, targeting concentrations of 14–20 mg/L. The concentrations were attained and maintained during a median follow-up of 36 months in only 63 patients (52%). These patients showed a prolonged RFS compared with the remainders [hazard ratio of recurrence 0.497, $P < 0.01$], while a nonsignificant increase in OS was observed (hazard ratio of death, 0.511, $P = 0.06$). The rather limited duration of follow-up and the low number of events may explain why OS was not significantly changed. Mitotane concentration of 14 mg/L, or higher, was a predictor of RFS in multivariable analysis, and this finding supports the strategy of targeting a cutoff value of 14 mg/L when giving mitotane for adjunctive purposes, which was previously recommended on an expert opinion basis [5, 53–55]. However, the study also demonstrated that maintaining mitotane concentrations at target for a long time is a difficult task requiring firm commitment by both patients and physicians. The patients included in this study were treated with different dosing regimens of mitotane, according to the policies at each center. However, there was no difference between low-dose and high-dose regimens in the probability of reaching the target concentrations after 3 months of treatment, suggesting that individual factors may be as important as pharmacologic ones [52]. Treatment-related toxicity was overall acceptable and manageable with

temporary treatment discontinuation or dose reduction. Although a retrospective analysis may underestimate adverse events, it is likely that the monitoring of mitotane concentrations contributed to limit severe unwanted effects, which may be linked to circulating mitotane levels exceeding 20 mg/L [5, 53–55].

Very recently, a retrospective analysis at the University of Michigan reported on 389 patients followed from 1979 to 2013, of whom 105 patients treated postoperatively with mitotane [44]. Despite the fact that the adjuvant group had a worse risk profile than the control group, mitotane treatment was associated with a significantly improved RFS (hazard ratio 0.7, P < 0.05). However, treatment failed to prolong significantly OS. The lack of effect on OS may be due to the relatively short follow-up duration (25.6 months in the overall series). Despite the usual limits of being a retrospective analysis, this study has the merit of including a large cohort of well-characterized patients from a single center. Lacking data from controlled prospective trials, the results of this study add further evidence in favor of the use of mitotane in an adjuvant setting.

Controversy on adjuvant mitotane is deemed to continue unless results of prospective controlled studies become available. Therefore, we have launched the first randomized trial in an adjuvant setting for ACC, the ADIUVO study (http://www.adiuvo-trial.org) under the endorsement of the European Network for the Study of Adrenal Tumors (ENS@T). The study's aim is to assess the efficacy of adjuvant mitotane treatment in prolonging RFS in ACC patients at low-intermediate risk of recurrence. Results of ADIUVO may not be expected before 2019.

Practical Guidelines to Adjunctive Mitotane Therapy

At San Luigi Hospital, we advise to start adjunctive mitotane treatment as soon as possible after surgery, at the very last within 3 months, in patients at high risk of recurrence, while the remainders are encouraged to enter the ADIUVO trial. Although our capability of predicting future risk of ACC recurrence after radical surgery and death is limited, it is generally agreed that stages III–IV ACC, margin-positive resection, and an elevated mitotic index are all factors portending an unfavorable prognosis [56]. Stage IV ACC may be susceptible to complete removal of the primary and metastatic tumor sites, but this condition may be assimilated to a margin-positive resection. Even if solid evidence is lacking, it is usually thought that these patients with stage IV tumors require postoperative medical treatment [57]. An elevated mitotic index is increasingly recognized as a negative prognostic factor, and studies showed that cutoff values of 10% for Ki-67 or nine mitoses per high-power microscopic field were able to categorize patients at high risk of recurrence [57, 58].

We do not institute mitotane therapy before surgery, as advocated by Dickstein et al. [59]. In our practice, we use a low-dose regimen (starting dose of 1 g daily with daily increments of 0.5 g every 4 days until the maximal tolerated dose, usually less ≤6 g/daily), because it is better tolerated with less impact on the quality of life

of the patients. In some centers, however, mitotane is currently administered at high, rapidly escalating doses (up to 6–9 g daily) [60]. Although a high-dose regimen is able to provide therapeutic plasma concentrations of mitotane within 1 month in about one-third of the patients [21], we are more cautious with dose escalation. A high-dose regimen requires an intensive follow-up, combining clinical and mitotane level monitoring, and may be more frequently associated with side effects, while our schedule is better tolerated.

The most common unwanted effects are gastrointestinal manifestations that appear early, independently on mitotane levels [60]. They can be managed with temporary dose reduction, or delay of dose increments, and supportive therapy. Elevated c-glutamyltransferase levels are also frequently observed but are not actually troublesome unless values are exceedingly elevated. Clinically significant liver toxicity is characterized by a marked increase in transaminases and bilirubin but is infrequently observed in the absence of predisposing conditions [3, 7]. Central neurologic toxicity (cerebellar symptoms, disturbed cognitive performance) is more closely associated with elevated mitotane concentrations (20 mg/L), but subtler symptoms, such as memory impairment or attention deficit, may be observed in some patients even when they are exposed to lower drug concentrations [7, 38, 39]. A great individual variability in the susceptibility to mitotane-related unwanted effects is apparent for causes that are still unknown. A general measure to deal with mitotane toxicity is a step-down to the previously tolerated dose or temporary drug withdrawal in the event of severe manifestations. However, well-informed and motivated patients are able to cope with side effects and maintain compliance to treatment [3, 61]. To accomplish this task, it is important to establish a close patient-physician relationship to induce and maintain adherence to treatment. Patients seek advice frequently, also because their local physicians are unfamiliar with mitotane use and its attendant complications, and it is necessary to give a timely counseling to keep patients on treatment.

Because of the adrenolytic effect of mitotane, all patients should receive glucocorticoid replacement to prevent adrenal insufficiency. Steroid doses are typically higher than in Addison's disease, due to an enhanced metabolic clearance rate of glucocorticoids induced by mitotane [3, 61, 62]. An inadequate treatment of adrenal insufficiency increases mitotane-related toxicity, particularly gastrointestinal side effects, and reduces tolerance [3, 38, 54]. Mineralocorticoid supplementation is not mandatory in all patients because the zona glomerulosa is partly spared by the toxic effect of mitotane [3, 54]. This may be also the result of the biphasic action of mitotane on aldosterone synthase, as previously mentioned. Moreover, mitotane affects thyroid and gonadal function in a complex way by mechanisms that are still to be completely elucidated. Mitotane administration is associated with low FT4 levels without a compensatory rise in TSH, an effect that becomes apparent early in the course of treatment. This prompts thyroxin replacement, even if the benefit of this measure is difficult to appreciate [54, 61]. In women, gonadal function is usually preserved, and most female patients have regular cycles unless PRL levels are significantly increased [54, 57, 61] due to a weak estrogen-like action of mitotane [63]. Conversely, in men mitotane treatment causes sexual dysfunction as a late but com-

mon unwanted effect, due to inhibition of testosterone secretion. Sex steroid replacement may become necessary to treat hypogonadism in some patients but may worsen gynecomastia [54, 57, 61].

The optimal duration of therapy remains undefined. The time to first recurrence after complete tumor resection is highly variable from some months to more than 10 years, but most recurrences occur within 2 years of primary surgery [1–3, 28, 31, 54, 57]. In our own series, about 70% of relapses took place in the first 2 years of follow-up, whereas the frequency of late (>5 year) relapses was less than 1% [40]. It is our current practice to accommodate patient preferences between a range of possibilities (2, 5, or even more years of therapy) in a shared decision-making depending on tumor and patient characteristics. However, we are eager to prolong treatment if well tolerated in patients at elevated risk.

Selection of Patients to Adjuvant Mitotane

Despite the limits of the available evidence, adjuvant mitotane therapy is currently recommended in many expert centers whenever the patients present an elevated risk of recurrence. Differences do exist in the criteria used to define a high-risk condition, as exemplified in a recent position of an international panel of experts who agreed on stages I–II, complete (R0) resection, and ki-67 < 10% as markers of good prognosis, but a consensus was not found on stage III R0 ACC [56]. In patients with good prognostic markers, the decision on adjuvant mitotane therapy may be individualized, whereas adjuvant mitotane is mandatory in the high-risk category [56]. Following the ENS@T ACC staging system, stage III applies to locally invasive tumors characterized by infiltration in surrounding tissue, positive regional lymph nodes, or a neoplastic thrombus in the vena cava or vena renalis [64]. It is biologically plausible that tumor spread in regional lymph nodes or in the vein system may portend to a higher risk of recurrence than local infiltration, and it is our opinion that subgroups at different risk of recurrence do exist among stage III ACC. Infrequently, a stage IV ACC, defined by presence of distant metastases [64], may be completely resected and has to be considered at a high risk of recurrence. The lowest risk applies to stage I and II ACC, being tumors localized in the adrenal gland with a size of ≤5 cm or >5 cm, respectively [64]. Recent data suggest that the proliferation activity of the tumor is the most important factor predicting risk of recurrence following R0 surgery. Assessment of the proliferation index Ki-67 is currently used to assess proliferation, despite some problems to harmonize immunohistochemical readings among different pathologists. In a European multicentric study, a threshold value at 10% was found to separate patients at good or worse prognosis with a hazard ratio of recurrence of 1.042 per each % increase [65]. Although the results of this study have still to be considered as preliminary, the availability of a large patient cohort totaling more than 500 patients represents a solid database to confirm the view that tumor proliferation is a strong determinant of patient survival. The value of ACC proliferation has been already appreciated in smaller series by the use of mitosis

count [31, 58], which is likely the single most predictive factor of Weiss score. Conversely, Weiss score as a whole does not clearly indicate the probability of tumor recurrence [58, 66]. Resection status is another established adverse risk factor, being Rx (unknown), R1 (microscopically positive margins), and R2 (macroscopically positive margins) associated with progressively reduced RFS irrespectively of other risk factors [57, 67–72]. A number of molecular markers, like matrix metallo-proteinase type 2 [73], glucose transporter GLUT1 [74], SF1 [75], and BUB1B and PINK1 [76], might potentially emerge in the future as powerful outcome predictors, but none of them has yet found a place in current management of ACC.

It would be interesting to identify a molecular signature that may predict mitotane efficacy. In a study by our group, the ribonucleotide reductase large subunit (RRM1) gene expression was able to predict efficacy of adjuvant mitotane [77]. The RRM1 gene encodes for an enzyme essential for the production of deoxyribonucleotides prior to DNA synthesis in S phase of dividing cells. It is located in an important tumor-suppressor gene region. Alterations in this region have been associated with the Beckwith-Wiedemann syndrome, Wilms tumor, rhabdomyosarcoma, adrenocortical carcinoma, and lung, ovarian, and breast cancer. This gene may play a role in the pathogenesis of such malignancies. High RRM1 gene expression was associated to shorter disease-free survival (DFS) and overall survival at both univariate and multivariate analysis. In patients with low RRM1 gene expression, adjuvant mitotane was associated with improved DFS, whereas this effect was lost in cases with high RMM1 expression. In vitro mitotane induced strong upregulation of RRM1 transcription (up to 25-fold increase) in mitotane-insensitive human ACC cell line SW-13 but not in mitotane-sensitive human ACC cell line H295R cells. Furthermore, RRM1 silencing in SW-13 cells induced sensitivity to mitotane. The efficacy of this marker for predicting response to mitotane still deserves validation in prospective studies.

Mitotane for Advanced Adrenocortical Carcinoma

The management of ACC patients with recurrent and metastatic disease is challenging and the prognosis is often poor. However, ACC is a heterogeneous disease and, a subset of patients bear a less aggressive tumor and may have longer survival perspective, although most patients are destined to die of disease progression within 1–2 years. Several prognostic factors such as time since diagnosis, presence of distant metastases, number of metastatic lesions and number of tumoral organs involved, high mitotic rate (20 per 50 high-power field), and atypical mitoses in the primary tumor have been found to predict survival in patients with metastatic ACC [78, 79]. Two previous reports identified cortisol secretion as a negative prognostic factor in metastatic ACC patients. In a large single-institution French series including 202 patients with different disease stages, cortisol excess was found to be an independent prognostic factor for OS and was predictive of subsequent metastatic disease in the subset of patients with stages I–III [32]. However, the study does not

provide demonstration that treating cortisol excess improves prognosis by itself. In clinical practice, it is difficult to discriminate between the effect of tumor shrinkage and cortisol reduction. Similar results were obtained from a series of 72 Italian patients with metastatic ACC submitted to chemotherapy with EDP (etoposide, doxorubicin, and cisplatin) plus mitotane [80].

The treatment of advanced/metastatic patients includes locoregional approaches such as surgery, radiofrequency ablation (RFA), and chemoembolization in addition to systemic therapies in patients with slowly progressive disease and low metastatic burden. RFA and chemoembolization have been found to be of potential utility in advanced ACC [81, 82], and we are currently using these techniques in association with mitotane in the more favorable clinical setting. In the presence of isolated locoregional recurrence or oligo-metastatic disease, surgery can lead to improved survival [25], so an aggressive surgical approach may be advisable whenever complete resection (R0) can be envisaged. Conversely, tumor debulking offers little benefit and may be considered in patients with functional tumors not controlled by medical treatment.

Mitotane alone or mitotane plus chemotherapy are the currently adopted systemic strategies. Chemotherapy plus mitotane is currently recommended for patients with aggressive disease and multiple metastases. However, in the presence of isolated locoregional recurrence, or metastatic disease involving a limited number of organs, mitotane monotherapy can be a reasonable systemic option. Single agent mitotane is active, and response rates between 13% and 31% have been reported (Table 13.2.). Most of the responses are of limited duration, and complete responses rarely occur. The key concept of mitotane treatment in patients with advanced/meta-

Table 13.2 Outcome of mitotane monotherapy in patients with advanced ACC

References	Daily dose (g)	Patients	OR (no, % and CI)	CR (no, % and CI)	Duration (months)
Retrospective studies					
Henley et al. [84]	NR	24	6 OR (25%, 7–43)	None	3–24
Venkatesh et al. [30]	NR	72	21 OR (29%, 18–40)	None	NA
Luton et al. [85]	3–20	37	5 OR (13%,2–24)	None	5–25
Pommier et al. [28]	NA	29	7 OR (24%, 8–40)	None	NA
Haak et al. [35]	4–8	55	15 OR (27%, 15–39)	8 CR (15%, 5–25)	2–190
Barzon et al. [36]	4–8	11	2 OR (18%, 0–41)	None	40–64
Williamson et al. [86]	4–10	16	2 OR (13%, 0–30)	None	NA
Total		244	58 OR (24%, 18–30)	8 CR (3%, 1–5)	
Prospective studies					
Decker et al. [87]	6	36	8 OR (22%, 8–36)	2 CR (6%, 0–14)	3–82
Baudin et al. [39]	6–12	13	4 OR (33%, 7–59)	1 CR (8%, 0–23)	10–48
Total		49	12 OR (24%, 12–36)	3 CR (6%, 0–13)	

OR overall response, *CR* complete response, *NA* not available, *NR* not retrieved

static disease is that plasma mitotane concentration ranging between 14 and 20 mg/L should be targeted in any patient. It was demonstrated that disease responses are mainly confined in patients attaining and maintaining over time serum levels within this therapeutic range [35, 39]. This concept has been validated more recently in a retrospective series of 91 patients receiving mitotane for unresectable or metastatic ACC [53]. In this study, mitotane level above 14 mg/L was associated with tumor response and better survival irrespective of whether mitotane was administered as monotherapy or in combination with chemotherapy. Besides its antitumor effect, mitotane is a strong inhibitor of adrenal steroidogenesis, and it has a compelling indication in patients with endocrine symptoms, although the rate of success in controlling hormone excess is not well known [57, 67]. Owing to the latency of mitotane to attain the therapeutic range, mitotane monotherapy is indicated in the management of patients with a low tumor burden and/or more indolent disease. For patients whose disease shows an aggressive behavior, cytotoxic chemotherapy is required. Chemotherapy in the management of advanced ACC is usually administered in association with mitotane not only in patients with treatment-naïve disease but also in patients with disease progression to mitotane therapy, when mitotane is usually maintained at the same doses if tolerated. Despite that combining mitotane with classic cytotoxic agents is a commonly used strategy, the evidence supporting a synergism between mitotane and chemotherapy is weak. Mitotane may have a synergistic effect on chemotherapy activity thanks to the ability to reverse multidrug resistance mediated by P-glycoprotein expression. ACC produces high levels of the multidrug resistance protein MDR1 (also known as P-glycoprotein) which functions as an ATP-dependent drug efflux pump, transporting out of the cell hydrophobic cytotoxic agents such as doxorubicin, vinblastine, and paclitaxel. Overcoming MDR gene, mitotane may enhance the cytotoxicity of anthracyclines, etoposide, and taxanes [83, 88] whose activity is hampered by MDR gene expression. However, the effect of mitotane on MDR has been questioned [89]. Indirect comparisons of response rates obtained in non-randomized Phase II trials showed greater activity of chemotherapy regimens, including mitotane, as recently reviewed [90]. However, no randomized study has tested prospectively the efficacy of mitotane plus chemotherapy vs chemotherapy alone.

The first prospective multinational trial on treatment of ACC (FIRM-ACT) ever published has recently set a standard of care for advanced/metastatic ACC [91]. In this trial, the association of etoposide, doxorubicin, and cisplatin plus mitotane (EDP-M) was found to be superior to streptozotocin plus mitotane (SZ-M) in terms of disease response rate and progression-free survival (PFS). On the bases of the results of this study, the EDP-M scheme is actually recommended as the standard approach for ACC patients by international guidelines [70]. The efficacy of EDP-M in this multinational Phase III trial, however, was modest: the response rate was low (23%), and the median PFS and OS were of only 5 and 14.8 months, respectively. The FIRM-ACT trial also provided some evidence that mitotane levels at target could improve patient outcome [91]. Mitotane efficacy is not immediate, and the so-called therapeutic range is usually attained within 2–3 months, so disease progression may precede the time when mitotane levels are at target. Chemotherapy

may be effective in the first weeks of therapy, and this is a pragmatic point favoring a functional synergism between mitotane and chemotherapy in patients with aggressive disease. On the other hand, mitotane may be also important in the long-term disease control. In the randomized trial FIRM-ACT, a few patients were free of progression after 4 years in both EDP-M and SZ-M arms. In these patients, mitotane could have contributed to the long-term delay of disease progression.

Conclusion

Whenever ACC is completely removed, we should face the dilemma to use adjuvant therapy or not. In our opinion, adjuvant mitotane is the preferable approach in most cases, because the majority of patients referred to our institution following adrenalectomy have an elevated risk of recurrent disease. A better understanding of factors that influence prognosis and response to treatment [92, 93] will help in stratifying patients according to their probability of benefiting from adjuvant mitotane, with the aim of sparing unnecessary toxicity to patients who are likely unresponsive. However, until significant advancements take place, we have to deal with uncertainty using our best clinical judgment and personal experience in the clinical decision process. Our current policy, then, is to recommend adjuvant mitotane after extirpation of ACC. Patients at low risk of recurrence (R0, stage I–II, Ki-67 < 10%) are offered to participate in the ADIUVO trial and are randomized between mitotane treatment and observation. A monitored mitotane treatment is followed targeting levels between 14 and 20 mg/L. Our scheme of low-dose mitotane treatment is given in Table 13.3. Minimal duration of treatment for high-risk patients is 2 years, but we strive continuing for 4–5 years in most cases. The strategy of treatment of advanced ACC is chosen considering a number of prognostic factors (tumor burden,

Table 13.3 Practical guidelines for giving low-dose adjuvant mitotane treatment

• Start with 1 g daily and increase mitotane dose every ≈ 4 days up to 6–8 g daily or the maximum tolerated dose. Give mitotane in split doses with meals or snacks
• Accommodate mitotane schedule to patient's tolerance aiming at serum mitotane concentrations of 14–20 mg/L (therapeutic levels)
• Check mitotane levels every 4–8 weeks to adjust dosage until reaching target levels
• At target, clinical assessment, biochemical and hormonal evaluation, and monitoring of mitotane levels every 3–4 months or in case of significant side effects. Adjust mitotane dose according to circulating levels and tolerability
• In case of slight unwanted effects, continue mitotane and treat symptoms (e.g., nausea, diarrhea)
• In case of moderate side effects, step down to the previously tolerated dose and use symptomatic therapy
• In case of severe side effects, discontinue mitotane and institute specific treatment. Duration of treatment stop depends on clinics and mitotane levels. After interruption, restart with a lower dose

type of progression, secretion, proliferation index) and the clinical conditions. If a patient is fit and carries bad prognostic factors, we recommend the polychemotherapy regimen EDP plus mitotane. In case of compromised conditions, platinum plus mitotane is an alternative. Patients at perceived good prognosis may be treated with mitotane monotherapy, and EDP is added on in case of disease progression.

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