Michael E. Symonds *Editor*

Adipose Tissue Biology

Second Edition

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Editor
Michael E. Symonds
Academic Division of Child Health
The School of Medicine, The University Hospital
Queen's Medical Centre
Nottingham, UK

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Contents

Chapter 1
The Evolution of Mammalian Adipose Tissues

Caroline M. Pond

Abstract Anatomical organization, genes and metabolic pathways in white, beige and brown adipose tissues are traced from their invertebrate origins through lower vertebrates to mammals and birds. Invertebrate storage organs and adipose tissues of lower vertebrates are also metabolic regulators. In large turtles, some depots are thermogenic or insulators. Reptilian, avian and mammalian adipocytes sort fatty acids, especially essential polyunsaturates. All mammals have numerous adipose depots, many with site-specific properties including thermoregulation, structural roles or paracrine interactions with contiguous tissues. Paracrine provisioning of lymph nodes with fatty acid sorting optimizes cellular nutrition during fasting or on deficient or imbalanced diets, averts competition with other tissues and utilizes scarce resources efficiently. The mechanisms may be defective in HIV/AIDS and Crohn's disease and some obesity-related diseases. Thermogenesis by shivering and non-shivering mechanisms in muscle occurs in some lower vertebrates and, in birds, is as effective as mammalian brown adipocytes. Facultative thermogenesis emerged gradually in birds and mammals, utilizing genes of reptilian ancestors, including some resembling uncoupling proteins. Mammalian thermogenic tissue evolved from muscle that lost contractile functions and expanded its mitochondria and lipid-storage capacity, thus generating confusing resemblances to white adipocytes. As well as storage and endocrine functions, adipose tissues' capacities for paracrine interactions, fatty acid sorting and thermogenesis supported the evolution of mammalian heterothermy (i.e. diet-induced thermogenesis, torpor and hibernation), lactation and their ability to exploit nutritionally imbalanced diets. These features probably appeared early in mammalian evolution enabling rapid colonization of new habitats, including efficient utilization of poorer quality diets, and metabolic support of lactation that enables fast-growing young to delay maturation of specialised dentitions. The contribution of 'grandmothers' to their descendants' evolutionary fitness drove selection for post-menopausal longevity, aided by larger lower-body superficial depots that protect cardiovascular and metabolic health. Sex differences in human adipose tissue distribution evolved under such sexual selection plus

C.M. Pond (✉)
School of Life, Health & Chemical Sciences, The Open University,
Milton Keynes MK7 6AA, UK

Department of Zoology, Oxford University, South Parks Road, Oxford OX1 3PS, UK
e-mail: C.M.Pond@open.ac.uk

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adaptations to heat dissipation. Natural obesity without metabolic impairment found in some arctic mammals evolved by numerous genetic modifications over at least a million years, much longer than human adjustments to modern diet, cooking, heating and clothing.

Keywords Comparative • Reptiles • Mammals • Birds • Primates • Apes • Bears • Paracrine interactions • Immune system • Fatty acids • Perinodal • Crohn's disease • Colitis • Lipid-soluble toxins • Hibernation • Diet-induced thermogenesis • Herbivory • Lactation • Thrifty genes • Sex differences • Cold adaptation

1.1 Introduction

For many centuries, comparative biology and medicine advanced in parallel, with many practitioners making important and mutually beneficial contributions to both fields. Increasing specialization in the twentieth century forced them apart until the rise of molecular phylogeny, medical genomics and developmental biology in the 1990s reunited the estranged partners. Adipose tissues have been one of the most spectacular beneficiaries of this rapprochement: comparative and medical biologists now recognise that their findings are as mutually supportive to each others' progress as they have even been. This chapter is a three-way synthesis of comparative concepts from wild animals in natural systems, experimental data from laboratory animals & *ex vivo* cultures and human studies to elucidate the normal functions and pathologies of adipose tissues.

Although research involving adipose tissues has expanded enormously during the past 50 years (Rosen and Spiegelman 2014), evolutionary and comparative studies lagged behind metabolism, endocrinology and human epidemiology. Both white (WAT) and brown (BAT) adipose tissues have been largely omitted from genetic and developmental investigations into the origins and evolution of tissues and cell types that complement the long-established discipline of comparative anatomy, functionality and adaptation because they appear too variable, too closely linked to diet and body condition to reveal any general principles determining their site-specific properties and anatomical distribution or phylogenetic relationships to 'lean' tissues.

Interest in its origins and evolution was stimulated by recognition of WAT's endocrine and paracrine relationships, its role in metabolic regulation and its value as a source of stem cells and in reconstructive surgery as well as lipid storage and recently accelerated by the study of the uniquely mammalian tissues BAT and beige or brite adipocytes (Cohen and Spiegelman 2015). Understanding of adipose tissues has progressed from its dismissal by comparative anatomists to its recognition as central to the evolution of the skin, immune system, thermoregulation, mammalian lactation and the metabolic control that underpins these systems. This chapter outlines the origins and evolution of the anatomy, physiology and many functions and

specializations of adipose tissues and their relevance to medical sciences; the evolution of the genes involved is left to experts (Caesar et al. 2010).

1.1.1 Comparative Perspectives on Obesity and Diabetes

Obesity and adipose tissues, almost synonymous in the mid-twentieth century, drifted apart as the focus of the former shifted to appetite control and inheritance and of the latter to adipokines (Dodson et al. 2014; Sanchez-Gurmaches and Guertin 2014), development (Chau et al. 2014; Sanchez-Gurmaches and Guertin 2014) and involvement in inflammation and immunity (Exley et al. 2014; Mraz and Haluzik 2014; Couturier et al. 2015).

Of several recent attempts to account for the evolution of obesity in humans, some hardly mention current understanding of the organisation and basic properties of adipose tissues (Power and Schulkin 2009; Isler 2014), while others recognise their central, distinctive roles in human appearance, social and sexual behaviour and metabolism (Wells 2006, 2010).

Obesity is unusual among human diseases in that very similar conditions are integral and essential components of the habits and life history of certain wild animals. Natural obesity, like pathological obesity, arises from 'overeating', periods in which animals become hyperphagic, in some cases aided by sedentary habits. But in wild animals, obesity is always transient and controlled: hyperphagia and fat deposition are followed by periods of anorexia and/or intensive exercise, leading to weight loss (Pond 1998). Adaptive obesity is never a direct cause of diabetes, cardiovascular disease or reproductive dysfunction. The study of natural obesity can reveal much about the 'ideal' structure, composition and anatomical distribution of adipose tissue, the neural and endocrine control of blood composition, appetite and energy expenditure and about the causal relationships between high levels of stored lipid and the adverse metabolic changes that are so frequently associated with obesity in humans.

The origins and incidence of Type 2 diabetes have been explored in many dimensions from metabolism, molecular signalling and immunity to human evolution, ecology and social behaviour (Watve 2012). A general theory of macronutrient nutrition, food selection and foraging (Simpson and Raubenheimer 2012) integrates the scattered and fragmentary information about wild species with human nutritional problems, including obesity. Validated by observations and experiments on organisms ranging from fungi and flies (Solon-Biet et al. 2015) to bears (Erlenbach et al. 2014), its tenets unite nutrition with an impressive range of topics in ecology, cell biology, physiology, immunology, psychology and lifespan (Simpson et al. 2015), though not yet with gross anatomy and the contributions of different organs and tissues. The evolution of adipose tissues, their gross anatomy and relations with other tissues, and, at the microscopic level, adipocytes and the many other cell types they incorporate, have until recently received little attention.

Comparative physiology and genomics during the past 20 years have demonstrated remarkable similarities in the relationships between diet, metabolic control, energy storage and key life history parameters including longevity and fecundity (Fontana et al. 2010). Concepts developed from the study of insects (*Sophophora*, formerly *Drosophila*), nematode worms (*Caenorhabditis*) and other 'lower' organisms have entered medical thinking (Blüher 2008) and the search for new drugs (Hofbauer and Huppertz 2002). Therefore, it is appropriate to begin with an evolutionary and comparative perspective on the structure and functions of adipose tissues.

1.2 Storage Tissues

Tissues and physiological control systems that enable animals to survive long periods of fasting, during which body fabric is depleted and metabolism adjusted, arose early in evolution, so many similarities, but also some important contrasts, are found among living phyla.

1.2.1 Invertebrates

Many invertebrates, especially those that undergo diapause or metamorphosis, have specialised liver-like tissues involved in whole-body metabolic regulation and energy storage. The most thoroughly studied is the insect 'fat body'. This irregularly shaped, sometimes relatively large, structure develops in the abdomen, an anatomical position that maximises contact with the haemolymph and permits large changes in volume with minimal impact on other organs. Its most abundant cell type, called 'adipocytes' by some authors, store glycogen and acylglycerols, releasing the breakdown products in response to metabolic demand from other tissues (Arrese and Soulages 2010). The basic mechanisms of fatty acid uptake and transport, lipogenesis and lipolysis are essentially similar in insect 'adipocytes' and vertebrate white adipose tissue.

The insect fat body also secretes several peptide metabolic regulators (Slaidina et al. 2009) that, at least in drosophila (Arthropoda, Insecta, Diptera), function remarkably like insulin-like growth factors in vertebrates (Okamoto et al. 2009). Neuropeptide Y belongs to an ancient family of peptides that mediate signals between storage cells and the nervous system in various invertebrates (de Jong-Brink et al. 2001; McVeigh et al. 2005).

Insulin is another ancient signal molecule known in *Caenorhabditis elegans* (Nematoda) (Michaelson et al. 2010) and in drosophila (DiAngelo and Birnbaum 2009) as well as all vertebrates. In lower vertebrates such as teleost fish, cells other than pancreatic β cells may be competent to secrete insulin (Roy et al. 2003).

Genes coding for and regulating these messenger molecules and their receptors are among the many gene families that diversified in early vertebrate evolution

(Larsson et al. 2008). Most of the signals and receptors shown to be regulators of appetite and energy storage in mammals are known in the sea squirt *Ciona* (Ascidia, Chordata), an invertebrate chordate (Kawada et al. 2010). The appetite-suppressing hormone leptin seems to be specific to vertebrates, probably appearing early in the evolution of fish (Gorissen et al. 2009), thus long preceding the evolution of adipocytes that are its major producers in higher vertebrates. Insects have analogous peptides that signal peripheral energy stores to the nervous system (Al-Anzi et al. 2009).

1.2.2 Vertebrate Adipose Tissues

Most animal cells contain small quantities of triacylglycerols that serve as energy reserves. Triacylglycerols spontaneously form homogeneous compartments in an aqueous environment. In most tissues that store substantial quantities (brown adipocytes, angiosperm seeds, etc.), the lipids form droplets a few microns in diameter, or around 1–10 fL (10^{-14}–10^{-15} L) in volume (Cinti 2007). Extending the interface between triacylglycerols and lipolytic enzymes may facilitate rapid mobilisation of the lipid stores that supports abrupt transitions between dormancy and vigorous activity. The evolution from yeasts to mammals has been traced for intracellular lipid droplets (Ottaviani et al. 2011) and wider aspects of the biochemistry of lipid storage and its metabolic control (Birsoy et al. 2013).

Single large lipid droplets, usually 0.1–1 nL (10^{-8}–10^{-9} L) in volume, are a special feature of vertebrate white adipocytes. The unusual arrangement is mediated by adipose-specific protein 27 (FSP27) (known in humans as cell death-inducing DFF45-like effector C (CIDEC)) that promotes lipid uptake and coalescence of droplets while reducing the maximum rate of lipolysis (Puri et al. 2007). Experimental reduction of CIDEC in isolated adipocytes increases lipolysis (Ito et al. 2010). The protein probably functions in conjunction with perilipin forming the interface between lipids and proteins (Brasaemle et al. 2000; Shen et al. 2009). FSP27/CIDEC is unique to vertebrates though structurally similar proteins are found in several invertebrate groups (Wu et al. 2008).

From a comparative perspective, these findings suggest that white adipose tissue evolved as a readily deposited, slowly mobilised lipid store suitable both for taking up circulating fatty acids following large, rich meals and for supporting prolonged fasts with low rates of energy expenditure. The evolution of jaws equipped early gnathostome vertebrates as top predators that probably ate relatively large, nutrient-dense prey irregularly and sometimes infrequently (Janvier 2009). The special features of white adipose tissue compared to invertebrate storage tissues exemplify its role as protection for other tissues against lipotoxicity due to excessive lipid accumulation as well as long-term storage (Unger 2002; Unger and Scherer 2010). White adipocytes may be among the novel cell types to appear during early vertebrate evolution, alongside diversification of cell types in the immune system such as mast cells (Crivellato and Ribatti 2010). Advances of vertebrate over invertebrate storage tissues include protection for other tissues against lipotoxicity due to

excessive lipid accumulation as well as long-term storage (Unger 2002; Unger and Scherer 2010) and its metabolic support of cellular immunity (van Niekerk and Engelbrecht 2015).

1.2.3 Fish, Amphibians and Reptiles

Many extant fish, especially the primitive groups, store large quantities of triacylglycerols in the liver and/or skeletal muscle as well as adipose tissue. Quite closely related species show distinct patterns of deposition and mobilization of lipids from the various depots (Weil et al. 2013) but the functions and mechanisms involved are poorly understood.

Almost all adipokines known from mammals have been identified in bony fish (Nishio et al. 2008; Murashita et al. 2009; Ronnestad et al. 2010). Rainbow trout (*Oncorhynchus mykiss*) migrate long distances, fuelled almost entirely by fatty acids that are stored in adipose tissue and transported to muscles by extremely efficient lipoproteins (Weber 2009). Under the highly artificial conditions of fish farms, salmon adipocytes display some of the pathological changes known in obese mammals (Todorčević et al. 2010), but there are no reports of similar effects in wild fish. Transgenic manipulation of the zebra fish (*Danio rerio*) has developed a teleost model of obesity that is remarkably similar to the mouse (Song and Cone 2007; Holtta-Vuori et al. 2010). Messenger molecules with some resemblance to mammalian leptin can be detected in this fish, of which one may have some involvement in energy metabolism (Gorissen et al. 2009), but in a related teleost, its main source is the liver, not adipocytes (Huising et al. 2006).

Most adult amphibians hibernate (or aestivate) for long periods supported by fat accumulated during (often brief) periods of food abundance. Much of the triacylglycerols are stored in paired fat-bodies that are loosely suspended in the abdomen, much like those of insects, and in some species, in and under the thin, distensible skin (Wygoda 1987). In these sites, expansion and shrinkage of the storage tissue avoid distorting adjacent organs.

Blood pressure is higher in reptiles and their body shape is more constrained by tougher, less distensible skin so adipose tissue is more compact and its anatomical arrangement is more varied. Most snakes and lizards have a few large depots but in Testudines (tortoises and turtles), adipose tissue is partitioned into numerous small depots that superficially resemble those of mammals (Pond and Mattacks 1984), an arrangement that may maximise storage capacity while minimising distortion of contiguous tissues.

In the enormous leatherback turtle (*Dermochelys coriacea*), the anatomical distribution and chemical composition of adipose depots seem to be specialized to thermal insulation (Davenport et al. 1990), perhaps extending the range of these partially endothermic reptiles to cooler seas. As well as ‘blubber’ under the carapace and around the viscera and muscles, the abundant adipose tissue in the turtle’s head and neck suggest that it insulates key neural, glandular and vascular structures

from the surrounding water and from the oesophagus, cooled by ingestion of large volumes of low-nutrient food (Davenport et al. 2009).

Very low rates of energy expenditure interspersed with brief periods of much higher metabolic rate are fundamental strategies in nearly all extant reptiles (Secor and Diamond 1997, 1999). They fatten readily and can withstand and recover completely from very prolonged fasts (McCue 2010). However, reptiles are nutritionally fragile, with poor capacity to rebalance dietary minerals and other micronutrients (Frye 1981; Allen and Ullrey 2004). Nutritionally imbalanced diets are a major cause of morbidity in captive reptiles, including severe obesity (Frye 1981). Adipose tissue triacylglycerols are particularly important for provisioning yolk-rich eggs (Warner et al. 2008) so female reptiles are often fatter than conspecific males just before the breeding season and more dependent upon accessing suitable diets.

1.3 White Adipose Tissue in Mammals and Birds

White adipose tissue was presumably named from post-mortem observations on wild insectivores and piscivores or young domestic livestock. It appears yellow to brown in older herbivores and their predators, and in human consumers of dairy products, as accurately illustrated in Rembrandt's 1632 masterpiece *The Anatomy Lesson of Dr. Nicolaes Tulp*. The colour arises from passive (i.e. non-enzymatic and probably non-functional) accumulation of carotenes and any other lipid-soluble residues, including synthetic toxins ingested with food (Polischuk et al. 2002). Thus sequestered, they are mostly harmless until released into the circulation during prolonged fasting or exercise, lactation, egg production or cachexia (Yordy et al. 2010; Fang et al. 2015). Their presence in mobilized and secreted lipids constitutes a major hazard to wildlife, especially during reproduction (De Andres et al. 2016), and in humans is implicated in infant health (Lignell et al. 2011), cardiovascular disease (Bergkvist et al. 2015), cancer (Irigaray et al. 2007) and dementia (Kim et al. 2015).

Dissectible WAT comprises >0.5–50% of the live body mass of free-ranging wild mammals, with an average of about 7% (Pond and Mattacks 1985c). Tissue from wild species generally contains less lipid and more protein, especially collagen, than homologous samples from people and laboratory and domesticated livestock (Pond and Mattacks 1989). Regardless of fatness, the white adipose tissue of large species is composed of fewer, relatively larger adipocytes than that of smaller species of similar dietary habits in both mammals (Pond and Mattacks 1985c) and birds (Pond and Mattacks 1985b). In this respect, adipocytes resemble neurons and contrast with most other cell types in mammals (Savage et al. 2007). Lipid droplet volume, the principal determinant of adipocyte size, is itself related to lipolysis (Ito et al. 2010). By controlling the rates of mobilisation of stored fatty acids and clearance of excess energy absorbed from the diet, white adipocytes are central to metabolic rate during feasting as well as fasting. This scaling of adipocyte volume to body size may reflect the complex and very controversial relationship between

body mass and basal metabolic rate (Kolokotrones et al. 2010). The topic has not been thoroughly investigated in reptiles or any other lower vertebrates.

Comparative biology shows that some functions of the liver in lower vertebrates take place in adipose tissue in mammals. Leptin was first described as a secretion from mammalian adipose tissue, the archetypal adipokine (Caro et al. 1996). Adipose tissue is its main source in all extant mammals including the most primitive (Doyon et al. 2001). Very similar molecules that regulate appetite and energy metabolism are known in all the major classes of vertebrates (Dridi et al. 2004). Although adipose tissue is present, sometimes in substantial quantities, the liver is the main source of leptin in teleost fish (Huising et al. 2006) and in birds (Taouis et al. 2001). Comparative data are too sparse to establish how many other hepatic functions have been 'taken over' by adipose tissue in mammals.

As well as its central role in lipid storage and metabolism, mammalian adipose tissue also participates in amino acid metabolism, particularly that of the non-protein, energy-supplying amino acid, glutamine (Curthoys and Watford 1995; Kowalski et al. 1997). Site-specific differences in glutamine synthesis and turnover suggest depot specialization comparable to that of fatty acid metabolism (Digby and Pond 1995; Digby 1998). Many years after these studies, the role of glutamine as a precursor to fatty acid synthesis (Crown et al. 2015) and in adipocyte differentiation and maturation (Green et al. 2016) are now being investigated.

White adipose tissue of mammals (Pond and Mattacks 1985b), and to a lesser extent that of birds (Pond and Mattacks 1985a), is partitioned into a few large and numerous small depots that merge only when greatly expanded. White adipose tissue metabolism and its neural and endocrinological controls are similar in both groups (Price et al. 2008) as are its involvement in immune function (see Sect. 1.6.1). Avian adipocytes mature much earlier in embryonic development, where they manage yolk lipids, directing appropriate fatty acids into structural lipids and others to oxidation (Speake et al. 1998).

1.3.1 Anatomical Distribution and Site-Specific Properties

In all mammals, white adipose tissue is distributed to a common pattern, though with substantial differences in relative mass between (Pond 1998), and to a lesser extent within species (Pond et al. 1995).

Depots were characterized first by site-specific differences in relative adipocyte volume and various biochemical features (Pond 1992, 1998). Then within-depot differences were shown to enable functionally important paracrine relationship with embedded lymphoid structures (Pond and Mattacks 1995, 1998, 2003; Pond 2007). Site-specific differences in human adipose tissues, until recently regarded as irrelevant, are now identified by a widening range of genetic, developmental and functional properties, many of significance to medicine (Sbarbati et al. 2010; Macotela et al. 2012; Pinnick et al. 2014; Sanchez-Gurmaches and Guertin 2014; Gil-Ortega et al. 2015; Karpe and Pinnick 2015) and livestock production (Dodson et al. 2014).

The largest depots in mammals are found inside the abdomen and between the skin and superficial musculature. Intra-abdominal depots include the mesentery and the omentum, a uniquely mammalian structure, and small quantities associated with the gonads. The adipocytes in these depots plus those surrounding the heart share common developmental origins distinct from that of the superficial sites (Chau et al. 2014). The epididymal depots are exceptionally large and easily dissected out in murid rodents (rats, mice & hamsters) and for this reason alone have been intensively studied. In other mammals, the depots on the inner walls of the abdomen extending around the kidneys and into the pelvis are usually bigger. Detailed study of adipose depots in domestic livestock reveals their cellular compositions and metabolism to be complex and often variable (Dodson et al. 2014); the same may also be true of humans.

The cellular composition of superficial adipose tissues is complex and diverse with functions other than lipid storage (Alexander et al. 2015). Comparison of mammals of body mass 0.1–500 kg and similar proportions of adipose tissue shows that the superficial depots are both thicker and more extensive in larger specimens than in smaller ones because the ratio of surface area to volume is lower (Pond and Ramsay 1992). The resulting confluence of depots that appear discrete in smaller species can impede identifying homologous depots with larger ones, including humans. Abdominal volume and body surface area decrease relative to body mass with increasing size, so superficial adipose tissue can be impressively thick in large mammals, creating the impression they are ‘fatter’. Total dissection is essential to establish body composition.

One of the largest such depots, the inguinal depot on the anterior thigh and abdominal wall (often just called ‘subcutaneous’ in lab rodents and ‘femoral’ in humans), is also the most consistently present in mammals (and birds) (Pond and Mattacks 1986b; Pond 1998). Genetic, physiological and epidemiological studies in humans (Karpe and Pinnick 2015) suggest an explanation: inguinal adipose tissue can accommodate additional lipid stores without promoting inflammation and increased risk of cardiovascular and metabolic disease. In other words, these specialized depots support rapid fattening without diminishing fitness in endothermic animals of high metabolic rate, a fundamental capability for mammalian reproduction (see Sect. 1.7.3).

Many birds and mammals become transiently obese during migration, breeding, moulting or before seasonal food shortages but most remain ambulatory and some perform prolonged, strenuous exercise. Some species of knot (small seabirds, Charadriiformes) carry relatively enormous fuel loads for long-distance migration by selective atrophy of non-essential organs and appropriate redistribution of adipose tissue (Piersma et al. 1999; Battley et al. 2000). In such ‘adaptively obese’ in animals, the additional body mass imposes surprisingly low, sometimes undetectable, additional energetic costs in flight and, perhaps even more surprisingly, in walking. For example, locomotion is unusually efficient in camels, partly through replacement of some limb muscles by non-energy consuming tendons (Alexander et al. 1982). Locomotory efficiency is unimpaired by adipose tissue that can reach 32% body mass in Svalbard rock ptarmigans (*Lagopus muta hyperborea*) (Lees et al. 2010).

After decades of confusion, the tangled relationship between adipose tissues and thermoregulation, both thermogenesis (Sect. 1.4.1) and thermal insulation, is becoming clearer. Many large, naturally obese mammals occur in areas that are seasonally cold, giving rise to the long-standing and widely disseminated belief that adipose tissue accumulates between the skin and underlying body muscles an adaptation to thermal insulation. However, comparative data on the partitioning of white adipose tissue between superficial and internal depots in the mammalian order Carnivora of similar body conformation but widely different sizes do not support this theory (Pond and Ramsay 1992). The superficial depots are simply the most convenient repository for large quantities of lipid regardless of habits and habitats.

The contributions of fur and superficial adipose tissue to body insulation have been studied in marine mammals (Cetacea, Pinnipedia, Sirenia). In those such as fur seals that retain body hair, its main function is energy storage as in Carnivora, but in whales and others with reduced hair, the outer layer is specialized to adjustable thermal insulation mainly by efficient control of blood flow, and the inner layer to storage (Liwanag et al. 2012). UCP1 has been detected in the inner layer of blubber of porpoises and other small cetaceans, suggesting it may thermogenic as well as insulatory (Hashimoto et al. 2015).

The recent identification in laboratory mice of dermal adipose tissue, a small (only a few adipocytes thick) layer distinct from the often more massive subcutaneous layer (Alexander et al. 2015) is consistent with these findings in aquatic mammals and with the site-specific differences identified in layers of subcutaneous adipocytes in pigs (Hausman et al. 2007; Klein et al. 2007) and humans (Ardilouze et al. 2004). Murine dermal adipocytes serve as an insulating sleeve that thickens up to fourfold following prolonged exposure to cold. Those around hair follicles support hair growth, have antimicrobial roles and contribute to wound healing (Alexander et al. 2015; Zhang et al. 2015). The possibility that they also detect cooling (Ye et al. 2013) should be investigated. Thermal insulation in endothermic mammals must be adjustable because the metabolic rate of small mammals is high and during energetically demanding activities such as lactation, dissipation of heat generated as a by-product of digestion and metabolism, is limiting (Król et al. 2007). In experimentally overfed mice, too much superficial adipose tissue decreases skin thickness and elasticity (Ezure and Amano 2010). Additional superficial adipose tissue would exacerbate these problems so in wild mammals, its abundance and distribution must be well controlled.

1.3.2 Cellular Structure of Adipose Tissue

The total number of white adipocytes scales to (Body Mass)$^{0.75}$, and they range in volume from 0.01 nL in bats and shrews, to up to 4 nL in well-fed baleen whales (Pond and Mattacks 1985c). Carnivorous mammals and ruminants have about four times more adipocytes than non-ruminant herbivores (whose energy metabolism is based mainly on glucose) of the same body mass but are not on average fatter,

because the adipocytes are smaller. By coincidence, the adipocytes of rats and mice, small non-ruminant herbivores, are about the same size (0.1–1 nL) as those of humans, large omnivores who these days eat a high-fat diet.

Wild mammals that naturally become obese have up to 5 times, usually only 2–3 times, more adipocytes than would be expected in comparable non-obese species. Western adults have at least ten times more adipocytes in proportion to their body mass than would be expected from the comparison with wild mammals (Pond 1998). The limited information on other primates suggests that their adipocyte complements can also become disproportionately large (Pond and Mattacks 1987; Pereira and Pond 1995). Thorough studies of wild mammals always reveal much inter-individual variation in the total number of adipocytes that cannot be attributed to age, sex or any obvious feature of dietary history, particularly in carnivores (Pond et al. 1995). The number of adipocytes does not seem to be a major determinant of the capacity for fattening even in naturally obese species. In these respects, humans (van Harmelen et al. 2003; Spalding et al. 2008) are similar to other mammals.

1.3.3 Structural Adipose Tissue

Small depots, consisting of large quantities of extracellular material enclosing pockets of metabolically inert adipocytes are found in all tetrapod vertebrates. The firm, resilient tissue absorbs impact forces during locomotion and distributes weight in the feet, especially those of large terrestrial mammals such as elephants (Weissengruber et al. 2006). Fatty tendons around the knee of emus and other large running birds may have a similar role (Regnault et al. 2014). The fetal development (Shaw et al. 2008) and adult functions (Theobald et al. 2006) of Kager's fat pads in the human heel and around the Achilles tendon have been studied in detail. As well as acting as shock absorbers, the adipose tissue protects blood vessels and facilitates movement (Theobald et al. 2006). Injury or atrophy of structural adipose depots in the extremities lead to pain and debilitation that can be exacerbated by diabetes (Chatzistergos et al. 2014). So the study of these tissues using modern biomechanical concepts (Mihai et al. 2015) and techniques (Payne et al. 2015) is timely.

Several small structural depots help shape the face in humans (Kahn et al. 2000), other primates and certain large birds (Pond 1998). The buccal (Bichat's) fat pads are particularly large in human and other higher primates where they contribute substantially to facial appearance from infancy to old age (Yousuf et al. 2010), and, for reasons that remain unclear, sometimes regress in HIV infection (Agarwal 2014).

The white adipose tissue in the orbit behind and around the eye is also primarily structural (Wolfram-Gabel and Kahn 2002) but it may be less metabolically inert and more like 'typical' depots than had been supposed. Adipocyte volume differs consistently in different parts of the orbit and the cell sizes of both samples scale to body mass in mammals ranging in size from whales to voles (Pond and Mattacks 1986a) as in the more abundant metabolically active depots (Pond and Mattacks

1985c). In adult guinea-pigs, total adipocyte complement in the intra-orbital depots correlates with that of the rest of the adipose mass, with corresponding differences in mean volume that enable the depot to occupy a constant space (Mattacks and Pond 1985). Lymph vessels permeate the tissue in certain chronic inflammatory conditions of the eye (Fogt et al. 2004) in which inflammatory cytokines and prostaglandins can be detected (Schäffler et al. 2006). Infiltration of immune cells and the formation of additional adipocytes in the intra-orbital depots are characteristic of Graves' ophthalmopathy (Heufelder 2001; Schäffler and Büchler 2007). Most innate and acquired lipodystrophies involve facial and intra-orbital depots (Garg 2000).

The use of such material, both whole tissue and the stem cells derived from it, for reconstructive and cosmetic surgery (Clauser et al. 2008; Stillaert et al. 2010) has reinvigorated the study of previously neglected tissues structural depots in the human face (Yousuf et al. 2010) and limbs (Panettiere et al. 2011) are being re-examined.

1.4 Brown and Beige Adipose Tissue

Brown adipose tissue (though not non-shivering thermogenesis) are unique to mammals (Cannon and Nedergaard 2004). The comparative anatomy and histology of white adipose tissue were studied in detail (Hoggan and Hoggan 1879) 40 years before similar investigation in brown 'adipose tissue' began (Rasmussen 1922, 1923). The similarities between the names of these tissues and their contrasting but apparently complementary contributions to obesity prompted biologists to emphasise their resemblances, an attitude that recent molecular and developmental findings reveal to be misleading.

The pattern of gene transcription in stem cells differentiating into brown adipocytes resembles that of muscle more closely than that of white adipocytes (Timmons et al. 2007). Brown adipocyte precursors can be detected in skeletal muscle (Crisan et al. 2008) and muscle-specific microRNAs can be found in such cells in tissue culture (Walden et al. 2009). Both muscle and brown adipose tissue have numerous mitochondria, rich blood perfusion and high capacity for uptake and oxidation of fatty acids, some of which may be stored as triacylglycerols in small droplets. In a further similarity to adipose tissue, skeletal muscle is now believed to secrete 'myokines' especially when strenuously active (Pedersen 2011). The resemblances between brown and white adipose tissue arose convergently and long-established histological methods emphasise their similarities more than their contrasts.

The situation is further complicated by the identification of beige or brite adipocytes, that arise from, and in intimate association with, white adipocytes (Wu et al. 2012) and occur in traditional 'brown' adipose depots (Lidell et al. 2013). Under beta-adrenergic stimulation, beige adipocytes may acquire thermogenic, energy dissipating properties similar to those of brown adipose tissue (Wu et al. 2013; McMillan and White 2015), though at rates well below those of brown adipose

tissue (Shabalina et al. 2015). Their presence in many intra-abdominal and superficial depots may contribute to the relationship between body fat patterning and metabolism (Sanchez-Gurmaches and Guertin 2014). Beige adipocytes may be the basis for tissues in laboratory rodents that appear to be mixtures of interconvertible brown and white adipocytes (Giordano et al. 2014). The presence of beige adipocytes may also explain the observations that 'white' adipose tissue of free-living wild mammals, particularly arctic species, contains a greater proportion of protein, even in obese specimens, than the corresponding depots of laboratory rodents or humans (Pond and Mattacks 1989).

The anatomical distribution of beige adipocytes is yet to be studied as thoroughly as that of white or brown and preliminary reports suggest that their physiological roles may extend beyond thermogenesis. Gene activation in beige adipocytes that accumulate around chronic rotator cuff tears indicate that they also promote muscle repair (Meyer et al. 2015).

1.4.1 Origins of Thermogenic Mechanisms

Various tissues and metabolic pathways contribute to whole-body metabolic rate and facultative thermogenesis in lower vertebrates, many of them with common endocrine control (Silva 2006). A recent synthesis of the evolution of thermogenesis in vertebrates (Rowland et al. 2015) concluded that most ancient form of heat generation shivering in skeletal muscles was supplemented in teleost fish by non-shivering thermogenesis 'futile' cycles of calcium ion transport across the sarcoplasmic reticulum. At least two lineages of fish have evolved specialised 'heater organs', derived from skeletal muscle with greatly reduced contractile proteins and extensive, often folded, sarcoplasmic reticulum (Rowland et al. 2015). Some fish, especially large deep-water species, are functionally endothermic (Wegner et al. 2015) with white adipose tissue insulating the brain (Runcie et al. 2009).

Proteins resembling mammalian uncoupling proteins are also expressed in a reptile (the common green lizard, *Lacerta vivipara*) (Rey et al. 2008) and teleost fish (Jastroch et al. 2005) but reptiles, including dinosaurs (Grady et al. 2014) and their descendent groups (including prototherian and metatherian mammals) generate heat (that incubates eggs and other functions) in their extensive musculature by shivering and non-shivering biochemical cycles similar to those of fish (Rowland et al. 2015). Beige adipocytes have been proposed as an intermediate stage in the evolution of brown adipose tissue in eutherian mammals (Li et al. 2014).

The internal body temperature of almost all adult birds is slightly higher than that of euthermic mammals (Schleucher 2004) and in both groups, endothermy uses energy at 5–10 times the rates measured in ectotherms of similar body mass (Hulbert and Else 2000). Many birds, including some very small species, live in polar climates and/or swim in very cold water and, although feather insulation is as good or better than that provided by hair, endogenous thermogenesis is likely during sleep

and other periods of inactivity. Many nestling birds, and adults of a few species, become torpid at night or during periods of fasting and re-warm themselves with a mixture of shivering and non-shivering thermogenesis (Schleucher 2004; Geiser 2008). In spite of much wishful thinking and fruitless searching (Oliphant 1983; Saarela et al. 1989), brown adipose tissue cannot be demonstrated in birds (Mezentseva et al. 2008). Nonetheless, birds do have an uncoupling protein (UCP) that is structurally similar to UCP1, the key component of thermogenesis in mammalian brown adipose tissue (Raimbault et al. 2001; Emre et al. 2007).

Birds' relatively massive muscles are the principal source of thermogenesis, not adipose tissue. As well as shivering muscle mitochondria are uncoupled by membrane protein, adenine nucleotide translocase (ANT) not UCP, increased Na^+/K^+-ATPase activity on the plasma membrane (Walter and Seebacher 2009). Thermogenic substrate cycle involving the Ca^{2+}-ATPase pump on internal membranes regulated by sarcolipin also occur in mammalian skeletal muscle (Bal et al. 2012). The decline in activity from the maxima in neonates can be delayed by cold exposure (Pant et al. 2015). Such cycles of calcium ion transport across the sarcoplasmic reticulum are also found in their poikilothermic ancestors (Rowland et al. 2015).

Substrate cycles ('futile' cycles) in liver, muscle and white adipose tissue were described more than 30 years ago as mechanisms of metabolic regulation and thermogenesis (Newsholme et al. 1984). In small hamsters, rates of adipose tissue cycles of triacylglycerol lipolysis and fatty acid re-esterification differ between adipose depots, highest in small intermuscular sites, and respond to exercise (Mattacks and Pond 1988). Such cycles continue using significant amounts of energy even during starvation in rabbits suggesting that they are fundamentally important (Weber and Reidy 2012). With new findings in brown adipose tissue, interest in non-UCP dependent thermogenesis in mammalian adipose tissues waned, until recently revived (Flachs et al. 2013).

UCP1-based thermogenesis in adipose tissues evolved first in eutherian (placental) mammals probably closely linked to reproduction (Oelkrug et al. 2015). Thus the current hypothesis is that UCP is an ancient protein that in mammals evolved to the new role of thermogenesis by uncoupling the mitochondrial respiratory chain (Hughes and Criscuolo 2008). Facultative thermogenesis in skeletal muscle became so important that the contractile components disappeared, though the very small, rapidly mobilisable lipid droplets remained, ATP synthesis was much reduced though mitochondria became numerous, thus diverting myogenic pathways to form brown adipose tissue (Timmons et al. 2007; Mezentseva et al. 2008). Gene transcription studies reveal similarities between beige adipocytes and smooth muscle (Long et al. 2014) suggesting parallel evolution from contractility to thermogenesis (Rowland et al. 2015). Muscle-derived tissue is the primary source of non-shivering thermogenesis as well as shivering in mammals, as it is in birds. Both inherited this fundamental role for muscle from their reptilian ancestors. The mammalian tissue's confusing resemblances to white adipose tissue arise from its specialisation to thermogenesis fuelled by locally stored lipids at the expense of contractility.

This evolutionary perspective on recent molecular and developmental findings reveals the name 'brown adipose tissue', chosen after careful consideration of a

wide range of evidence from wild animals as well as humans (Rasmussen 1923), to be inappropriate leading to decades of the mistaken belief in its close resemblance to white adipose tissue, and later confusion with beige adipose tissue. A new name, perhaps 'thermogenic tissue', reflecting function regardless of developmental origin, would clarify the situation.

1.5 The Specificity of Fatty Acids

Since leptin was discovered in the early 1990s, the secretion and reception of adipokines has been centre stage in adipose tissue research, emphasising its similarities to other tissues of the immune and endocrine systems (Fantuzzi and Mazzone 2007; Galic et al. 2010). Nonetheless, improvements in equipment and techniques for separating, characterizing and quantifying lipids have greatly advanced understanding of adipose tissue's specialised roles in the sequestration, sorting and selective management of fatty acids and triacylglycerols.

1.5.1 Structural Lipids

All living cells are bounded by fatty membranes and most can oxidise fatty acids or their derivatives. After many years focussed on heritable information and protein synthesis, lipid membranes as barriers and in cell proliferation are now well recognized as central to the evolution of cellular life (Szostak et al. 2001; Stano and Luisi 2010).

Plants and algae synthesise fatty acids from primary photosynthetic products as and when they need them but animals obtain most of theirs from food. In vertebrates, most fatty acids are derived from the diet, with only minor metabolic modifications. For most animals most of the time, *de novo* synthesis contributes only a little, the main exceptions being those that fatten rapidly on a low-fat diet, often prior to reproduction, migration, diapause, hibernation or other prolonged fast.

Membrane fluidity is closely linked to the cells' capacity to support channels and receptors and to deform during movement. Failures in these processes are the principal mechanism of death during hypothermia in mammals such as humans that cannot hibernate (Boutilier 2001). Temperature modulation of membrane fluidity is determined mainly by fatty acid composition of the phospholipids, though the exact relationships are complex (Hayward et al. 2007). Several essentially similar mechanisms that adjust the fatty acid composition of membrane lipids to temperature are found in microbes, plants and animals (Guschina and Harwood 2006). Heterothermic animals most clearly demonstrate the relationships of dietary lipids and their metabolic modifications and anatomical organisation to physiological capacities. For example, the diurnal desert iguana, *Dipsosaurus dorsalis*, can tolerate a wide range of body temperatures (<5 to >40 °C); feeding experiments demonstrate that the fatty

acid composition of dietary lipids determines the temperature at which the lizards choose to rest (Simandle et al. 2001). The effects develop over several weeks and presumably involve alterations in the fatty acid composition of lipid membranes, though the neural links between diet, membrane composition and behaviour are unknown.

Structural lipids are also becoming more important in biomedical sciences. The fatty acid composition of membrane lipids has been implicated as a determinant of natural longevity in several lineages (Hulbert et al. 2014; Galván et al. 2015) and dietary fats correlate with certain psychiatric conditions including long-term cognitive impairment among elderly humans (Solfrizzi et al. 2010).

Although it is generally assumed that some, perhaps many, of the fatty acids in an animal's structural lipids have been components of its own or its mother's storage lipids, trafficking between neutral lipids and phospholipids has been little studied. An exception is the demonstration of the resemblance between the compositions of fatty acids in newly formed lymphoid cells and the triacylglycerols in contiguous adipocytes, suggesting that specialised adipocytes supply fatty acids to adjacent immune cells (Pond and Mattacks 2003; Mattacks et al. 2004a; Pond 2009).

1.5.2 Storage Lipids as Fuels

As well as providing fatty acids appropriate to structural lipids in various kinds of cells operating under various physiological conditions, the composition of triacylglycerols is important to their role as energy stores during strenuous exercise, immune responses and thermogenesis. Biomechanical and metabolic studies show that human running is not very efficient compared to that of animals adapted to fast long-distance travel (Alexander 2004). However, exercise physiologists recognize that comparative studies can offer tips on improving athletic performance.

Long-distance migration in birds, especially small species, is among the most metabolic demanding of all activities, fuelled almost entirely by fast, sustained mobilisation of storage lipids (Weber 2009). Sandpipers (*Calidris pusilla*) demonstrated selective incorporation of dietary fatty acids into structural or storage lipids and evidence for adaptive desaturation that maximises energy density and efficient mobilisation of the storage lipids during prolonged flight (Maillet and Weber 2006). However, studies of another species of sandpiper (*Philomachus pugnax*) produced no evidence for similar selectivity of fatty acids mobilised during shivering elicited by prolonged exposure to cold (Vaillancourt and Weber 2007). This comparison suggests that active lipid management entails some physiological cost: the process is essential preparation for migration (Weber 2009) which requires precise coordination between muscles during flight but is dispensable for shivering, a more chaotic activity. Similar investigations on mammals have not yet been performed.

1.5.3 Fatty Acid Sorting

In mammals including humans, selective deployment and transport of fatty acids begins as dietary lipids are absorbed from the gut (Hodson et al. 2009; Hodson and Fielding 2010). Both brown and white adipose tissue can harbour triacylglycerols of a wide range of compositions and various lipid-soluble substances, including potentially toxic contaminants and metabolic waste products. As well as storing and mobilizing metabolically useful lipids and glutamine, adipose tissue is a repository for such unexcretable end-products, especially in elderly.

The capacity of rat adipocytes for selective release or retention of fatty acids that differ in chain length and degree of saturation was identified more than 20 years ago (Raclot and Groscolas 1993). The process has been demonstrated in several mammals including humans and the cellular mechanisms are now well understood (Raclot 2003). Fatty acids released from adipocytes into the circulation contain more highly unsaturated fatty acids and fewer long-chain saturated and monounsaturated fatty acids than the triacylglycerols from which they are derived. Raclot (2003) concludes that 'the observation that the molecular structure of fatty acids seems to govern their release does not support the idea of a particular demand of the body for specific fatty acids.' Comparative studies in a broader context reveal this conclusion to be unduly pessimistic. When supplemented by fatty acid synthesis and modification, dietary choice and selective intake, these mechanisms contribute to lipid deployment and storage appropriate to temperature and other conditions.

This important biochemical mechanism has been little studied in other vertebrates. Experimental starvation of diamondback rattlesnakes (anatomically advanced, physiologically versatile snakes) kept at temperatures at which they would normally feed found some evidence for selective retention of essential polyunsaturated fatty acids in whole-body homogenates (McCue 2007). Studies of egg formation and embryonic development in the viviparous lizard *Pseudemoia entrecasteauxii* also reveal some capacity for fatty acid sorting in reptiles (Speake et al. 1999).

The process is much more specific and efficient in birds (Speake and Thompson 1999). Avian embryos oxidise mostly carbohydrate in the early stages of development, later switching to lipids. In domestic chickens, the cells lining the embryonic gut start 'eating' droplets of yolk around the twelfth day of incubation and pass its lipids into the blood as lipoproteins. At the same time, mature white adipocytes appear (early compared with mammalian fetuses) and take up the yolk-derived lipids. The adipocytes and the lipoproteins manage the embryo's irreplaceable lipid provisions, incorporating appropriate fatty acids into structural lipids while others are oxidized (Speake et al. 1998). For example, most polyunsaturated fatty acids in the yolk lipoproteins of king penguin eggs are preferentially incorporated into structural lipids in the brain and eyes, while the more abundant saturates are used in energy production (Groscolas et al. 2003). The composition of yolk lipids is similar in several species of penguin in contrasting habitats (Polito et al. 2012).

This capacity for fatty acid sorting is one of the major advances of avian embryos over their reptilian ancestors and is essential to the growth and maturation of the large complex brain and eyes (Speake and Thompson 1999). For example, only 0.24% of the key neural polyunsaturate, docosahexaenoic acid (22:6*n*-3), in the egg yolk of water pythons ends up in the structural lipids of the hatchlings' brains compared to nearly 20% in bird embryos (Speake et al. 2003).

By adjusting the relationship between diet and egg composition, fatty acid sorting facilitates utilization of new foods and extension of range, including breeding in captivity. The avian capacity for fatty acid sorting may be retained into adult life, contributing to selective incorporation of certain polyunsaturated fatty acids into adipocyte triacylglycerols and muscle membranes during the fattening period that precedes long-distance migration, thereby improving the efficiency of prolonged, strenuous exercise (Maillet and Weber 2006; Weber 2009). The fact that fatty acid sorting by adipose tissue has been investigated thoroughly only recently, more than 100 years after its role as a lipid repository was recognised, reflects the progress of scientific concepts and instrumentation.

1.6 Paracrine Interactions with Adipose Tissue

Functional interpretation of the anatomy of brown adipose depots was established long ago: its thermogenesis warms essential organs by direct conduction into contiguous tissues and by convection via the blood (Heaton 1972; Rothwell and Stock 1984; Cannon and Nedergaard 2004). But attempts to interpret the anatomy of the many minor depots of white adipose tissue that are intimately associated with the vasculature, skeletal and cardiac muscle, skin and the immune system have lagged far behind.

Until the 1990s, physiological studies of white adipocytes concentrated heavily on the large depots, especially epididymal and perirenal, which provide enough 'pure' adipose tissue for most biochemical analyses. Adipocytes in the small and large depots are histologically similar, so were assumed to be physiologically and functionally similar as well. Doubts raised by the observation that lymph nodes (in neonatal guinea-pigs) are firmly attached to the surrounding adipose tissue were ignored (Gyllensten 1950). The anatomical arrangement attracted little interest until site-specific properties indicating paracrine interactions between minor adipose depots and contiguous tissues were demonstrated, first in perinodal adipose tissue about lymph nodes (Pond and Mattacks 1995), then in 'adventitious' perivascular tissue around blood (Löhn et al. 2002) and lymph vessels (Dixon 2010).

The concept of 'paracrine' was originally, and largely still is, associated with control systems rather than cellular nutrition (Grossman 1979), reflecting the emphasis on informational mechanisms that has prevailed since the 1960s. Evidence for 'paracrine' interactions between mature adipocytes and other tissues was presented in the mid-1990s (Pond and Mattacks 1995, 1998) but the universality of the mechanism was not recognised until the late 1990s (Trayhurn and Beattie 2001).

These days, the paracrine relationships involving white adipose tissue are mainstream (Rosen and Spiegelman 2014) and are investigated as routes for drug delivery (Trevaskis et al. 2015).

The best understood are with muscle, lymphatics and blood vessels, but in mammals, 'yellow' bone marrow adipocytes secrete several adipokines and may interact locally with osteocytes (Hardouin et al. 2014; Devlin and Rosen 2015). The adipose tissue surrounding the prostate may also modulate its properties (Sacca et al. 2012). Even the epididymal depot of murid rodents, so widely studied as 'archetypal' white adipose tissue that it seemed to have evolved for scientists' convenience, has been recognised as essential to spermatogenesis in the contiguous testes (Chu et al. 2010). Recently, beige adipocytes have been implicated in paracrine mechanisms of tissue repair (Meyer et al. 2015).

1.6.1 The Immune System

The involvement of adipose tissue in immune function was inferred 70 years ago from developmental and anatomical observations (Gyllensten 1950) but became widely recognised in the 1990s, with reports of localized interactions around lymph nodes (Pond and Mattacks 1995) and systemic effects (Grünfeld et al. 1996). Other chapters address the exchange of signal molecules and the role of macrophages in inflammation of adipose tissue in obesity. This section concerns the evolution of functional, non-pathological relationships between adipose tissue and immune structures.

According to a recent theory (van Niekerk and Engelbrecht 2015), the capacity of white adipose tissue to support the metabolic costs of the cellular responses to pathogens was more important for the evolution of adaptive immunity in early vertebrates (i.e. jawless and jawed fish) than gene evolution or selective pressures. Many invertebrate lineages have the necessary genes and are similarly exposed to pathogens (Downs et al. 2014), but inadequate metabolic scope prevented the evolution of adaptive immunity as efficient as that of vertebrates.

The evolution of relationships between adipose and immune tissues can be traced through fish and poikilothermic tetrapods, but has been most thoroughly studied in mammals. At all levels from gross anatomy to molecular complexity, both the immune system and adipose tissues are more elaborate and diverse in mammals than in reptiles. Mammalian lymphoid organs are more numerous and elaborate, and involve more genes, proteins and cell types than those of other vertebrates, and many components are efficiently deployed only in association with membranes of appropriate composition (Zapata and Amemiya 2000). Although anatomically complex lymph nodes widely distributed throughout the body were described long ago as a characteristic feature of eutherian (placental) mammals, immunologist and lymphologists took longer to recognise their functional relationships to adipose tissue (Harvey et al. 2005; Harvey 2008).

Comparative studies show that associations between the immune system and adipose tissue evolved early in mammalian evolution (Pond 2003b). In the echidna

(*Tachyglossus*), a primitive prototherian mammal that lays large eggs (but feeds its nestlings on secreted milk), tiny lymph nodules embedded in fatty tissue are present throughout the chest, neck and pelvic regions (Diener and Ealey 1965). The larger, more complex lymph nodes of Metatheria (marsupials) are surrounded by adipose tissue in adult kangaroos (Old and Deane 2001). Although the authors do not mention adipose tissue, their images of developing lymph nodes in another small metatherian, the quokka (*Setonix brachyurus*), reveal adipocytes surrounding lymphoid tissue by the age of 2 weeks (Ashman and Papadimitriou 1975).

Parallel advances in the anatomical, and probably physiological, relations between adipose and immune tissues also evolved in birds, endothermic descendents of a different group of reptiles. Lymph nodes in birds are smaller, simpler and less abundant than those of mammals, but are nonetheless associated with adipose tissue: 'The simplest [lymph nodes in birds] represent non-encapsulated lymphoid infiltrates embedded in the fat tissue' (Zapata and Amemiya 2000). In the more complex lymph nodes of domestic chickens, lymphoid cells are intimately associated with adipocytes in various ways (Oláh and Glick 1983). Thus close association between lymphoid and adipose tissues seems to be a fundamental feature of endothermic vertebrates.

1.6.2 Perinodal Adipose Tissue Around Lymph Nodes

Investigations into the adipose tissue surrounding lymph nodes were prompted by the observation that these small clumps of adipocytes retained their lipid content in very lean but otherwise healthy wild mammals in which most other adipose tissue—cardiac depots being another important exception—had been depleted to invisibility.

Apart from slightly smaller volume and more extracellular and vascular material, perinodal adipocytes are anatomically indistinguishable from those elsewhere in the same individual and are identified only by biochemical properties (Pond and Mattacks 1995; Pond 2005). All such properties are most pronounced in the adipose tissue nearest to nodes and diminish with distance from them. Perinodal adipose tissue is arbitrarily defined as within a radius of 10 mm around a lymph node. Many, possibly most, of the fatty acids incorporated into lipids in lymph node lymphoid cells that are newly formed in response to immune stimulation are derived from triacylglycerols in perinodal adipocytes (Pond and Mattacks 2003). *In vitro* studies demonstrate that adipose stromal cells migrate from perinodal adipose tissue into adjoining lymph nodes where they interact with indigenous cells (Gil-Ortega et al. 2013).

The adipocytes in depots containing lymph nodes, especially perinodal adipocytes, seem to be partially emancipated from supplying lipolytic products to more remote tissue. Although such adipocytes respond *in vitro* more strongly to maximal noradrenalin, *in vivo*, they contribute less lipolytic products to the circulation during fasting than those in depots containing few or no lymphoid structures (Mattacks and Pond 1999). The basal rate of lipolysis in perinodal adipocytes is slightly lower than

that of other adipocytes but significant increases can be detected within an hour of an experimentally elicited immune response (Pond and Mattacks 1998). Increased release of fatty acids from perinodal adipocytes around the lymph node(s) draining the site of the immune stimulus reaches a maximum after about 6 h and then wanes, disappearing totally after about 24 h, unless prolonged by further stimulation. With repeated immune stimulation, increased lipolysis and responses to interleukin-4 and tumour necrosis factor-α spread to adipocytes situated further from the simulated lymph node within 12 h and to perinodal adipocytes around other, remote, lymph nodes within 24 h (Pond and Mattacks 2002).

The appearance of more receptors for tumour necrosis factor-α on perinodal adipocytes follows a similar time course in response to mild immune stimulation (MacQueen and Pond 1998). Perinodal adipocytes respond much more strongly than those not anatomically contiguous to lymphoid structures to tumour necrosis factor-α, interleukin-4 and interleukin-6 and probably other cytokines (Mattacks and Pond 1999). These signal molecules may mediate the paracrine interactions between adipocytes and the lymphoid cells that they supply.

The popliteal perinodal adipose tissue is most frequently studied only because these depots are easily accessible and being paired facilitates experimental design. The responses of perinodal adipocytes around other lymph nodes are qualitatively similar but differ quantitatively. The largest and most sustained responses are consistently found in the mesentery and omentum of rodents (Pond and Mattacks 2002; Mattacks et al. 2004a; Sadler et al. 2005), and probably also in humans, in which the patterns of site-specific differences in adipocyte triacylglycerol composition (the property most easily measured in preserved samples) are similar (Westcott et al. 2005).

Many of the site-specific differences in gene expression in murine mesenteric adipose tissue compared to epididymal or inguinal (Caesar et al. 2010) can be explained as adaptations to interactions with lymphoid cells within or emanating from lymph nodes. Human visceral depots include more blood vessels, especially in obesity, and are more susceptible to inflammation than superficial adipose tissue (Villaret et al. 2010). The gene products mediating the relationship between lymph vessels and adjacent adipocytes have been identified (Harvey et al. 2005). Chronic inflammation and induced genetic defects in lymph vessel growth can stimulate adipose tissue formation in quantities amounting to obesity (Harvey 2008). Perilymphatic adipose tissue (PLAT) exchanges signal molecules with cells in the lymph vessels it surrounds (Souza-Smith et al. 2015).

1.6.3 Permeating Dendritic Cells

Dendritic cells interact with adjacent adipocytes. Those extracted from the adipose tissue stimulate lipolysis, while those from adjacent lymph nodes inhibit the process, though the effects are strong only in perinodal and milky spot-rich samples and minimal in the adipocytes extracted from adipose sites more than 10 mm from

lymph nodes (Mattacks et al. 2005). Inducing mild inflammation by injection of lipopolysaccharide amplifies these effects, suggesting that they are integral to immune responses. Switching from anti-lipolytic to pro-lipolytic secretions seems to be among the transformations that dendritic cells undergo as they migrate from the lymph nodes through the adjacent adipose tissue, and thus should be considered as part of the maturation process (Mattacks et al. 2005). The lymph vessels that permeate the perinodal adipose tissue facilitate the uptake of dendritic cells from among the adipocytes and return them to the nodes, where they contribute to the inflammatory responses (Kuan et al. 2015).

The fatty acid compositions of lipids in intercalated dendritic cells closely resemble those of adjacent adipocytes (Mattacks et al. 2004a). Site-specific differences and experimental changes of the dietary lipids alter the fatty acid composition of both types of cells, but the similarities between cells that were contiguous *in vivo* remain. The simplest explanation for this resemblance is that maturing dendritic cells acquire fatty acids (and perhaps other precursors) from adjacent adipocytes, rather than from remote sources via the blood or lymph, as was previously assumed (Mattacks et al. 2004a). Structural lipids are the most easily traced, but those used for the production of signal molecules or ATP are probably of similar origin.

In all normal monogastric mammals that have been investigated, the triacylglycerols of adipocytes near to lymph nodes are disproportionately rich in polyunsaturated fatty acids, including the specific precursors of eicosanoid and docosanoid signal molecules that are integral to lymphoid cell function (Mattacks and Pond 1997; Pond 2003c). These differences in composition presumably arise by selective uptake and/or release of fatty acids that differ in chain length and degree of unsaturation (Raclot 2003). The site-specific differences in adipocyte-derived fatty acids thus conferred on intercalated dendritic cells add another source of structural, and perhaps also functional, diversity to these cells that hitherto have been classified by genes activated and proteins synthesised (Gehring et al. 2008).

In rats fed unaltered or sunflower oil-supplemented diets, prolonged experimental inflammation alters the composition of fatty acids in lipids of perinodal adipose tissue, and hence that of fatty acids incorporated into permeating dendritic cells (Mattacks et al. 2004a). But the fatty acid composition of phospholipids in such dendritic cells from unstimulated and immune-stimulated rats whose diet over the previous 6 weeks has been supplemented with fish oils are indistinguishable from those of immune-stimulated rats eating standard diets and hardly change under experimental inflammation. These data imply that diets enriched with fish oil create membrane compositions in dendritic cells that are ideal for supporting the immune response, thus eliminating the need for further adaptation in response to immune stimulation. Over a period of several weeks, the ratio of *n*-6/*n*-3 fatty acids in triacylglycerols in the perinodal adipose tissue surrounding the locally inflamed lymph node also changes, partially rectifying the composition imposed by dietary imbalances (Mattacks et al. 2004a). This mechanism may be among the ways that perinodal adipocytes minimise the impact of fluctuations in dietary lipids on whole-body immune function and may be physiologically important, especially during fasting and hibernation (Pond 2009).

The involvement of perinodal adipocytes in immune responses not only begins within minutes but can persist for months. In a rat experiment to explore recovery from simulated low-level chronic inflammation, the numbers of dendritic cells recovered from the locally stimulated lymph node and its perinodal adipose tissue were found to rise at least tenfold within 4 weeks of local subcutaneous injection of 20 μg of lipopolysaccharide three times a week and remained high for as long as this regime was applied (Sadler et al. 2005). Dendritic cell numbers were still significantly above baseline 12 weeks after termination of the regime of simulated low-level chronic inflammation. These effects were observed in node-associated adipose tissue remote from the site of stimulation as well as that adjacent to it with parts of the mesentery and omentum being among the most responsive. The mesenteric lymph nodes and their contents atrophy in mice made obese by a high-fat diet, apparently poisoned by high concentrations of fatty acids and lipoproteins (Kim et al. 2008). These findings have implications for slow, deleterious changes in both the immune system and adipose tissue induced by chronic stress and prolonged inflammation.

1.6.4 Adipose Tissue in Normal Immune Function

Immune cells of the innate and adaptive systems, including macrophages, neutrophils, B cells and T cells permeate adipose tissue at normal body composition and, in greater numbers, in obesity (Grant and Dixit 2015; Travers et al. 2015). But 'ordinary' subcutaneous white adipocytes respond to infections in adjoining skin by secreting antimicrobial peptides, supplemented by local proliferation and maturation of preadipocytes (Zhang et al. 2015). Inflammatory processes in metabolically active adipocytes are an integral component of adipose tissue's response to demand for increased fat storage (Asterholm et al. 2014). Although impaired interactions between the tissues are fundamental to obesity (Grant and Dixit 2015), the attitude that adipose tissue is controlled by the immune system (Brestoff and Artis 2015) is questionable.

Perinodal adipose tissue is specialized for more precise, localized paracrine interactions with the immune system, as summarized in Fig. 1.1. Many immunologically important fatty acids are dietary essentials, and hence can be limiting, especially during anorexia associated with major inflammatory diseases (Johnson 2002). By ensuring that the immune system has priority access to essential lipids, this mechanism complements sickness-induced anorexia, an ancient mechanism that has been demonstrated in arthropods (Adamo et al. 2010) and lower vertebrates as well as in mammals (Johnson 2002; Straub et al. 2010).

Without effective lipid management, key precursors may not be available when and where they are needed and could be squandered by increased oxidation of lipids during anorexia. By releasing appropriate fatty acids to lymphoid cells when and where they are required, the perinodal adipose tissue promotes efficient utilization of essential fatty acids and partially emancipates immune function from fluctuations

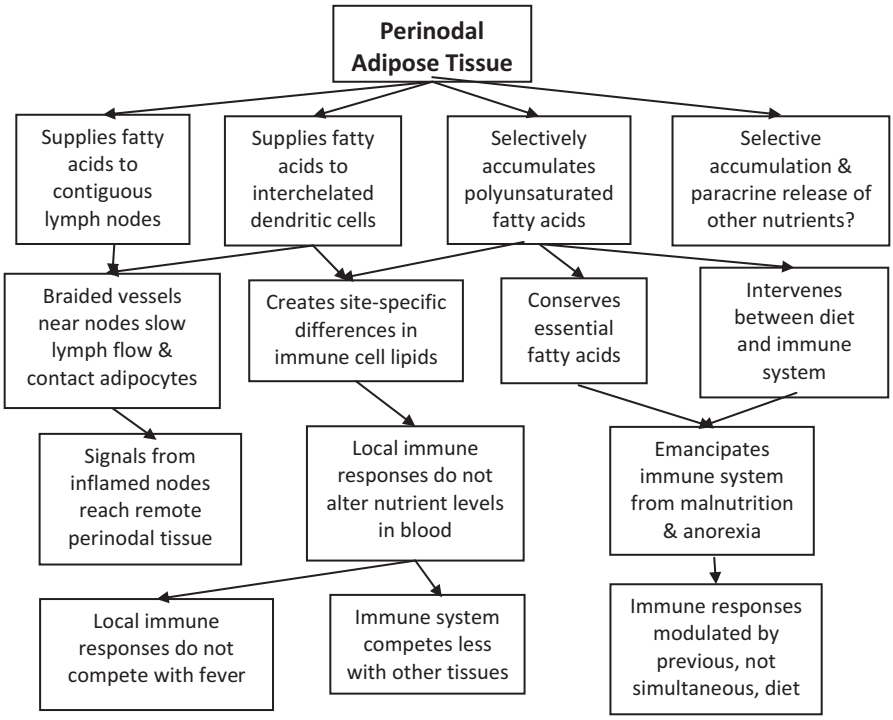

Fig. 1.1 Summary of the structure, properties and functions of mammalian perinodal adipose tissue and their roles in metabolism during immune responses

in the abundance and composition of dietary lipids (Pond 2003b). In rats, the selective accumulation of polyunsaturated fatty acids that generates the *n*-6/*n*-3 ratio appropriate for lymphoid cells is quite slow (Mattacks et al. 2004a) and can probably be overwhelmed by prolonged dietary deficiencies or excesses. Nothing is known about the extent to which the efficiency and robustness of these mechanisms differ between individuals or between species, thus making their immune systems more, or less, susceptible to impairment by dietary imbalance or insufficiency.

Paracrine control of lipolysis by lymphoid cells reduces competition with other tissues for specific, essential lipids, thus enabling fever and other energetically expensive defences against pathogens to take place simultaneously with proliferation, maturation and activation of lymphoid cells and with functions such as lactation and exercise, even during anorexia or starvation (Pond 2007). Under some circumstances, notably prolonged anorexia nervosa, immune function remains surprisingly efficient in spite of massive reduction in adipose tissue mass (Nova et al. 2002), less fever in response to infection (Birmingham et al. 2003) and altered plasma cytokines (Brichard et al. 2003). As long as local interactions between adipose and lymphoid tissues are unimpaired, the mammalian immune system can probably function over a wide range of body compositions. Obvious cachexia with extensive muscle

depletion occurs about the same time as perinodal adipose tissue disappears. Deficiencies in its capacity for preferential support of immune function, rather than reduction in whole-body energy supplies *per se*, may be the mechanism by which nutritional 'stress' impairs immune function.

Paracrine supply from specialised adipocytes to the immune system ensure supplies while minimizing lipid traffic in blood and its associated actions on metabolism and appetite and risk of damage to blood vessels. The concept is a special case of the hypothesis proposed by Unger (2003), Unger and Scherer (2010): adipocytes store fuel reserves safely, protect other tissues from fluctuations in the quantity and quality of dietary lipids and ensure that their 'client tissues' are appropriately supplied. Although more difficult to demonstrate experimentally, adipocytes may supply other nutrients to lymphoid cells. Glutamine is a likely candidate in view of its importance in nutrition of the immune system (Ardawi and Newsholme 1985) and its site-specific metabolism in adipose tissue (Digby and Pond 1995; Digby 1998).

Paracrine interactions with adipocytes may also account for some features of the anatomy of lymph vessels and nodes (Gyllensten 1950; Pond 1996; Harvey et al. 2005). The branching of fine lymphatics near nodes would slow the passage of lymph and bring a greater surface area of vessels into contact with adipocytes, thus facilitating the exchange of signals, nutrients and metabolites. Adipocytes specialised to interact with adjacent immune cells have been demonstrated in a variety of monogastric mammals but seem to be absent or at least to have very different properties in ruminants (Pond 2003c). Ruminant artiodactyls pass much more globulins and other components of passive immunity to neonates in the colostrum than most other mammals (Langer 2009). The functional and phylogenetic relationships between this habit and ruminants' unusual perinodal adipose tissue would be interesting.

A notable feature of naturally lean mammals (other than ruminants) is the retention of a small amount of perinodal adipose tissue around major lymph nodes, probably because prolonged fasting does not raise lipolysis in perinodal adipocytes as much as in adipocytes not anatomically associated with lymphoid tissue (Mattacks and Pond 1999). Lymphoid-associated adipose tissue also regenerates sooner. After experimental lipectomy of the epididymal fat pads of adult rats, compensatory regrowth of adipose tissue is significant 16 weeks later in the node-containing mesenteric and inguinal depots but not in perirenal (Hausman et al. 2004). All these site-specific properties are consistent with the indispensible paracrine support of immune function by specialized adipocytes.

The importance of membrane lipids to prompt, efficient immune responses (Heller et al. 2003; Serhan et al. 2008) and the local interactions hypothesis (Knight 2008) are becoming more widely accepted among immunologists but have been criticised by Schäffler et al. (2006) for lack of evidence that 'perinodal adipocytes and derived adipokines can directly influence the lymph node function in a paracrine manner during local inflammatory processes'. This comment misses the point common to most nutritional deficiencies and therapies. In providing appropriate membrane composition and precursors, perinodal adipocytes may equip lymph node lymphoid cells to respond appropriately and promptly to other signals, rather

than themselves generating short-term signals that can be easily measured in the laboratory. Although ill-defined, slow-changing and difficult to quantify, nutrition may be as important to well coordinated and regulated immune responses as the transiently-acting adipokines.

With the rise of lipidomics (Ivanova et al. 2004; Quehenberger et al. 2008) and better understanding of the roles of dietary lipids in immune function (Enke et al. 2008), the contribution of adipocytes to lymphoid cell diversity and function merits further investigation. Reports of translocation of lipid from adipocytes to human tumour cells in culture (Gazi et al. 2007) and its roles in human bowel disease (Zulian et al. 2013; Kruis et al. 2014) should prompt further study of paracrine mechanisms.

1.6.5 Human Perinodal Adipose Tissue

Perinodal adipose tissue is now recognized as an integral part of the lymphatic system, and as such, is under investigation as a potential drug target, especially for lipid-soluble agents (Trevaskis et al. 2015), and for conditions in which the tissue is directly involved.

Dietary lipids have long been implicated in both ulcerative colitis and Crohn's disease (Ananthakrishnan et al. 2014), as have adipocytes in their capacity to modulate interchelated macrophages by adipokine secretion (Kredel et al. 2013), but the relationships prove complex. Increased incorporation of *n*-3 polyunsaturated fatty acids into complex lipids usually suppresses inflammatory markers both *in vitro* and in chronic inflammatory diseases (Calder 2007). But blood-borne mononuclear cells from Crohn's disease patients contain more, not less, *n*-3 polyunsaturated fatty acids than those of the controls, and are deficient in arachidonic acid (Trebble et al. 2004). The site-specific differences in fatty acid composition of lipids in the mesenteric adipose tissue expected from animal studies (Pond 2003c) are absent from patients with Crohn's disease, though they were found in similar samples from the controls (Westcott et al. 2005). The composition of lymphoid cells in mesenteric lymph nodes resembles that of the adjacent perinodal adipose tissue in the controls, but not in the Crohn's diseased patients, which suggests that their adipocytes are not supplying fatty acids to cells in the adjacent lymph nodes. In the sample studied, the lymph node lymphoid cells from the Crohn's disease patients contained only 23% as much of the eicosanoid precursor arachidonic acid (C20:4*n*-6) as the controls. Its major fatty acid precursor, linoleic acid, and linolenic and docosahexaenoic acids, the precursors of docosanoids, were also significantly depleted. Such defects in lipid metabolism are not reflected in the fatty acid composition of superficial adipose tissue (Westcott et al. 2006) so would be difficult to identify without abdominal surgery.

Insufficiencies in the synthesis of eicosanoid and docosanoid signal molecules may contribute to the inappropriate inflammation characteristic of Crohn's disease and to its anomalous responses to anti-inflammatory drugs (Gassull et al. 2002;

Trebble et al. 2004). Induced colitis in rats increased the proportion of *n*-6 fatty acids in mesenteric perinodal adipocytes as well as modulating adipokine secretions (Acedo et al. 2011). General defects in perinodal adipose tissue leading to impaired immune function could explain the association between the bowel disorders and other chronic diseases such as arthritis, eczema and rhinitis (Book et al. 2003). Ingesting excess alcohol can disrupt its relationship in mesenteric lymphatics, causing inflammation in the adipose tissue and promoting onset of the metabolic syndrome linked to alcohol abuse (Souza-Smith et al. 2015).

'Fat wrapping' is local hypertrophy of mesenteric adipose tissue around the inflamed intestine, although nearly all patients undergoing laparotomies for Crohn's disease are lean following prolonged disruption to appetite, digestion and absorption (Westcott et al. 2005). As expected from the animal studies (Pond and Mattacks 2002), visceral adipose tissue remote from the diseased intestine as well as the contiguous 'wrapped fat' are inflamed in chronic Crohn's disease (Zulian et al. 2012). In rats, prolonged inflammation causes maturation of additional adipocytes and hence permanent enlargement in adipose tissue in the lymph tissue-rich intra-abdominal depots (Mattacks et al. 2003a; Sadler et al. 2005). The anomalous growth of adipose tissue in Crohn's disease may be induced by signals arising from adjacent immune cells unable to access sufficient, appropriate fatty acids to support inflammatory responses.

The roles of bacterial translocation into adipocytes (Kruis et al. 2014), inflammation in adipose tissue (Kredel et al. 2014; Gonçalves et al. 2015), impaired adipocyte apoptosis (Dias et al. 2014) and connective tissue changes (Shelley-Fraser et al. 2012) in inflammatory bowel disease are under active investigation. Site-specific differences in adipocyte susceptibility to bacterial colonization have been demonstrated between ulcerative colitis and Crohn's disease (Zulian et al. 2013). The roles of adipose tissue and the origins of its pathognomonic appearance are still hotly debated (Kredel et al. 2014).

HIV-associated adipose redistribution syndrome (HARS) is another chronic disease in which prolonged inflammation causes site-specific atrophy of some adipose depots alongside adipocyte hyperplasia and hence permanent enlargement of others, especially those that incorporate infected lymphoid cells (Pond 2003a). The condition is exacerbated by long-term treatment with antiretroviral drugs (Domingo et al. 2012) but such slow effects on adipose tissue are difficult to demonstrate *in vitro* (Mattacks et al. 2003b). HIV proliferates as an intracellular parasite in lymphoid cells, particularly dendritic cells (Lehmann et al. 2010). Mesenteric lymph nodes are important reservoirs of quiescent HIV (Estaquier and Hurtrel 2008). Recent findings reveal that adipose tissue harbours HIV and its simian counterpart in macaque monkeys (Damouche et al. 2015) and HIV-infected memory CD4+ T cells selectively accumulate in perinodal adipose tissue (Couturier et al. 2015).

Comparisons between node-containing depots show that paracrine interactions between perinodal adipocytes and dendritic cells are strongest in those around the numerous mesenteric lymph nodes and omental lymphoid tissue (Mattacks et al. 2004b, 2005; Sadler et al. 2005). Perinodal and omental adipocytes may proliferate (i.e. the depots enlarge) as part of their response to 'garbled messages' emanating

from HIV-infected dendritic cells (and other lymphoid cells including macrophages). HARS has been attributed to impairment of adipocyte mitochondria and regarded as a form of premature ageing (Caron-Debarle et al. 2010) but irreversible hypertrophy induced by prolonged paracrine interactions between parasitized lymphoid cells and adipocytes specialized to support immune function can explain the manifestation of the syndrome in drug-naïve as well as treated patients. Expert opinion on the mechanism of HARS now favours site-specific differences in inflammation of HIV-infected adipose tissue over the mitochondrial impairment hypothesis (Gallego-Escuredo et al. 2013).

1.6.6 Paracrine Interactions with Muscle

Lipolytic products as fuels for skeletal muscle have a long history and are well understood (Frayn 2010). Metabolic processes within adipocytes, such as intracellular re-esterification, as well as those in adipose tissue regulate levels in the circulation. In humans, mobilisation of local sources of lipid fuels within the muscle itself can make a substantial contribution. Intra- and inter-muscular adipose tissue and intramyocellular lipids are generally more conspicuous in large mammals and in muscles adapted to very frequent, sustained use, suggesting that these findings may apply generally to large species. Intramuscular adipocytes have distinctive site-specific properties (Gardan et al. 2006), though in early investigations, some were confused with features arising from proximity to lymph nodes embedded in small peripheral depots near skeletal muscle (Pond et al. 1984; Mattacks et al. 1987; Pond and Mattacks 1991).

Intramuscular lipids increase in athletes trained for sustained exercise and seem to be more quickly metabolised (van Loon and Goodpaster 2006). Paradoxically, intramuscular lipids increase in the leg muscles of healthy young people after a few weeks of experimental inactivity (Manini et al. 2007) and many reports link their presence to insulin resistance (Machann et al. 2004). Muscle satellite cells, stem cells essential to muscle repair and plasticity, can acquire features of adipocytes that could explain the enormous increase in such adipose tissue in humans (Vettor et al. 2009) and domestic mammals bred and raise for meat (Hocquette et al. 2010).

1.6.7 Cardiac Adipose Tissues

Until the 1990s, the adipose tissue in the human heart and pericardium was dismissed as pathological, irrelevant to normal function (James et al. 1982; Szczepaniak et al. 2007). Its gross anatomy and basic properties were introduced alongside appeals for further research (Iacobellis et al. 2005; Sacks and Fain 2007). Understanding of the normal function and pathology of cardiac adipose tissues has

advanced amazingly fast, aided by improvements to echocardiography, MRI and increased availability of biopsy samples (Iacobellis 2015). Cardiologists recognise a role for paracrine interactions involving musculature of the heart and major vessels and the small quantities of adipose tissue surrounding them (Hassan et al. 2012; Fitzgibbons and Czech 2014).

Both epicardial and pericardial adipose tissue are found in most lean, healthy wild mammals, especially large species (Marchington et al. 1989), and large birds such as swans (*Cygnus olor*), but are absent in the huge marine turtles (Braz et al. 2016) and probably other lower vertebrates. They are selectively spared in starvation, so both emaciated and very lean, healthy specimens may have lipid-filled adipocytes only in the cardiac and perinodal depots (see Sect. 1.6.2). As in humans (Sacks and Fain 2007), epicardial adipocytes are not bounded by fascia and always adhere tightly to the myocardium. In species that naturally become obese, no correlation between the masses of these depots and those elsewhere in the body is found (Pond et al. 1992, 1993, 1995) and the much more thorough studies of humans reveal surprisingly weak associations (Rabkin 2007).

Epicardial and pericardial adipose tissue are minimal in murid rodents so can only be studied experimentally in guinea-pigs or larger animals or *in vitro* (Marchington and Pond 1990; Swifka et al. 2008), until improved techniques enabled the depots to be studied in mice (Yamaguchi et al. 2015). Preliminary experiments 25 years ago suggested that these small depots are specialised to protect the heart from toxic levels of fatty acids by uptake and esterification, as well as to supply the cardiac muscle with fuel (Marchington and Pond 1990). The range of adipokines secreted from these specialised depots (Iacobellis and Barbaro 2008) and the finding that isolated rat heart muscle exports excess fatty acids *in vivo* (Park et al. 2004) are consistent with this concept. Fatty acids exchanged between the contiguous tissues may be accompanied by lipid-soluble pollutants that may be toxic to the heart (Bergkvist et al. 2015).

Comparing human epicardial adipocytes and myocardium with the developmental origins and maturation of homologous tissues in mice explains its appearance early in life and much of the contrasts between species (Yamaguchi et al. 2015). Transcriptome data from human biopsy samples indicate site-specific properties within epicardial adipose depot (Gaborit et al. 2015), another example of structural complexity within apparently amorphous adipose masses. Thermogenesis is now recognised as an important property of epicardial adipose tissue. Brown adipose tissue is clearly visible in these depots in neonates and hibernators (Nedergaard et al. 1986; Cannon and Nedergaard 2008) and in some adult humans (Cypess et al. 2009). In epicardial adipose tissue of Americans undergoing cardiac bypass surgery, particularly younger patients, the mRNA for the mitochondrial uncoupling protein (UCP1) is detectable (Sacks et al. 2009). Further examination of human biopsy samples indicates that the epicardial depots include some beige adipocytes (Sacks et al. 2013).

These and other aspects of recent research into these small, idiosyncratic but medically important adipose depots are thoroughly reviewed elsewhere (Iacobellis 2015).

1.6.8 Perivascular Adipose Tissue

Twenty years ago, the study of neurohumoral activity of perivascular adipose tissue around rat aorta was prompted by the observation that 'virtually every blood vessel in the (human) body is surrounded to some degree by adipose tissue' (Soltis and Cassis 1991). Like the epicardial adipocytes and those around lymph vessels, perivascular adipocytes are not separated by a fascia from the underlying tissue (Ouwens et al. 2010), an anatomical arrangement that facilitates paracrine interactions. These specialised white adipocytes are widespread and heterogeneous, with many site-specific differences (Gil-Ortega et al. 2015). They receive and secrete a wide range of signals (Fitzgibbons and Czech 2014; Ozen et al. 2015) and contribute to paracrine control of vascular smooth muscle (Ozen et al. 2015), tissue repair (Takaoka et al. 2010), immune processes (Omar et al. 2014), inflammation and thermogenesis (Brown et al. 2014; Fitzgibbons and Czech 2014). Defects in these interactions are implicated in various human pathologies, including atherosclerosis, blood pressure abnormalities and type II diabetes (Fitzgibbons and Czech 2014).

Aided by the development of animal models and *in vitro* systems (Brown et al. 2014), the physiology and medical implications of these small but active adipose depots is progressing rapidly (Fitzgibbons and Czech 2014), including adipokine secretion and paracrine control of vascular tone (Ozen et al. 2015). Gene expression and some physiological properties of murine perivascular adipose tissue resemble that of brown as well as adipocytes (Fitzgibbons et al. 2011). Thermal imaging combined with CT scanning reveals the presence of thermogenic adipose tissue around major blood vessels, as well as the oesophagus, upper regions of the spinal cord and vital organs of the thorax (Sacks and Symonds 2013).

1.7 Adipose Tissues for Mammalian Habits and Habitats

Several distinctive features of mammalian adipose tissues are described above: distributed anatomical arrangement, site-specific properties, fatty acid sorting, participation in multiple signalling pathways and paracrine as well as endocrine interactions. These properties can be related to some of the most fundamental features of mammals: herbivory, thermogenesis, variable, often high body temperature, lactation, allometric growth and sociality.

1.7.1 Diet

Selective foraging for foods that together form a balanced diet is essential for herbivores, but becomes less important if predator and prey have similar body composition (Kohl et al. 2015). Most extant reptiles are snakes, the great majority of which

prey on other vertebrates that they eat whole and within minutes of death (i.e. before rancidity and putrefaction impair its nutritional quality). Thus the chemical composition of the prey is unusually similar to that of the predator. All adult crocodiles and most large lizards are also predators on other vertebrates and they eat at least some of it very fresh. Such prey may be intermittently available and demanding to obtain but a single meal is close to supplying a 'balanced diet'.

Large herbivorous reptiles became extinct at the end of the Mesozoic and failed to re-establish themselves in the face of competition from mammals and birds. The only reptilian herbivores to survive into the modern era are the tortoises and the adult stages of a few tropical lizards (the juveniles eat small prey, as do the chicks of most herbivorous birds). Flight and climbing enable birds to access a varied diet of highly nutritious, energy-dense foods that may be widely dispersed. In all extant species, the teeth are entirely replaced by a beak, digestion is quick and water requirements usually low.

In contrast, the great majority of mammals are and have been throughout the Tertiary specialist consumers of fruit, seeds, grasses and other vegetation, abundant but nutritionally imbalanced foods that can be successfully exploited with good teeth, efficient digestion and means of detoxifying plant anti-herbivory compounds. Many mammals have highly specialized dentition and/or digestion and restricted ranges so at least at certain seasons, their diets are more homogeneous than those of similar-sized birds. From a nutritional point of view, such diets are far from ideal, often low in minerals and essential amino and fatty acids, though efficient chewing and digestion greatly improve absorption (Langer 2002). Small mammals, particularly the large ubiquitous groups such as rodents and bats (Chiroptera), owe their abundance and diversity to the ability to breed prolifically on monotonous, nutrient-poor diets. Eating more of poor quality but abundant forage to obtain these components generates too much energy, which may be stored in white adipocytes or dissipated by diet-induced thermogenesis (Cannon and Nedergaard 2004). In other words, 'burning off' excess energy can help to correct nutritional imbalances in monotonous or barely adequate diets, distilling out scarce nutrients including amino acids, essential fatty acids, vitamins and minerals from energy-rich but nutrient-poor foods.

The principal mediator of such facultative thermogenesis is probably mitochondrial uncoupling, but especially in large mammals that have little brown adipose tissue, other substrate cycles in muscle or liver (Dulloo et al. 2004; Wijers et al. 2009; Rowland et al. 2015) and thermogenic processes demonstrated in subcutaneous white adipose tissue of UCP1-knock-outs (Meyer et al. 2010) may contribute. Such processes may underlie the finding that lipodystrophic but not 'healthy normal' humans also respond to excess fat intake by substantially increasing their total daily energy expenditure (Savage et al. 2005). Those familiar only with lab animals and people on modern, nutritionally balanced diets fail to recognise the central role of such processes (Kozak 2010).

The presence of brown adipose tissue in superficial depots on the back and neck of adult mammals, including humans (Nedergaard et al. 2007), is also consistent with heat dissipation. Thermogenic adipose tissue in internal depots inside the

abdomen and thorax works best for heat retention, as required for rewarming after birth, and for hibernation and torpor. Diet-induced thermogenesis was among the first roles of brown adipose tissue to be investigated in adults (Stirling and Stock 1968; Rothwell and Stock 1979), but proved less convenient than cold exposure for studying the cellular and molecular mechanisms in laboratory rodents (Cannon and Nedergaard 2004; Xue et al. 2009). Spectacular physiological feats such as rewarming of adult mammals following hibernation and tiny neonates achieving euthermy attracted more thorough investigation, leading to the notion that these functions may be the original roles of brown adipose tissue. This conclusion overlooks the importance of adjusting metabolism to diet and digestion in conferring many of the ecological advantages of mammals over reptiles and birds. Nonetheless, facultative thermogenesis is now seen as central to maintaining energy balance (Wu et al. 2013) and recent accounts of thermogenesis in brown and beige adipocytes recognise control from 'other stimuli' as well as cold exposure (Cohen and Spiegelman 2015) (Fig. 1.1). Feeding has been shown to increase non-shivering thermogenesis involving several different pathways in sheep skeletal muscles (Clarke et al. 2013).

The capacity to deal with imbalanced or nutrient-poor diets may be transferred to offspring, probably through epigenetic mechanisms, as 'fetal programming' (Barker 2002; Mostyn and Symonds 2009; Symonds et al. 2009). Brown as well as white adipose tissues are particularly susceptible to such maternal influences (Symonds et al. 2003).

1.7.2 Controlled Heterothermy and Thermogenesis

Endothermy has long been regarded as the principal physiological advance of mammals over ancestral mammal-like reptiles, but it evolved slowly and is far from 'perfect' in some living species.

Many living reptiles are poikilothermic, warmed by solar radiation and by heat generated by exercise, protein synthesis and digestion, suggesting the same of the extinct ancestors of mammals and birds including dinosaurs, for which the term 'meosthermy' has been coined (Grady et al. 2014). Implanted probes reveal deep-body temperatures in the large South American tegu lizards (*Salvator merianae*) that cannot be fully explained by these processes alone but arise from seasonal changes in metabolic rate and in whole-body thermal conductance (Tattersall et al. 2016). The highest temperatures were found in breeding females, as is also true of primitive eutherian mammals (Levesque and Lovegrove 2014).

Although many different biochemical processes contribute to body heat, mammals and birds maintain core body temperature very precisely during euthermic periods (Silva 2006).

Controlled heterothermy (i.e. hibernation, torpor) takes several distinct forms in mammals (Ruf and Geiser 2015) but some general principles emerge. Hypothermia entails selective gene activation and dedicated neural pathways that set minimum body temperature which protects body tissues and maintains adequate but low

energy expenditure during fasting, followed by rewarming by BAT and muscle-based thermogenesis. At maximum, thermogenesis is among the most energy-demanding of all biological activities (Cannon and Nedergaard 2004) and entails rapid mobilization and transport of lipids from cool tissues.

Although mammals can oxidise almost all animal-derived (and most plant-derived) fatty acids when euthermic, efficient hibernation depends upon appropriate fatty acid composition of storage lipids. Experimental feeding of captive mammals and observations on diet selection in free-ranging specimens show that some hibernators can achieve low body temperatures, and hence minimal energy expenditure, only if they have access to adequate quantities of lipids containing low melting-point fatty acids (Dark 2005; Frank et al. 2008). The functional bases of this relationship are not fully explored but optimizing membrane fluidity and lipid transport in cold, slow-moving blood are among the possibilities.

Such experiments demonstrate the importance of different fatty acids for various aspects of metabolic well-being. While diet selection is the principal mechanism by which blood-borne lipids acquire compositions appropriate to the tissues' requirements, the adipose tissues can help. During the fattening period preceding hibernation, the adipose tissue of Alpine marmots (*Marmota marmota*), a strictly herbivorous rodent, selectively retains unsaturated fatty acids (Cochet et al. 1999). The echidna (*Tachyglossus aculeatus*), one of the most primitive extant mammals, also selectively utilises monounsaturated fatty acids during prolonged hibernation (Falkenstein et al. 2001). Fatty acid sorting ensures that saturates are oxidised while the body is euthermic, reserving the lower melting-point fatty acids to support metabolism at low temperature. The capacity partially emancipates mammals from the necessity of ingesting a diet containing a large proportion of monounsaturated and polyunsaturated fatty acids just before hibernation and thus extends the range of foods that hibernators can exploit.

Immune responses to pathogens acquired during or just before hibernation are fully effective only after arousal and rewarming (Prendergast et al. 2002). The slow transport of nutrients in the blood and lymph that delay immune responses at low temperatures would be alleviated by paracrine provision of lipids from perinodal described above (Sect. 1.6.4).

Thus fatty acid sorting in adipose tissue, even if slow and only partially efficient, enables mammals to adapt to ecological fluctuations and species to diversify into new niches. Many students of physiological evolution believed that 'constitutional eurythermy' was the norm for primitive Mesozoic mammals i.e. torpor and hibernation are very ancient habits (Grigg et al. 2004). If so, more efficient fatty acid sorting and paracrine provision may be early and fundamental properties of mammalian white adipose tissue, and indeed do occur in prototherians (Falkenstein et al. 2001). Hibernation implies both controlled cooling and active warming; shivering and activation of brown adipose tissue, both fuelled by lipolytic products released from white adipose tissue, are the main mechanisms of additional thermogenesis in mammals. Palaeontological perspectives on the evolution of endothermy recognise a role for more abundant and leakier mitochondria (Kemp 2006), but not of the adipose tissues that manage the physiologically risky process of thermogenesis (normal body is perilously close to dangerous hyperthermia) as well as hold and dispense the fuel.

1.7.3 Pregnancy and Lactation

Birds are endothermic and the majority provision their hatchlings, mostly by gathering appropriate foods that may differ significantly from those of the parents, but a few, notably pigeons and doves, produce 'crop-milk', a mixture of deciduous tissue and secretions from the upper digestive tract. Thus comparisons between these two advanced groups can reveal something of the origins and physiological relationships of these traits (Farmer 2003), both predicated on properties of mammalian adipose tissues.

Comparative anatomists and physiologists have long emphasised that lactation is an ancient and fundamental habit of mammals (Pond 1977; Farmer 2000), a conclusion consistent with modern genomics (Lefèvre et al. 2010) and evo-devo analysis (Oftedal and Dhouailly 2013). Lactation enables mammals to breed efficiently on any diet that can support the adults, supplemented as necessary by body reserves, in contrast to birds and reptiles, especially large species, that need access to foods suitable for all growth stages (Pond 1983). Ecological modelling of this reproductive strategy (Dall and Boyd 2004; Kunz and Hosken 2009) fail to recognise the importance of lipids, proteins and minerals reclaimed from adipose tissue, bone etc. to supporting lactation and thus rapid growth of suckling offspring through periods of food shortage. Adipose tissue's role in managing lipid supplies to the mammary gland has been demonstrated in laboratory animals and livestock (Vernon and Pond 1997) and in wild mammals (Fowler et al. 2016).

Reliable supplies of nutritionally balanced milk support rapid post-natal growth and remove the need for diet-induced thermogenesis, thus releasing brown adipose tissue in suckling mammals for cooling-induced thermogenesis. By transferring the physiological demands of obtaining and digesting food from neonates to mother, functionality of some systems, notably the teeth, can be postponed until the body has grown large enough to support them. Figure 1.2 summarizes the causal relationships of these apparently disparate features and habits to thermogenic adipocytes and those involved in storage, fatty acid sorting and signalling.

Milk synthesis and secretion have long been recognized as energetically demanding processes, especially for small mammals that have large litters and/or nutrient-poor diets (Langer 2003). The mother's gut, liver and pancreas enlarge during lactation to meet the additional metabolic requirements but the composition of the food does not usually change: the mother just needs more of her usual foods, thus permitting the evolution of specializations of teeth, digestion and metabolism to particular diets and largely eliminating the need for seasonal migration to habitats that can support breeding. Energy and nutrients from body stores and greatly increased food intake contribute to milk synthesis but competing metabolic demands, including some immune processes, may be compromised (McClellan et al. 2008; Speakman 2008) and human mothers experience extreme tiredness.

The finding that shaving mice increases milk secretion suggests that the capacity to dissipate metabolic heat, not nutrient availability, is limiting, at least for small mammals (Król et al. 2007). The capacity to support such high metabolic rate and

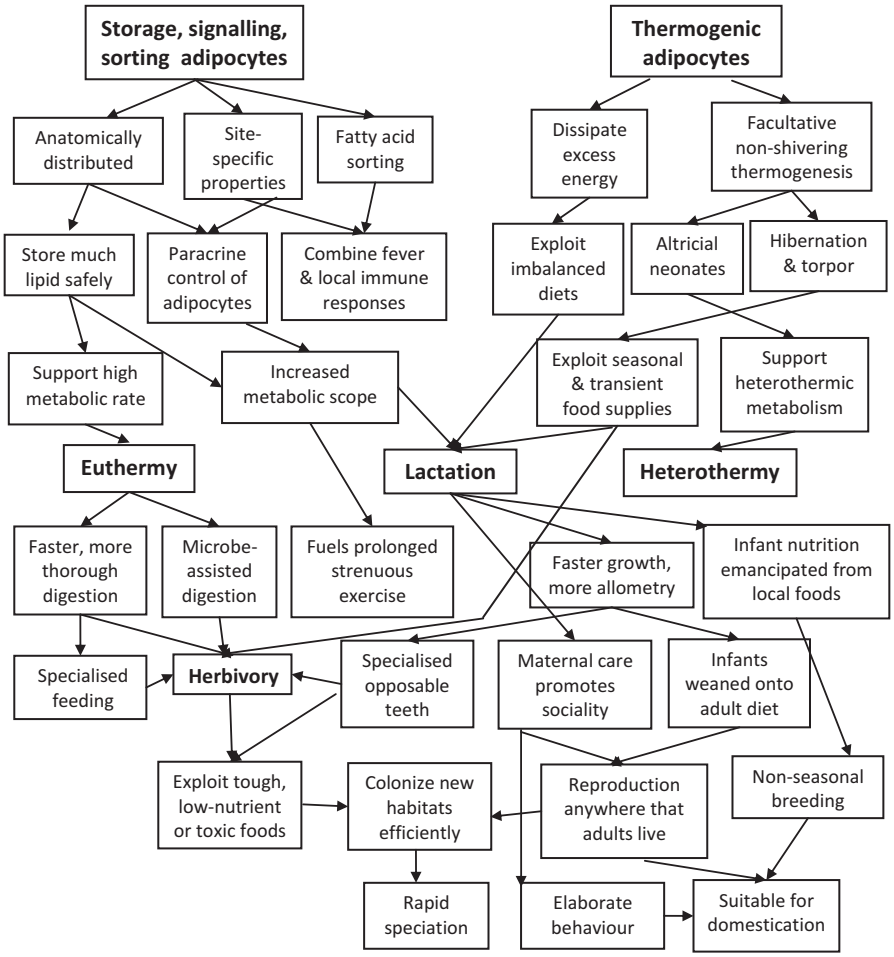

Fig. 1.2 Summary of the contributions of storage, signalling & sorting (*white*) adipocytes and thermogenic (*brown*, *beige*) adipocytes to the evolution of mammalian structure, habits and reproductive strategy

to tolerate high body temperature, at least transiently during lactation, must have evolved alongside the evolution of mammary glands, secretory mechanisms and milk proteins. Genomic data on the latter show that lactation evolved very early in mammalian ancestry and that genes, proteins and cellular processes derived from the immune system make a major contribution (Goldman 2002; McClellan et al. 2008). Milk may have been as important to protection from pathogens (many of them derived from the nest and/or the parents) as to nutrition, as it is modern eutherian mammals (Langer 2008, 2009). In both cases, adipose tissue is strongly implicated.

Many wild mammals fatten during pregnancy with the stored nutrients supporting milk synthesis. Mammals that eat little or nothing during lactation become massively obese before parturition: for example, polar bears give birth to up to four relatively very small offspring in inland dens and suckle them for several months without eating or drinking until the young are mature enough to accompany the mother to the coast where she has access to her normal diet of seals (Ramsay and Stirling 1988). Somehow, females adapted to such reproductive strategies avoid the complications of pregnancy and parturition found in obese women (Davis and Olson 2009).

Thus metabolic adaptations enabling storage of large quantities of lipid during pregnancy can evolve among wild mammals, but are weak or absent in women, suggesting that humans are adapted to support pregnancy and lactation mainly from current diet. The gluteo-femoral depots, that are always extensive in women of reproductive age but regress after menopause (Wells et al. 2010), extract fatty acids from the circulation slightly more slowly than other subcutaneous adipose tissue so may be an adaptation to long-term, more metabolically inert lipid storage (McQuaid et al. 2010). Nonetheless, lactation for a substantial period has clear benefits for women's lipid metabolism and body composition after giving birth (Stuebe and Rich-Edwards 2009). The observation that obese women breast-feed less competently than comparable women of normal body composition (Kitsantas and Pawloski 2010) is also consistent with the conclusion that, from an evolutionary point of view, obesity during human breeding is an aberration not an adaptation.

Metatherian and eutherian mammals produce very small, almost yolk-free eggs and the fetus is supplied entirely by the mother after development begins. Nutrient uptake is continuously regulated by the placenta and by the fetal tissues, processes that, at least in the most primitive eutherians may entail thermogenic adipose tissue. The hedgehog tenrec (*Echinops telfairi*) of tropical Madagascar is among the most phylogenetically ancient extant eutherians in which the unusual anatomical arrangement of brown adipose tissue suggests it may warm the gonads. Like many primitive mammals, its body temperature fluctuates except during pregnancy when non-shivering thermogenesis maintains euthermy, suggesting that thermogenic adipose tissue was fundamental to reproduction in early eutherians (Oelkrug et al. 2013, 2015). In euthermic rodents of similar size, the perigonadal depots are among the last to develop thermogenic adipocytes under experimental conditions (Sanchez-Gurmaches and Guertin 2014).

During gestation, glucose is the main energy source and most fatty acids are incorporated into cell structures, but immediately after birth, the roles reverse. Birth also triggers major changes in the immune system that adapt the neonate to symbiotic and pathogenic microbes, not least those from their own parents (Calder et al. 2006). In contrast to lower vertebrates and birds, the development of adipocytes is delayed until shortly before birth. Even the exceptionally large quantities of white adipose tissue in neonatal humans does not form until the last trimester of gestation (Kuzawa 1998). Lack of involvement in fetal metabolism may have enabled the specialization of adipose tissues to perinatal thermogenesis and paracrine interactions.

1.7.4 Primates and the Origins of Human Obesity

Despite its obvious relevance to humans, primate adipose tissue has only recently been studied from comparative and evolutionary perspectives, mainly to explore the propensity for obesity and its relationship to reproductive biology, including sex differences in its distribution and the demands of gestation and infant care.

The energetic cost of reproduction is lower in primates than in other advanced eutherians (such as rodents and ungulates) and is particularly low in humans (Dufour and Sauther 2002; Prentice et al. 2005). Using evidence from anthropology, reproductive biology and diet, Wells (2010) concluded that in the great apes, encephalisation (disproportionately large brain) and omnivory are among the characteristics closely associated with increased adiposity. More than half of the dry weight of the brain is lipid, a high proportion of which contains long-chain fatty acids derived from dietary essentials. So the metabolic bases of both these features require efficient digestion and internal distribution of dietary and synthesised lipid. Another dimension to the evolutionary relationships between brain and adipose tissue in primates is the hypothesis that physiological flexibility in energy storage is complemented, to varying degrees, by cognitive flexibility that entails reasoning and social organization and thus a large brain (Isler 2014; Heldstab et al. 2015).

For more than 50 years, the evolution of exceptionally high average fatness of humans was attributed to 'thrifty' genes that enable people to cope with food supplies that, until very recently, were irregular and unpredictable (Wells 2009, 2010; Watve 2012). Adaptations to recurrent starvation were claimed for the human genome and patterns of gene imprinting *in utero* (Prentice et al. 2008). Attractive though it is, this hypothesis is inconsistent with much human demographic data and genetic theory (Speakman 2007), and with information from other primates. A recent critique (Higginson et al. 2016) directs attention away from external factors affecting food supplies and towards the evolution of internal factors that regulate body mass, and, by implication, appetite.

The ranges of several other species of long-lived, slow-maturing apes were closely similar to those of *Homo* and its ancestral genera for much of their evolutionary history, so would be similarly affected by prolonged, severe famines caused by climate fluctuations etc. Comparisons of body composition, metabolism and habits can illuminate the origins of human obesity. Even after many years in humane captivity, the average proportion of dissectible adipose tissue in bonobos (*Pan paniscus*) is much greater than the minimum compatible with health found among indigenous peoples following traditional lifestyles, though, as in humans, females are consistently fatter than males (Zihlman and Bolter 2015). Gorillas (*G. G. gorilla*) and orang-utans (*Pongo*) in captivity can become fatter (Zihlman and McFarland 2000) but in the absence of data from free-living specimens, the norms for wild apes are difficult to estimate.

Three metabolically active body constituents, the skin, skeletal muscle, especially of the arms, and the digestive system, are proportionately more massive in these great apes than in modern humans (Zihlman and McFarland 2000; Zihlman

and Bolter 2015). Nonetheless, measurements on healthy young adult orang-utans in a large, semi-natural enclosure in Iowa reveal the lowest daily energy expenditure ever recorded from a higher primate (Pontzer et al. 2010). Their idiosyncratic locomotion through dense forest is unusually efficient (Thorpe et al. 2007). This ape, which evolved under selective pressures similar to those acting on the ancestors of modern humans and chimpanzees (Enard et al. 2010), has responded to unreliable food supplies by improving mechanical and metabolic efficiency and breeding slowly.

The human diet has probably been as diverse as it is throughout the modern world for much hominid evolution (Bellisari 2008). Such adaptability contributes greatly to efficient colonization of new habitats (Wells and Stock 2007) and to coping with rapid fluctuations in climate (Wells 2012c). Hunting animals, especially in large social groups, requires advanced cognition and communication, and prolonged strenuous activity but provides nutrient-dense food. Cooking and manual food preparation increase the efficiency of nutrient extraction, enabling the jaws, teeth and digestive system to become smaller, and to exploit a greater variety, but not quantity, of different foods (Wrangham 2009). Most chimpanzees' diets include some animal food and some actively hunt other mammals. Chimps apparently understand the advantages of cooked food (Warneken and Rosati 2015), thus supporting the hypothesis that cooking evolved earlier among the ancestors of modern people than previously believed (Wrangham 2009).

Such advances in diet and food processing allow for smaller stomach and intestines, contributing, with the radical changes to the pelvis and lumbar region (Warrener et al. 2015), to the distinctively human shape of the abdomen and waist. Smaller abdominal cavity would mean less space for intra-abdominal adipose tissue, thus promoting expansion of the superficial depots without altering overall body composition, as has happened in Carnivora (Pond and Ramsay 1992).

Diet, foraging strategy, food processing and digestion are intimately related to the evolution of hair reduction and hence of superficial adipose tissue. Diurnal pursuit of prey in the tropics generates much body heat, and many unique features of human anatomy, including hair reduction, evolved adaptations to heat dissipation (Wheeler 1991; Lieberman 2015). Body hair became thicker and more extensive in elephants, rhinoceroses and other large mammals as they colonized cooler areas, but not in *Homo*, which exploited fire and animal skins instead. A review of alternative hypotheses for the evolution of hair reduction favoured the theory that it protects against ectoparasites and the diseases they transmit (Rantala 2007).

The human integument not only has greatly reduced hair over most of the body, it is also much less massive than that of other great apes (Zihlman and Bolter 2015). Humans have more white adipose tissue, especially in superficial depots, than other mammals from late gestation onwards (Kuzawa 1998). Even non-obese western adults have about ten times as many white adipocytes as would be expected in a wild mammal of similar size and diet (Pond and Mattacks 1985c). Although similar in general organization and relative thickness to that of other (furred) primates, human superficial adipose tissue is unusually extensive and supports various skin functions (Klein et al. 2007). The unusual thinness of human skin may contribute to

the tendency of adipose tissue to form, sometimes in substantial quantities, on limbs with impaired lymph drainage (Brorson et al. 2008) and perhaps some forms of generalised obesity (Harvey 2008).

Such theories cannot explain why human ancestors evolve thinner, softer skin overlying thicker superficial adipose tissue. The identification of beige adipocytes in subcutaneous adipose tissue under cold-exposed skin (Lidell et al. 2014) suggests that the tissue may be capable of thermogenesis as well as insulation (Alexander et al. 2015). Thermal insulation entails restriction of blood flow, while heat dissipation and active thermogenesis require a rich blood supply. The extensive vasculature and its neural control combined with reduced pigmentation enable the skin to acquire roles in social and sexual signalling, as it has in other higher primates (Street et al. 2016). From about 2.3 My ago, visibility of such indicators of social and sexual status was enhanced by reduction in body hair (Dror and Hopp 2014), contrasted against denser hair on the head and external genitalia. Exposed skin enabled sex and ethnic differences and age-related changes in adipose tissue distribution to evolve under sexual as well as natural selection (Pond 1998).

Adipose tissue is central to such intra-specific communication and its roles in flexible habits and life history strategies in human evolution (Wells 2012b). Sexual dimorphism in the distribution of adipose tissue is not more extensive in macaque monkeys and lemurs (prosimians) than would be expected from differences in body size (Pond and Mattacks 1987; Pereira and Pond 1995), but contributes to the signalling of age and social status in most apes, including the conspicuous fatty cheeks in mature male orang-utans (Caillaud et al. 2008). These small but conspicuous depots on the face and head are species-specific and are presumably composed of adipocytes arising from the neural crest, another example of its plasticity (Billon et al. 2007).

The interpretation of sex differences in the distribution of superficial adipose tissue does not consider the contribution of site-specific properties to whole-body metabolism. The rounded buttocks created by pelvic adaptations to faster, more efficient running are a key evolutionary advance of *Homo sapiens* that in women are emphasised by adipose tissue to create enlarged, rounded buttocks and smooth, thickened thighs. This distribution of adipose tissue, conspicuous and sometimes massive at least since the Palaeolithic, emphasises the sex differences in pelvic anatomy that enable women to combine running fully erect with giving birth to neonates with large heads (Pond 1998; Gruss and Schmitt 2015). The intrinsic metabolic properties of these depots have also contributed to human evolution: enlargement of these lower-body depots entails less risk of metabolic and cardiovascular disease than expansion of intra-abdominal and upper body depots (Karpe and Pinnick 2015). In other words, natural selection has promoted the evolution thick thighs and large buttocks that enable women to maximise energy reserves without compromising longevity. Sexual selection for these depots in younger, reproducing women (Furnham et al. 2004) promotes social bonding that is essential for the role of post-reproductive ‘grandmothers’ in advancing the evolutionary success of their descendents (Wells 2012b), and also entails high post-menopausal life expectancy.

Adipose tissues thus have several important roles in the anatomy and metabolism of modern humans, and have evolved to continuously support more fat than is found in most mammals. Body insulation from clothing during the past 70,000 y would undermine the thermoregulatory importance of superficial adipose tissue, freeing it to evolve under different selection pressures, or none, and producing the observed variations in the modern population (Wells 2012d). A wide range of other habits and social factors contribute to the pathogenicity of large quantities of adipose tissue (Wells 2012a).

The only large-scale, long-term study of spontaneous obesity in large primates is that of macaque monkeys (*Macaca mulatta*) 'ranched' in large enclosures in USA. The resemblances to human populations are striking: not all apparently similar monkeys on similar diets gain weight, and of those that become obviously obese, not all develop metabolic complications (Schwartz et al. 1993; Wells 2009).

1.7.5 Rapid Adaptation to Modern Diets and Lifestyles

In a few taxa, notably whales (Cetacea), large quantities of adipose tissue are widespread and ancient enough for positive selection for 'obesity' to be detected for scores of genes involved in lipid metabolism and its control (Wang et al. 2015). Such studies demonstrated that mammals can evolve adaptations to obesity, but are only obliquely relevant to the current epidemic of human obesity and other metabolic disorders.

Bears, Svalbard reindeer and other mammals too big to hibernate and not subject to heavy predation deal with similar fluctuations in food intake by both long periods of low energy expenditure and by impressive levels of obesity, at least for part of the year. Their total adipocyte complements are only 2–3 times larger than those of related lean species (Pond 1998), mostly in superficial depots (Pond et al. 1993), and there is no evidence that they suffer from the complications of pathological obesity found in modern people and in many apes and large monkeys in captivity. These and other evolutionary and comparative points are among the most persuasive evidence that humans are not naturally and adaptively obese. But the observations on bears show that metabolic adjustments to high-fat or poor quality diets and improved thermogenesis can evolve over many millennia, not decades, and presumably under intense natural selection.

The present rate of change of human diets and habits is apparently the fastest in history and probably in pre-history (Wells 2006, 2010, 2012a; Morin 2012). Arctic mammals may be the only wild animals that have undergone comparably rapid adaptation. Bears (Carnivora, Ursidae) ranged over much of the northern hemisphere during the Pleistocene glaciations when *Homo* was colonizing the temperate regions of Europe and Asia. Several of the species that shared human habitats (sometimes featuring in Palaeolithic art) are now extinct (Morin 2012), but the polar bear (*Ursus maritimus*) and its direct ancestor, the brown bear (*Ursus arctos*) are fortunately still extant, though their ranges are much reduced. Some adaptations

that evolved, probably relatively rapidly, to changes in diet and climate have been recently described and offer some interesting parallels with human metabolic adjustments.

Comparison of the genomes of *U. maritimus* with *U. arctos* shows that adaptations of thermoregulation and thermogenesis in polar bears entail changes to the nuclear and mitochondrial genes fundamental to cellular respiration (Welch et al. 2014). They are also heterothermic to a degree unusual for a large mammal (Whiteman et al. 2015). Very low daily energy expenditure, including reduction in vital organs, adapts another ursid, the giant panda (*Ailuropoda melanoleuca*) to its indigestible, nutritionally poor diet of bamboo (Nie et al. 2015).

Brown bears, like most ursids, are omnivores, selecting diets that supply about 15–20% of the metabolizable energy as protein, much the same as humans, but in choosing far more lipid over carbohydrate, they resemble dogs—and laboratory mice artificially selected for obesity (Erlenbach et al. 2014). This preference is taken to extremes by polar bears, whose basic diet of seal blubber (80% fat, 20% protein) comprises one of the highest in fat known for any mammal (Thiemann et al. 2011). They accumulate enough replete adipose tissue to support up to 8 months of ambulatory fasting (Atkinson et al. 1996) or 4 months suckling twins (sometimes triplets) (Robbins et al. 2012), without detectable impairment of locomotory capacity (they travel huge distances), or nutritional, metabolic and cardiovascular health. Studies of the gut microbiota in wild *U. arctos* during summer fattening and winter fasting suggest that symbiotic microbes may facilitate such nutritional and metabolic feats (Sommer et al. 2016).

Another factor is anatomical distribution of the adipose tissue: depots in the relatively small abdominal cavity enlarge in proportion to the viscera, so up to 85% of the dissectible adipose tissue is superficial. As in healthy women (Karpe and Pinnick 2015), depots over the lower back, pelvis and hind limbs expand most with increasing obesity but, although the metabolic demands of reproduction are far greater for female bears, sex differences in adipose tissue distribution are undetectable (Pond et al. 1992). The partitioning between intra-abdominal and superficial depots is similar to that of other terrestrial carnivores, refuting the hypothesis that it is an adaptation to thermal insulation (Pond and Ramsay 1992).

Woolly mammoths (*Mammuthus primigenius*) are another species of tropical ancestry that colonised northern Eurasia 1–2 million years ago, sharing their cold habitat with Upper Palaeolithic people during the final 10% of that time. Comparisons of mammoth genomes extracted from subfossil tissues preserved in ice with those of Asian elephants reveal differences in 54 genes that directly influence the anatomical distribution, abundance or metabolism of adipose tissues and a similar number acting on hair growth and temperature sensation (Lynch et al. 2015). *Homo sapiens* had much less time in which to evolve similar adaptations to cold climate, and the changes have been abruptly reversed during recent millennia with technological advances in clothing, fire use and shelter. Interestingly, data from native Greenlanders suggest that adaptations to diets high in protein and ω-3 fatty acids have evolved faster than those to the cold climate (Fumagalli et al. 2015).

1.8 Conclusions

Mammalian adipose tissues are physiologically more diverse, have more complex anatomical relations to non-adipose tissues and make a wider range of fundamental contributions to activities at all stages of the life cycle than those of lower vertebrates. Partitioning white adipose into numerous depots, many with site-specific properties, is a fundamental feature of mammals. Paracrine interactions avert competition between tissues and enable adipose tissue to support the specific requirements of contiguous tissues, notably the heart and immune system during hypothermia as well as euthermia. Depots that support lymphoid tissues demonstrate capacities for selective uptake and/or retention of certain fatty acids thus directing scarce essentials to where they are most needed. Paracrine interactions between adipocytes and the immune system have central roles in infectious diseases including HIV/AIDS, digestive disorders such as Crohn's disease and the metabolic complications of obesity. Early-developing adipocytes in avian embryos also sort fatty acids, ensuring that the limited lipid resources in the yolk are efficiently partitioned between oxidation and structural roles (especially in the nervous and immune systems).

Thermogenesis in adipose tissues involves several different biochemical mechanisms some of which resemble non-shivering thermogenesis in muscle. Facultative thermogenesis and its many control systems evolved gradually, starting in reptilian ancestors of mammals and paralleled in birds. Diet-induced thermogenesis enables mammals to dissipate excess energy taken in to obtain scarce proteins, vitamins and minerals. These fundamental metabolic roles of adipose tissue may have appeared early in the evolution of mammals as adaptations to rapid colonization of new habitats, including efficient digestion and utilization of poorer quality diets, and metabolic support of lactation that enables fast-growing young to delay fully functional dentition until after weaning.

Selection for post-menopausal longevity as well as sexual selection and heat dissipation may have contribution to the evolution of sex differences in the distribution of human adipose tissue. Genomic analysis of the evolution of natural obesity in arctic mammals reveals scores of genetic changes that appeared in around a million years ago. Several interspecific comparisons indicate that modern human are not (yet) similarly adapted to obesity and that intermittent famine and cold climate have been at most minor factors in recent evolution.

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Chapter 2
Adipocyte Progenitors from Human Pluripotent Stem Cells

A.-L. Hafner and C. Dani

Abstract The current epidemic of obesity and overweight has caused a surge of interest in the study of adipose tissue formation. Much progress has been made in defining the transcriptional networks controlling the terminal differentiation of preadipocytes into mature adipocytes. However, the early steps that direct pluripotent stem cells down the adipocyte lineage remain largely unknown. Similarly, the study of the developmental origins of adipocytes has been largely overlooked until now. Induced Pluripotent Stem Cells (iPSCs) provide a novel cell model for investigating human adipocyte ontogenesis. From a clinical standpoint, many issues have to be resolved before using hiPS-derived adipocyte progenitors in cell-based therapeutic for obesity. However, the differentiation of iPS cells towards the adipogenic lineage offers a unique opportunity to purify brown adipocytes from patients, which could lead to the development of autologous transplantation procedures to counteract obesity.

This chapter summarizes recent work on the developmental origins and the therapeutic potential of adipocyte progenitors from human pluripotent stem cells.

Keywords Adipocyte progenitors • Pluripotent stem cells • Adipose tissue • Adipocyte development

2.1 Introduction

In mammals, three types of adipocytes coexist, i.e. brown, beige/brite and white adipocytes, which are all involved in regulation of the energy balance while having opposite functions. White adipose tissue (WAT) is dispersed throughout the body and is mainly involved in energy storage. In contrast to WAT, brown adipose tissue (BAT) is specialized in energy expenditure. Activated BAT consumes metabolic substrate and burns fat to produce heat thanks to the Uncoupling Protein (UCP)1. Beige/brite adipocytes have been recently described as brown-like adipocytes and represent a third type

A.-L. Hafner • C. Dani (✉)
Faculty of Medicine, University Nice Sophia Antipolis, iBV CNRS/Inserm,
Nice Cedex 2 06107, France
e-mail: dani@unice.fr

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of adipocytes that are in WAT (Wu et al. 2012). Activation of BAT or recruitment of beige/brite adipocytes leads to powerful anti obesity and anti-diabetic effects in mice. Independent laboratories have reported that adult BAT transplantation could reverse metabolic disorders in mice, making brown/brite adipocytes transplantation a credible approach to treat obesity and type 2 diabetes (Stanford et al. 2013; Gunawardana and Piston 2010; Liu et al. 2015). More recently, it has been shown that beige/brite adipocytes derived from the capillaries of human subcutaneous adipose tissue were able to enhance glucose tolerance after implantation into mice (Min et al. 2016). Therefore, the notion of transplanting brown or beige/brite adipocyte progenitors (BAPs) in obese patients as a therapeutic approach to counteract obesity and its associated metabolic complications has recently emerged. However, BAT represents a minor fraction of adipose tissue in humans and disappears from most areas with age, persisting only around deeper organs (Frontini and Cinti 2010). Human BAPs are hard to isolate in this regard. Therefore, a cellular source is urgently needed to amplify and characterize human BAPs. Pluripotent stem cells appeared recently as a powerful model to decipher human adipocyte ontogenesis and as an unlimited source of autologous BAPs for transplantation.

2.2 Potential of Human PSCs to Generate Adipocytes

Following the pioneer work of Yamanaka's group on the generation of patient-specific induced pluripotent stem cells (hiPSCs) by reprogramming fibroblasts (Takahashi et al. 2007), hiPSCs emerged as an unlimited source of APs for autologous cell-based therapy to treat obesity. The capacity of hiPSCs to generate functional white adipocytes was first reported by Nakao's group. Interestingly, adipocytes generated from hiPSCs can maintain their functional properties for several weeks after transplantation into nude mice (Taura et al. 2009; Noguchi et al. 2013). These data revealed that hiPSC-adipocytes could potentially be used to correct metabolic parameters in lipodystrophic patients. In these experiments, differentiated hiPSCs, but not purified APs, were transplanted into mice. Indeed, differentiated hiPSC cultures are enriched with adipocytes after adipogenic induction, but also contain several other cell types that are undesirable for transplantation, including undifferentiated iPSCs that can form teratomas. This indicates that purification of hiPSC-APs with a high adipogenic capacity is required prior to proposing an hiPSC-based therapeutic approach. Nishio et al. (2012) developed a procedure to generate functional brown adipocytes at a high frequency from hiPSCs using a hematopoietic cocktail to induce hiPSC differentiation. Remarkably, hiPSC-brown adipocytes were able to improve glucose tolerance after transplantation in mice. This report indicated that hiPSCs could potentially be used to generate brown adipocytes with therapeutic properties. Finally, Ahfeldt et al. (2012) were able to generate pure brown and white adipocytes from hiPSCs, but only following transduction with master genes of adipogenesis. The need to genetically modify hiPSC-APs to generate adipocytes clearly illustrates the low adipogenic potential of hiPSC-derived APs.

2.3 Adipogenic Capacity of hPSC-Derived Adipocyte Progenitors

Chen et al. first underlined the limited capacity of hiPSC-derived mesenchymal cells to undergo adipocyte differentiation, a feature that has often been observed but not always highlighted (Chen et al. 2012). The reasons of this feature are unknown and hamper their use both in cell-based therapy and basic research. Interestingly, some authors claim that the low differentiation capacity is limited to adipogenesis since hiPSC-derived mesenchymal cells are able to differentiate towards chondrogenic and osteogenic lineages at high levels (Xu et al. 2004; Boyd et al. 2009; Karlsson et al. 2009). The low adipogenic capacity of hiPSC-derived APs is unlikely to be due to the derivation method. In fact, there are two main approaches to differentiate pluripotent stem cells into adipocyte progenitors. One strategy involves embryoid body (EB) formation. In this approach, suspension cultures allow pluripotent stem cells to form 3-dimensional structures called EB. This step, models the *in vitro* embryonic development with the commitment of cells into the three primary germ layers. EBs are then seeded onto culture dishes and, after a proliferation step, outgrowth cells are maintained in a mesenchymal culture medium. Subsequently, adherent cells display a fibroblast-like morphology and acquire specific mesenchymal markers after serial passages (Brown et al. 2009; Lee et al. 2010). An alternative strategy involves direct differentiation of pluripotent stem cells without the EB step (Hynes et al. 2013). Another version of this protocol relies on the spontaneous differentiation of pluripotent stem cells into mesenchymal cells (Diederichs and Tuan 2014). The low adipogenic potential was observed in these reports. Finally, mesenchymal cells derived from different hiPSC lines generated from different donors, or from the same hiPSC clone using different derivation approaches, have the same adipogenic features (Hynes et al. 2013; Diederichs and Tuan 2014). Interestingly, the low differentiation potential is not restricted to hiPSC since mesenchymal cells derived from human embryonic stem cells (hESCs) display the same feature, thus ruling out the possibility that the low hiPSC-AP adipogenic capacity could be due to the reprogramming process or to an epigenetic mechanism. Several hypotheses could be put forward to explain the low adipogenic capacity of hiPSC-APs compared to adult adipocyte progenitors. We favoured the hypothesis that the range of factors or culture conditions required to induce adipocyte differentiation of adipocyte progenitors derived from adult tissues and from embryonic-like cells could differ (Hafner and Dani 2014). The low hiPS-AP adipogenic capacity is reminiscent of an earlier observation reported by Han and colleagues (2011). The authors observed that epididymal adipose tissue, which undergoes postnatal development in mouse, is composed of multipotent progenitor cells but lack an adipogenic capacity *in vitro*. In contrast to cells derived from other fat pads, epididymal fat cells require three-dimensional culture conditions and a different micro-environment to undergo differentiation. These results emphasise that the micro-environment has a critical role in differentiation but could differ between adult and embryonic-like cells. Recently, Mohsen-Kanson et al. have selectively generated white and brown

adipocyte progenitors from hiPSCs (Mohsen-Kanson et al. 2014). Then, they have identified a set of factors, including angiogenic factors, capable of governing hiPSC-BAP differentiation at a level similar to that of BAPs derived human adult adipose tissue (Hafner et al. 2016). Altogether, these data highlighted that a specific micro-environment is requirement to switch on hiPSC-brown adipogenesis and opens new opportunities to develop alternative strategies to counteract obesity.

2.4 Developmental Origins of Human Brown Adipocyte Progenitors

A better knowledge of the developmental origins of the different adipose progenitors is central to understand the diversity of adipose tissues. The emerging picture arising from lineage tracing tools is that brown, beige/brite and white adipocytes are generated from cells having multiple origins. It has been first proposed that classical brown and not beige/brite adipocytes, originate from a precursor expressing myogenic transcription factor-5 (Myf-5) (Seale et al. 2008). However, other studies revealed that all colours of adipocytes also originate from either Myf5-positive or negative progenitors (Sanchez-Gurmaches et al. 2012). It has been also further proposed that a subpopulation of beige/brite adipocytes derives from smooth muscle cells (Long et al. 2014). Recently, Sanchez-Gurmaches et al. have been nicely reviewed the complexity of adipocyte origins (Sanchez-Gurmaches et al. 2016). We have also demonstrated that white adipocytes have two embryonic origins, i.e. neuroectoderm and mesoderm, depending on their body localization in rodents (Billon and Dani 2011). APs derive from mesenchymal stem cells, which themselves are thought to arise from mesoderm only. However, it is worth noting that during development of higher vertebrates, the mesoderm is not the only germ layer source of mesenchymal cells. In the head, for instance, the facial bones have been shown to derive from the neural crest (NC). The NC is a vertebrate cell population that arises from the neuroectoderm. After neural tube closure, NC cells undergo an epithelio-mesenchyme transition and migrate to diverse regions in the developing embryo, where they differentiate into various cell types. In the head and neck, the NC also yields mesenchymal precursors differentiating into connective tissue cells (reviewed in Dupin et al. 2006). Adipogenesis of mouse Embryonic Stem (mES) cells *in vitro* provided the first model to investigate the earliest steps of adipocyte development and revealed the surprising conclusions regarding the ontogeny of such cells in the NC, better known to generate neuronal cells and glial cells.

In the mouse ES cell system, the generation of adipocytes is dependent on an early and transient exposure of EBs to retinoic acid (RA) and a subsequent treatment with conventional adipogenic factors. In a first attempt to unravel the events underlying the formation of mesenchymal derivatives in RA-treated mES cells, Kawaguchi et al. examined the expression of various mesodermal and mesenchymal markers in early EBs. Surprisingly, they noticed that treatment with RA resulted in

a sharp reduction in several mesodermal markers, as well as in the suppression cardiomyocyte formation, suggesting that RA reduces overall mesoderm formation in mESCs (Kawaguchi et al. 2005). They showed that *sox9*, *sox10*, *foxD3*, and *runx2*, which all play an important role in NC formation and/or mesenchymal condensation, were upregulated upon RA-treatment. Together, these data suggest that neuroectoderm/NC is the major source of mesenchymal cells in RA-treated mESCs. To test this hypothesis with respect to adipocytes, we checked whether adipocytes could be obtained from NCCs using a lineage tracing approach in mouse. This study revealed adipocytes derived from NC in craniofacial adipose depots. In contrast, no NC-derived adipocytes could be detected in truncal adipose depots, including subcutaneous, perirenal, periepididymal, and interscapular tissues. These data therefore provide new information about the ontogeny of the adipocyte lineage and demonstrate that during normal development, a subset of adipocytes in the craniofacial region originates from NC, and not from mesoderm (Billon et al. 2007). However, the white or brown phenotype of adipocytes derived from the cranial neural crest was not investigated at that time (see below). The role of RA in the early steps of adipocyte development remains to be demonstrated *in vivo* in mouse. Interestingly, RA has recently been shown to be required for differentiation of cephalic NCCs into adipocytes in developing zebrafish embryos (Li et al. 2010), which is a reminiscence of the role of RA in mESC adipogenesis.

It is now well documented that adipose tissues having different impact on the metabolic complications of obesity possess distinct developmental gene signatures suggesting that developmental origins may contribute to adipocyte properties (Tchkonia et al. 2007; Gesta et al. 2006). Therefore, studies on the origins of APs open at least two questions: are adipocytes derived from different developmental origins functionally different? And what are the developmental origins of APs in humans? As lineage tracing is impossible in humans, only specific marker expression could be used to propose an embryonic origin of human samples. *Pax3* expression was shown to be associated with neural crest development in humans and to be maintained in mesenchymal progenies (Betters et al. 2010; Fukuta et al. 2014). *Pax3* thus represents a unique marker to follow the neural crest fate in humans. Interestingly, hiPSC-Brown APs, but not hIPSC-White APs, express *PAX3* and the generation of BAP during hiPSC differentiation is preceded by expression of craniofacial neural crest markers (Hafner unpublished data). *PAX3* expression is maintained in human adults, and microarray analysis revealed that *Pax3* expression marks preferentially UCP1-expressing adipose tissue depots localized in the face (Kouidhi et al. 2015). Altogether, these reports strongly suggest that human BAPs originate from neural crest.

In conclusions, adipogenesis of human pluripotent stem cells is a new area that has tremendous interest to understand how energy-dissipating cells are generated. A thorough understanding of the signals that govern the generation and the differentiation of PAX3+ cells during pluripotent stem cell development could open new opportunities to better know human brown-like adipocyte ontogenesis and to develop alternative strategies to counteract obesity.

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Chapter 3
Adipocyte Differentiation

José María Moreno-Navarrete and José Manuel Fernández-Real

Abstract Adipocyte differentiation is a highly controlled process that has been extensively studied for the last 25 years. Two different kinds of in vitro experimental models, essential in determining the mechanisms involved in adipocyte proliferation, differentiation and adipokine secretion, are currently available: preadipocyte cell lines, already committed to the adipocyte lineage, and multipotent stem cell lines, able to commit to different lineages including adipose, bone and muscle lineage. Many different events contribute to the commitment of a mesenchymal stem cell into the adipocyte lineage, including the coordination of a complex network of transcription factors, cofactors and signalling intermediates from numerous pathways. New fat cells constantly arise from a preexisting population of undifferentiated progenitor cells or through the dedifferentiation of adipocytes to preadipocytes, which then proliferate and redifferentiate into mature adipocytes. Analysis of adipocyte turnover has shown that adipocytes are a dynamic and highly regulated population of cells. Adipogenesis is a multi-step process involving a cascade of transcription factors and cell-cycle proteins regulating gene expression and leading to adipocyte development. Several positive and negative regulators of this network have been elucidated in recent years. This review is focused in the main molecular and cellular processes associated with adipocyte differentiation, including transcriptional factors and cofactors and extranuclear modulators. The role of epigenetic factors, microRNAs and chronobiology in adipogenesis is also summarized.

Keywords Adipocyte • Adipogenesis regulatory factors • PPAR-γ • C/EBP-α • Preadipocyte cell lines • Adipose-derived stem cells • Mesenchymal stem cells

J.M. Moreno-Navarrete • J.M. Fernández-Real (✉)
Section of Diabetes, Endocrinology and Nutrition Hospital of Girona Dr Josep Trueta, Institut d'Investigació Biomédica de Girona (IDIBGi), Carretera de França s/n, Girona, 17007, Spain

CIBEROBN (CB06/03/010), Instituto de Salud Carlos III, Madrid, 17007, Spain
e-mail: jmfernandezreal.girona.ics@gencat.cat

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3.1 Introduction

Adipose tissue is characterized by a marked cellular heterogeneity: among its cellular components, we can find adipocytes, preadipocytes, fibroblasts, endothelial cells and multipotent stem cells able to differentiate into several cell types. Overall, fat tissue consists of approximately one-third of mature adipocytes. The remaining two-thirds are a combination of small mesenchymal stem cells (MSCs), T regulatory cells, endothelial precursor cells, macrophages and preadipocytes in various stages of development. Preadipocytes have the ability to proliferate and differentiate into mature adipocytes, conferring adipose tissue a constant functional plasticity, which determines its ability to expand throughout the entire lifespan.

Adipocytes, also known as fat cells and lipocytes, are found in stereotypical depots throughout the body and mixed with other cell types in some other positions, such as loose connective tissue. There are two kinds of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT), both of which differ in a few significant properties. Most of our understanding about adipocyte differentiation and adipogenesis comes from in vitro studies of fibroblasts and preadipocytes (Rosen and MacDougald 2006). White adipocytes contain single, large lipid droplets that appear to comprise the majority of cell volume, while the cytoplasm and nucleus are found at the cell periphery. Preadipocytes that resemble fibroblasts are cultured and after differentiation is induced, the cell cultures may be used for metabolic studies. Brown adipocytes, which are characterized by multilocular lipid droplets and high mitochondrial content, are derived from distinct adipose tissue depots that are highly vascular and innervated.

Obesity can be characterized into two main types, hyperplasic (increase in adipocyte number) and hypertrophic (increase in adipocyte volume). Hypertrophy, to a certain degree, is characteristic of all overweight and obese individuals. Hyperplasia, however, is correlated more strongly with obesity severity and is most marked in severely obese individuals (Hirsch and Batchelor 1976). Prolonged periods of weight gain in adulthood may result in an increase in adipocyte number. Indeed, animal studies suggest that increases in adipocyte size precede increases in adipocyte number. Adipose hypertrophy might be diabetogenic, with two independent prospective studies showing that adipose hypertrophy is an independent risk factor for developing type 2 diabetes (Weyer et al. 2000; Lonn et al. 2010).

At the cellular level, obesity was originally considered an hypertrophic disease resulting from an increase in the fat cell number or the size of individual adipocytes. New fat cells constantly arise from a preexisting population of undifferentiated progenitor cells or through the dedifferentiation of adipocytes to preadipocytes, which then proliferate and redifferentiate into mature adipocytes. In both cases, the generation of new fat cells plays a key role in the development of obesity. Given that in adulthood, adipocyte number stays constant, and weight changes are predominantly accompanied by changes in adipocyte volume, one may conclude that at some critical point in development, the final fat cell number is attained, and after this point, no fat cell turnover occurs. Analysis of adipocyte turnover using carbon-14 dating, however, has recently shown that this is not the case, but rather that adipocytes are

a dynamic and highly regulated population of cells. New adipocytes form constantly to replace lost adipocytes, such that approximately 50% of adipocytes in the human subcutaneous fat are replaced every 8 years (Spalding et al. 2008).

Adipogenesis is a multi-step process involving a cascade of transcription factors and cell-cycle proteins regulating gene expression and leading to adipocyte development. Several positive and negative regulators of this network have been elucidated in recent years (Lefterova and Lazar 2009). The first hallmark of the adipogenesis process is the dramatic alteration in cell shape as the cells convert from fibroblastic to spherical shape. These morphological modifications are paralleled by changes in the level and type of extracellular matrix (ECM) components and the level of cytoskeletal components (Gregoire et al. 1998). Mediation of the proteolytic degradation of the stromal ECM of preadipocytes by the plasminogen cascade is required for cell-shape change, adipocyte-specific gene expression and lipid accumulation (Selvarajan et al. 2001). Ectoderm-Neural Cortex-1 (ENC-1), a Drosophila kelch-related actin-binding protein, may also play a regulatory role early in adipocyte differentiation by affecting cytoskeletal reorganization and cell-shape change. In preadipocytes, ENC-1 colocalizes with actin filaments, and its mRNA levels are transiently increased 8–12-fold early in adipocyte differentiation, preceding peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT/enhancer binding protein-α (C/EBP-α) gene expression (Zhao et al. 2000).

During the terminal phase of differentiation, activation of the transcriptional cascade leads to increased activity, protein and mRNA levels for enzymes involved in triacylglycerol synthesis and degradation. Glucose transporters, insulin receptor number and insulin sensitivity also increase. Synthesis of adipocyte-secreted products including leptin, adipsin, resistin and adipocyte-complement-related protein (Acrp30) begins, producing a highly specialized endocrine cell that will play key roles in various physiological processes.

We here review the main molecular and cellular processes associated with adipocyte differentiation. First, we summarize the main cellular models to study and characterize these fascinating cellular changes.

3.2 In Vitro Experimental Systems to Study Adipocyte Differentiation

Two different kinds of cell lines are currently available: preadipocyte cell lines, already committed to the adipocyte lineage, and multipotent stem cell lines, able to commit to different lineages including adipose, bone and muscle lineage.

3.2.1 Preadipocyte Cell Lines

3T3-F442A and 3T3-L1 cells, isolated from the Swiss 3T3 cell line, derived from disaggregated 17–19-day-old Swiss 3T3 mouse embryos, are the most frequently

used preadipocyte lines (Green and Meuth 1974; Green and Kehinde 1976). Importantly, clonal cell lines are homogenous in terms of cellular population, and their cell types are all at the same differentiation stage. This allows a homogeneous response to treatments. In addition, these cells can be passaged indefinitely, which provides a consistent source of preadipocytes for study. For all these reasons, clonal cell models are an interesting and complementary tool to animal models for the study of relevant biological questions. 3T3-F442A are generally regarded as a model with a more advanced commitment in the adipose differentiation process than 3T3-L1 (Gregoire et al. 1998). During proliferation, all preadipose cell models show a similar morphology to fibroblasts. Induction of differentiation triggers deep phenotypical changes of preadipocytes that become spherical and filled with lipid droplets, displaying many morphological and biochemical characteristics of adipocytes differentiated in vivo.

Ob17 cells, derived from adipose precursors present in epididymal fat pads of genetically obese (ob/ob) adult mice, are employed less frequently. In comparison to 3T3-F442A and 3T3-L1 cells, adult derivation of Ob17 cells represents a later preadipocyte stage. The derivation from an obese animal could also confer properties different from those of embryonic origin (Negrel et al. 1978).

Most available models of murine preadipocyte (3T3-L1, 3T3-F442A and Ob17), once they reach confluence and growth arrest, upon opportune hormonal induction, re-enter cell cycle and undergo several rounds of postconfluent mitosis, known as mitotic clonal expansion (MCE). This is a fundamental requirement for terminal adipocyte differentiation. In fact, blocking the entry of 3T3-L1 cells into S phase at the time of MCE completely inhibits the adipose conversion program (Tang et al. 2003). Also, inhibition of DNA synthesis in 3T3-F442A cells prevents formation of fat cells (Kuri-Harcuch and Marsch-Moreno 1983). However, confluent 3T3-F442A cells shifted to suspension culture maintain their ability to differentiate, suggesting that growth arrest but not confluency is required for adipocyte formation (Pairault and Green 1979). Similarly, C3H10T1/2 cells treated with bone morphogenetic protein-4 (BMP-4) that triggers commitment to adipose lineage undergo MCE in the presence of differentiation inducers (Tang et al. 2004).

The availability of adipose clonal cell lines and primary preadipocytes has allowed us to investigate the adipogenic or antiadipogenic potential of hormones, growth factors and various pharmacological compounds. Confluent 3T3-L1 preadipocytes can be differentiated synchronously by a defined adipogenic cocktail. Maximal differentiation is achieved upon early hormonal induction for 48 h with a combination of insulin, GCs and methylisobutylxanthine (MIX), which elevates intracellular cAMP levels, in the presence of fetal bovine serum. Dexamethasone (DXM), a synthetic GC agonist, is traditionally used to stimulate the GC receptor. After the first 48 h, insulin alone is required to continue the differentiation program. Interestingly, DXM is a powerful inductor of adipogenesis at early stages of differentiation, but displays antiadipogenic effects when added at later stages of adipose maturation, indicating that the effects of hormones are strictly time dependent (Caprio et al. 2007).

Differentiation of 3T3-F442A preadipocytes does not require early induction with GCs, since their commitment in adipogenesis is more advanced compared to

3T3-L1 cells. It is worthy to note that treatment of 3T3-442A cells with DXM represses adipogenesis, confirming that observed in 3T3-L1 cells exposed to GC at a later stage of adipose conversion.

3.2.2 Mature Adipocyte-Derived Dedifferentiated Fat Cells

Recently, several authors showed that mature adipocytes derived from fat tissue retain the ability to dedifferentiate in vitro into fibroblast-like cells. The culture technique developed to dedifferentiate adipocytes is known as ceiling culture (Sugihara et al. 1986; Yagi et al. 2004; Matsumoto et al. 2008; Nobusue et al. 2008). In this protocol, floating unilocular mature adipocytes adhere to the top inner surface of a culture flask filled completely with medium. After about 7 days of culture, the adipocytes change morphology, spread and show fibroblast-like shape with no lipid droplets. These cells, known as dedifferentiated fat (DFAT) cells, retain remarkable proliferative ability and are able to differentiate again into mature adipocytes both in vitro and in vivo. Human DFAT cells from human subcutaneous adipocytes do not express adipocyte markers such as LPL, leptin, glucose transporter-4 (GLUT-4) and C/EBP-α, showing low levels of PPAR-γ, C/EBP-β and C/EBP-δ transcripts. Interestingly, these cells express RUNX2 and SOX9, critical factors for osteogenesis and condrogenesis respectively, and are able to undergo osteogenic and chondrogenic differentiation in vitro in the presence of appropriate culture conditions. Moreover, they are able to form osteoid matrix when implanted in nude mice, after osteogenic induction in vitro (Matsumoto et al. 2008). The ability of DFAT cells to proliferate and differentiate into multiple mesenchimal lineages confers to these cells the characteristics of adult stem cells.

3.2.3 Mesenchymal Stem Cells

C3H10T1/2 cells, established in 1973 from 14- to 17-day-old C3H mouse embryos, are MSCs which, following treatment with 5-azacytidine, can be differentiated into cells showing morphology and biochemical features of muscle, bone, cartilage and adipose tissue. Unlike 3T3-L1 cells, pluripotent C3H10T1/2 stem cells do not differentiate into adipocytes in the presence of adipose differentiation inducers (Konieczny and Emerson 1984). Treatment of proliferating C3H10T1/2 cells with BMP-4 is required to induce commitment to adipocyte lineage cells, which can differentiate into adipocytes when exposed to adipocyte differentiation inducers.

3.2.4 Adipose-Derived Stem Cells (ADSCs)

Adipose-derived stem cells (ADSCs) show a cell surface antigen profile similar to that observed on MSCs in adult bone marrow, but are more simple to purify, given

that their source is easily available. MSCs and ADSCs are characterized by a heterogeneous population that contains also differentiated cells, contaminating the stem cell preparation. Removal of the contaminating differentiated cells requires several passages. In fact, flow cytometer analysis shows that DFAT cells are more homogeneous than ADSCs, representing an interesting cell source for cell engineering and regenerative medicine applications (Matsumoto et al. 2008). Thanks to the adipose differentiation potential of DFAT cells, they represent a valuable cell system to study adipocyte development and metabolism, which could potentially replace conventional primary preadipocyte cultures.

ADSCs can be isolated and differentiated in vitro into mature adipocytes. Primary preadipocyte cultures may better reflect the context of adipose function in vivo, representing a suitable cellular system to confirm data deriving from preadipocyte lines. In addition, primary preadipocytes do not undergo continuous passages, hence they keep a diploid status, better reflecting the context in vivo. Interestingly, proliferation and differentiation of primary preadipocytes is clearly influenced by the anatomic site of the depots as well the age of the donor. In particular, aging reduces replicative ability of primary preadipocytes in cell culture. Subcutaneous ADSCs replicate and differentiate better than visceral ADSCs (Djian et al. 1983).

Cells corresponding to the adipose-derived stromal cells are defined by the following phenotype: $CD31^-$, $CD34^+$, $CD45^-$, $CD90^+$, $CD105^-$, $CD146^-$, and represent 70–90% of the total $CD45^-$ adipose cells. Stromal Vascular Fraction (SVF) also includes endothelial cells, defined as $CD34^+/CD31^+$ cells, and machrophages, which express CD14 and CD31. Cells capable of differentiating into adipocytes are included in the $CD34^+/CD31^-$ cell fraction and do not express the MSC marker CD105 (Sengenes et al. 2005). For this reason, adipose committed preadipocytes express a specific pattern of cell surface markers, allowing selective purification by immune-magnetic beads or by flow cytometric cell sorting.

3.3 Stages of Adipocyte Differentiation

Two phases of adipogenesis have been extensively characterized:

Determination phase: This stage results in the conversion of the stem cell to a preadipocyte, which cannot be distinguished morphologically from its precursor cell but has lost the potential to differentiate into other cell types.

Terminal differentiation phase: In this stage, the preadipocyte takes on the characteristics of the mature adipocyte. It acquires the machinery that is necessary for lipid transport and synthesis, insulin action and the secretion of adipocyte-specific proteins. The molecular regulation of terminal differentiation is more extensively characterized than determination because most studies have used cell lines that have a restricted potential to differentiate into other cell types. Some preadipocyte models (such as the mouse cell lines 3T3-L1, 3T3-F442A) need one or two rounds of cell

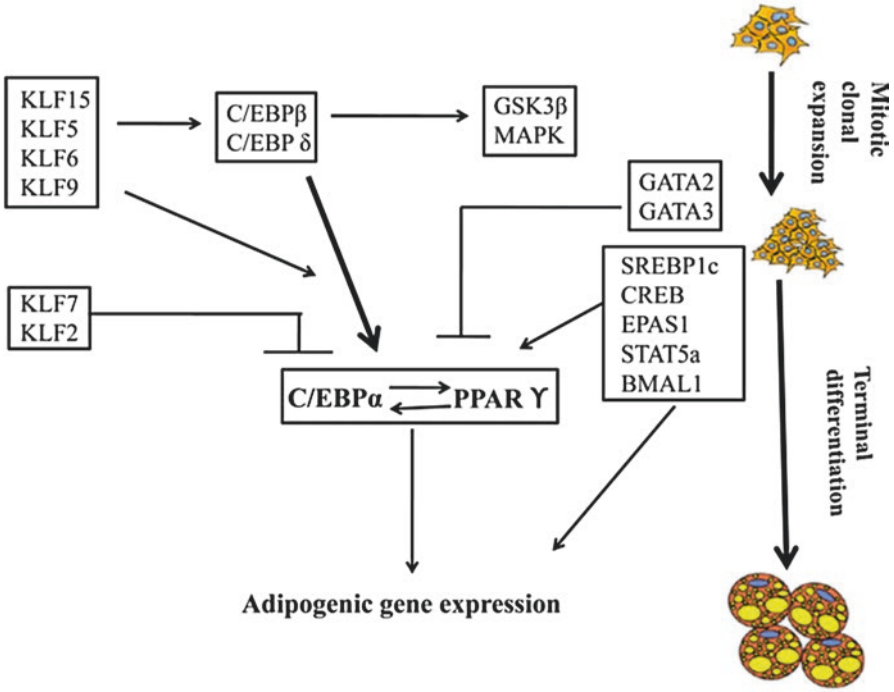

Fig. 3.1 Transcriptional regulation of adipocyte differentiation during 3T3-L1 mitotic clonal expansion and terminal differentiation

division prior to differentiation, whereas others (such as mouse C3H10T1/2 and human preadipocytes) differentiate without postconfluence mitosis. In MCE of preadipocytes, cells re-enter the cell cycle and undergo several rounds of supplementary cell divisions (Ntambi and Young-Cheul 2000). These events depend on a complex coordinated cascade of cell-cycle proteins, such as members of E2F and retinoblastoma protein, that are necessary for terminal adipocyte differentiation of murine preadipocytes (Fajas et al. 2002a, b). The mitosis is believed necessary to unwind DNA, allowing transcription factors access to regulatory response elements present in genes involved in adipocyte differentiation (Cornelius et al. 1994). Growth arrest is followed by expression of final adipogenic genes. It is clear that some of the checkpoint proteins for mitosis also regulate aspects of adipogenesis.

The course of adipocyte differentiation has been well studied using cell lines and primary preadipocyte cell cultures (reviewed above). In the presence of a hormonal cocktail consisting of insulin, DXM, and 3-isobutyl-1-methylxanthine, 3T3-L1 and 3T3-F422A preadipocytes can differentiate into mature adipocyte cells, expressing specific adipocyte genes and accumulating triacylglycerol lipid droplets (Cornelius et al. 1994). Differentiation requires the activation of numerous transcription factors which are responsible for the coordinated induction and silencing of more than

2,000 genes related to the regulation of adipocyte in both morphology and physiology (Farmer 2006) (Fig. 3.1).

3.4 Nuclear Regulation of Adipocyte Differentiation

3.4.1 Transcriptional Regulation of Adipocyte Differentiation

Terminal adipocytes differentiation involves a series of transcriptional processes. The first stage of adipogenesis consists of the transient dramatic induction of C/EBP-β and C/EBP-δ, stimulated in vitro by hormonal differentiation cocktail (Ramji and Foka 2002). C/EBP-β and C/EBP-δ begin to accumulate within 24 h of adipogenesis induction and the cells re-take the cell cycle and execute MCE synchronously (Tang et al. 2003). In the conversion from G1 to S stage, C/EBP-β is hyperphosphorylated and sequentially activated by glycogen synthase kinase-3β and mitogen-activated protein kinase (MAPK). Then, both C/EBP-β and C/EBP-δ directly induce expression of PPAR-γ and C/EBP-α, the key transcriptional regulators of adipocyte differentiation (Tang et al. 2005). PPAR-γ and C/EBP-α initiate positive feedback to induce their own expression and also activate a large number of downstream target genes whose expression determines the adipocyte. By day 2 of the differentiation course, C/EBP-α protein initiates to accumulate, and then is phosphorylated by the cyclin D3, inducing a proliferation inhibition effect on the cells, which allow to begin final differentiation phase of adipogenesis (Wang et al. 2006). By day 8 after differentiation induction, more than 90% of the adipocytes are already mature (Huang and Donald 2007) (Fig. 3.2).

C/EBP-α induces many adipocyte genes directly, and in vivo studies indicate an important role for this factor in the development of adipose tissue. PPAR-γ is a member of the nuclear receptor superfamily of ligand-activated transcription factors and is a prerequisite for the differentiation of both brown and white adipocytes (Kajimura et al. 2008). All the studies performed on PPAR-γ gain and loss of function models confirmed that PPAR-γ is both necessary and sufficient for fat formation (Farmer 2006). Ectopic expression of C/EBP-α in fibroblasts can induce adipogenesis only in the presence of PPAR-γ (Freytag et al. 1994). Accordingly, PPAR-γ ectopic expression can induce adipogenesis in mouse embryonic fibroblasts lacking C/EBP-α, but C/EBP-α cannot rescue adipogenesis when PPAR-γ is not expressed, showing that PPAR-γ is a master regulator of adipogenesis (Rosen et al. 2002). No factor has been discovered that promotes adipogenesis in the absence of PPAR-γ, and most pro-adipogenic factors seem to function at least in part by activating PPAR-γ expression or activity. The action of PPAR-γ is mediated through two protein isoforms: PPAR-γ1 and PPAR-γ2. PPAR-γ1 is constitutively expressed, and PPAR-γ2 expression is restricted to adipose tissue. Expression of each isoform is driven by a specific promoter that confers distinct tissue-specific expression and regulation (Zhu et al. 1995). Both isoforms are strongly induced during preadipocyte differentiation in vitro, and both are highly expressed in adi-

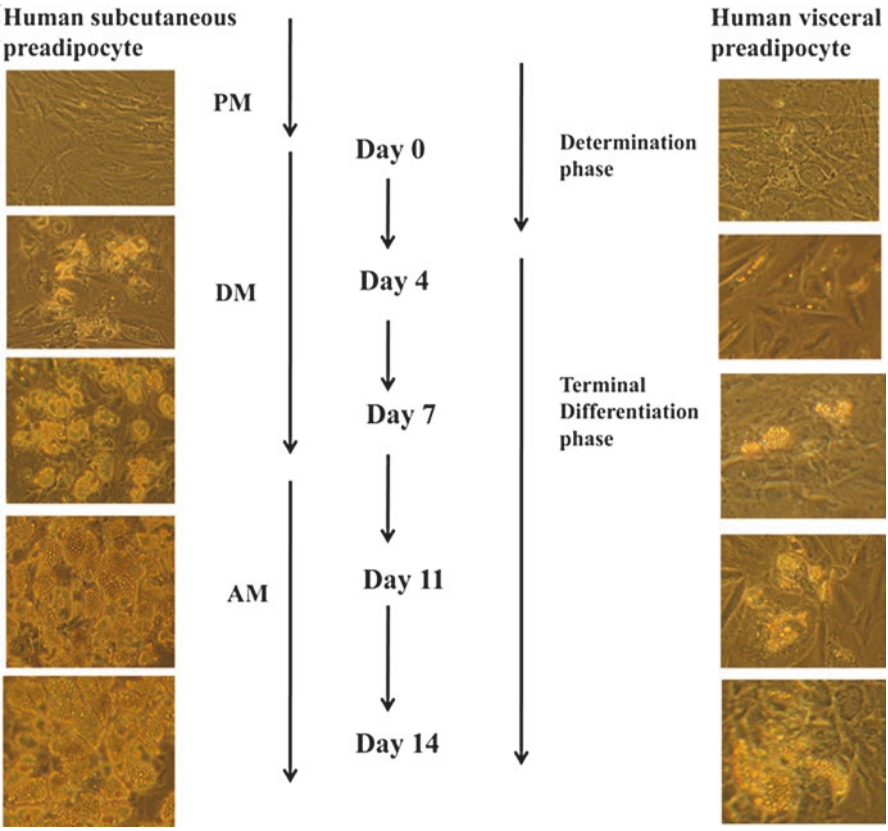

Fig. 3.2 Adipogenesis phases of human subcutaneous and visceral preadipocytes. PM is proliferatium medium and composed of DMEM/Nutrient Mix F-12 medium (1:1, v/v), HEPES, FBS, penicillin and streptomycin. DM is differentiation medium and composed of PM, human insulin, DXM, isobutylmethylxanthine and peroxisome proliferator-activated receptor-γ agonists (rosiglitazone). AM is adipocyte maintenance medium and composed of DMEM/Nutrient Mix F-12 medium (1:1, v/v), HEPES, FBS, biotin, panthothenate, human insulin, DXM, penicillin, streptomycin and amphotericin

pose tissues in animals. PPAR-γ1 is induced earlier than PPAR-γ2 and is maintained at high levels during adipocyte differentiation (Morrison and Farmer 1999). PPAR-γ is also required for maintenance of the differentiated state. Adenoviral introduction of a dominant-negative PPAR-γ into mature 3T3-L1 adipocytes causes dedifferentiation with loss of lipid accumulation and decreased expression of adipocyte markers (Tamori et al. 2002).

In addition to PPAR-γ and C/EBPs, several other transcription factors are likely to play an important role in the molecular control of adipogenesis. These proteins include pro- and anti-adipogenic transcription factors, and the adipocyte differentiation process is thus the result of an equilibrium between these intervening factors.

The Kruppel-like factors (KLFs) are a large family of C2H2 zinc-finger proteins that regulate apoptosis, proliferation and differentiation. The range of KLF genes that are expressed in adipose tissue, the variability in their expression patterns during adipocyte differentiation and their effects on adipocyte development and gene expression indicate that a cascade of KLFs function during adipogenesis. For example, KLF15 promotes adipocyte differentiation (Mori et al. 2005) and induces expression of the insulin-sensitive GLUT-4 (Gray et al. 2002). KLF5 is induced early during adipocyte differentiation by C/EBP-β and C/EBP-δ and activates the *Pparg2* promoter, functioning in concert with the C/EBPs. KLF6 inhibits the expression of preadipocyte factor-1 (Pref-1) in 3T3-L1 cells and fibroblasts. Although overexpression of KLF6 is not sufficient to promote adipocyte differentiation, cells with reduced amounts of KLF6 show decreased adipogenesis (Li et al. 2005). Recently, KLF9 has been reported as a key pro-adipogenic transcription factor through regulation of PPAR-γ2 expression with C/EBP-α at the middle stage of adipogenesis. The expression of KLF9 was markedly upregulated during the middle stage of 3T3-L1 adipocyte differentiation and inhibition of KLF9 by RNAi impaired adipogenesis (Pei et al. 2011). However, not all KLFs promote adipocyte differentiation. KLF2 and KLF7 are both anti-adipogenic factors, and KLF2 represses the *Pparg2* promoter (Wu et al. 2005; Kanazawa et al. 2005a). KLF factors would presumably be functioning through the differential recruitment of co-repressors and co-activators to the *Pparg2* promoter.

Sterol regulatory element binding transcription factor 1 (SREBP1c) was identified as a pro-adipogenic basic helix–loop–helix transcription factor that induces PPAR-γ expression and possibly generation of an as-yet-unknown PPAR-γ ligand (Kim et al. 1998a; Kim and Spiegelman 1996). SREBP1c also mediates the induction of lipid biosynthesis by insulin in adipocytes increasing the gene expression of the main lipogenic genes, as fatty acid synthase and acetyl-CoA carboxylase (Kim et al. 1998b).

Cyclic AMP response element-binding protein (CREB) also seems to have a possible role in the control of adipogenesis. CREB expression in 3T3-L1 preadipocytes is necessary and sufficient to induce adipogenesis, whereas silencing of CREB expression blocks adipogenesis (Reusch et al. 2000; Zhang et al. 2004). Other transcription factors that promote adipogenesis include Endothelial PAS domain Protein 1 (EPAS1) (Shimba et al. 2004), the signal transducer and activator of transcription-5a (Nanbu-Wakao et al. 2002; Floyd and Stephens 2003) and the circadian regulator Brain and Muscle ARNT-like Protein 1 (BMAL1) (Shimba et al. 2005).

Many transcription factors repress adipogenesis, including several members of the GATA-binding and forkhead families (Forkhead Box O1 (FOXO1) and Forkhead Box A2 (FOXA2)). GATA2 and GATA3, two members of the Guanine, Adenine, Thymine and Adenine (GATA) family of transcription factors which are zinc-finger DNA-binding proteins involved in developmental processes, are expressed in preadipocytes and downregulated during terminal maturation (Tong et al. 2000). Forced expression of GATA2 reduces adipogenesis, and GATA2-deficient embryonic stem cells displayed enhanced adipogenic potential. Constitutive expression of GATA2 and GATA3 blunts adipocyte differentiation and traps cells at the preadipocyte

stage. This inhibitory effect on adipogenesis could be mediated through reduced PPAR-γ promoter activity. Although GATA factors can bind to and inhibit the *Pparg2* promoter, a mutant GATA2 protein that does not bind to DNA retains anti-adipogenic activity by binding to C/EBPs and inhibiting their ability to transactivate *Pparg* (Tong et al. 2005).

3.4.2 Transcriptional Cofactors in Adipogenesis

Nuclear cofactors do not bind to DNA directly but participate in the formation of large transcriptionally active (co-activator) or inactive (co-repressor) complexes that link transcription factors to the basal transcription machinery.

Some cofactors modify chromatin directly, such as the histone acetyltransferases (HATs) and the ATP-dependent chromatin remodeling proteins of the SWI/SNF family, whereas other cofactors that do not have enzymatic activity function as platforms for the recruitment of chromatin modifiers. Many co-activators, including members of the p160 family, function as scaffolds and also have some HAT activity.

TRAP220 (or PPAR-binding protein) is a known binding partner of PPAR-γ, and the absence of this protein prevents adipogenesis (Ge et al. 2002), as well as the absence of a related co-activator called PPAR-interacting protein (Qi et al. 2003).

Another interesting example involves TATA binding protein-associated factor-8 (TAF8), which is a member of the TFIID complex of basal-promoter binding factors. TAF8 expression is upregulated during adipogenesis, and its expression is necessary for adipocyte differentiation (Guermah et al. 2003).

Several checkpoint-control proteins might also function as cofactors in adipogenesis. The cyclin D3–cyclin-dependent kinase-6 (CDK6) complex binds to and phosphorylates PPAR-γ and leads to increased transcriptional activity of PPAR-γ, which promotes adipogenesis (Sarruf et al. 2005). CDK4 also interacts with and activates PPAR-γ through the kinase domain of CDK4 (Abella et al. 2005). Conversely, cyclin D1 represses PPAR-γ activity and inhibits adipocyte differentiation (Fu et al. 2005). TAZ (transcriptional co-activator with PDZ-binding motif), represses PPAR-γ activity in adipocytes but activates RUNX2 activity in osteoblasts (Hong et al. 2005).

Some co-repressors recruit histone deacetylases (HDACs) to target promoters, thereby blocking transcription. HDACs repress adipogenesis and show coordinated reduction of expression during adipocyte differentiation. Mammalian sirtuins (SIRT1) with HDAC activity represses 3T3-L1 adipogenesis through its interaction with PPAR-γ. Other co-repressors, such as the nuclear receptor co-repressor and silencing mediator of retinoid and thyroid hormone receptors, are anti-adipogenic, and their reduction promotes differentiation (Yu et al. 2005).

3.5 Extranuclear Regulation of Adipocyte Differentiation

Adipogenesis can be influenced in a positive or negative way by many hormones, cytokines, growth factors and some pharmacological compounds.

3.5.1 Adipogenic Factors

It is well known that insulin, insulin-like growth factor-1 (IGF-1), thyroid hormones, GC s, mineralocorticoids and PPAR-γ agonists promote differentiation.

Insulin has marked effects on adipogenesis. Downstream components of the insulin/IGF-1 signalling cascade are also crucially important for adipogenesis. The loss of individual insulin-receptor substrate (IRS) proteins inhibits adipogenesis (Smith et al. 1988; Bluher et al. 2002). Downstream effectors of insulin action cascade, such as phosphatidylinositol-3 kinase, AKT1/2 and mammalian target of rapamycin, have been shown to be involved in adipogenesis (Garofalo et al. 2003; Kim and Chen 2004). IRS signalling also promotes CREB phosphorylation, which is important for adipogenesis of cultured cells (Klemm et al. 2001).

Thyroid hormone (T3) plays a central role in normal development, differentiation and metabolic homeostasis. It is well known that thyroid hormone stimulates basal metabolic rate and adaptive thermogenesis. In mammals, there are two major thyroid receptors isoforms, thyroid receptor α1 (TRα1) and thyroid receptor α2 (TRα2), which are functionally antagonistic. T3 induced adipogenesis through TRα1-induced lipogenic gene expression, whereas TRα2 antagonizes T3 action. In obese subjects, subcutaneous fat, with higher expression of TRα1, is more T3 responsive than visceral fat (Ortega et al. 2009).

GCs are potent inducers of adipogenesis in vitro, and hypercortisolism is associated with obesity and disturbances in fat tissue distribution (Joyner et al. 2000). GC receptors are present in human preadipocytes, and GCs activate the expression of C/EBP-δ and PPAR-γ (Wu et al. 1996). The enzyme 11-β-hydroxysteroid-dehydrogenase 1 (11BHSD1), which ensures the conversion of inactive cortisone to active cortisol (or corticosterone in rodent), is expressed in preadipocytes and adipocytes, and is thus able to sensitize adipose tissue to GCs. Interestingly, mice overexpressing 11BHSD1 in adipose tissue exhibit metabolic disturbances, including visceral adiposity, insulin resistance, dyslipidaemia and hypertension (Masuzaki et al. 2001). In contrast, mice lacking 11BHSD1 have reduced adiposity (Stewart and Tomlinson 2002). Moreover, obesity is associated with increased 11BHSD1 expression in adipose tissue in both rodents and humans (Rask et al. 2001). Locally produced cortisol may thus act in a paracrine manner to promote adipogenesis in visceral fat tissue.

Several studies have reported the effects of MAPK family members on adipogenesis with conflicting results. ERK1 is required in the proliferative phase of differentiation, and blockade of ERK activity in 3T3-L1 cells or in mice inhibits adipogenesis. Conversely, in the terminal differentiation phase ERK1 activity leads

to phosphorylation of PPAR-γ, which inhibits differentiation (Bost et al. 2005). p38 MAPK is required for adipogenesis in 3T3-L1 but not in primary preadipocytes (Aouadi et al. 2006).

Some fibroblast growth factors (FGFs), as FGF1, FGF2 and FGF10, show pro-adipogenic activity on human preadipocytes, and their neutralization inhibits adipogenesis (Hutley et al. 2004).

In recent years, the influence of environmental factors on adipogenesis is being increasingly reognized. For instance, infection with human adenovirus type 36 (Ad-36) has been demonstrated to promote adipogenesis, increasing adipose tissue-induced glucose uptake in the context of increased insulin action, similar to the effects of thiazolidinodiones. Ad-36 modulated regulatory points that covered the entire adipogenic cascade ranging from the upregulation of cAMP, phosphatidylinositol 3-kinase and p38 signaling pathways, downregulation of Wnt10b expression, and increased expression of CEBPβ and PPAR-γ2 and consequential lipid accumulation via its E4 orf-1 gene (Rogers et al. 2008a, b).

3.5.2 Antiadipogenic Factors

The Wnt family of secreted glycoproteins act through autocrine or paracrine mechanisms to influence the development of many cell types. Wnt completely blocks induction of the key adipogenic transcription factors C/EBP-α and PPAR-γ. In contrast, inhibition of Wnt signalling in preadipocytes results in spontaneous differentiation, indicating that preadipose cells produce endogenous Wnt that is a potent inhibitor of differentiation. Ectopic expression of the Wnt gene potently represses adipogenesis (Ross et al. 2000). In particular, the constitutive expression of *WNT10b*, a gene which is highly expressed in preadipocytes and downregulated during the course of differentiation, inhibits adipogenesis (Longo et al. 2004). Ectopic expression of *WNT10b* stabilizes free cytosolic β-catenin and is a potent inhibitor of adipogenesis. In vivo, transgenic expression of *WNT10b* in adipocytes results in a 50% reduction in WAT mass and the development of BAT is absent. In this sense, WNT10a and WNT6 have also been identified as determinants of brown-adipocyte development.

β-catenin functions as a Wnt effector, binds to the androgen receptor and is translocated to the nucleus in response to testosterone where it interacts with the TCF/LEF transcription factors to inhibit adipogenesis. Loss of β-catenin in myometrial tissue causes its conversion to adipose tissue, which shows that the Wnt–β-catenin pathway is an important regulator of adipogenesis and mesenchymal-cell fate in vivo (Kanazawa et al. 2005b; Singh et al. 2006).

The transforming growth factor β (TGFβ) superfamily members, TGFβ, BMPs and myostatin regulate the differentiation of many cell types, including adipocytes. TGF-β is a cytokine that stimulates preadipocyte proliferation and inhibits adipogenesis in vitro. TGFβ and its signalling components are expressed in cultured adipocytes and adipose tissue. Transgenic overexpression of TGFβ impairs the development of adipose tissue (Clouthier et al. 1997). Blockade of endogenous

TGFβ signalling by inhibition of SMAD3 increases adipogenesis. SMAD3 binds to C/EBPs and inhibits their transcriptional activity (Choy and Derynck 2003). Exposure of multipotent mesenchymal cells to BMP4 commits these cells to the adipocyte lineage, allowing them to undergo adipose conversion. The effects of BMP2 are more complex and are dependent on the presence of other signalling molecules. BMP2 stimulates adipogenesis of multipotent C3H10T1/2 cells at low concentrations, but favors chondrocyte and osteoblast development at higher concentrations. Myostatin, positively or negatively regulates adipogenesis in vitro, depending on the type of cell and culture conditions (Rebbapragada et al. 2003).

Pref-1 is a transmembrane protein that belongs to a family of epidermal-growth-factor-like repeats containing proteins and is activated by proteolytic cleavage (Villena et al. 2002). Pref-1 cleavage releases an extracellular moiety that inhibits adipogenesis, possibly through interaction with Notch. Expression of Pref-1 is high in preadipocytes and normally declines during differentiation, and forced Pref-1 expression in 3T3-L1 cells blocks adipogenesis. A soluble form of Pref-1 is sufficient to decrease adipose tissue mass and insulin sensitivity (Lee et al. 2003). Pref-1 is implicated in the regulation of adipogenesis by FOXA2 (Wolfrum et al. 2003), KLF2 (Li et al. 2005) and KLF6 (Wu et al. 2005).

Exposure of preadipocytes to pro-inflammatory cytokines inhibits adipogenesis by reducing PPAR-γ and C/EBP-α expression and by blocking insulin action. TNF-α and IL-1 suppress adipose conversion by activation of the TAK1/TAB1/NIK cascade, which in turn inhibits PPAR-γ activity (Suzawa et al. 2003). In fact, cytokines have the potential to decrease adipocyte numbers through multiple points in the adipogenic program and by activation of several distinct intracellular signalling pathways (Constant et al. 2006; Lumeng et al. 2007; Yarmo et al. 2009).

Some drugs show a strong influence on adipogenesis. Highly active antiretroviral therapy on human immunodeficiency virus (HIV) infection, has been associated with metabolic syndrome including insulin resistance, dyslipidemia, peripheral lipoatrophy and visceral adiposity (Leow et al. 2003). Studies in cell culture have shown that several protease inhibitors, for example nelfinavir and indinavir, decrease preadipocyte differentiation and lipogenesis, while increasing apoptosis and lipolysis (Dowell et al. 2000; Lenhard et al. 2000; Zhang et al. 1999). In addition, studies in patients with HIV-associated lipoatrophy display an increase in pro-inflammatory cytokines in adipose tissue, suggesting that the reducing effects of protease inhibitors on adipogenesis could be the consequence of the local overproduction of these cytokines (Bastard et al. 2002; Kannisto et al. 2003).

Metformin, a widely prescribed drug in the treatment of patients with type 2 diabetes, inhibited the differentiation of mouse 3T3-L1 cell line and primary human preadipocytes, decreasing lipogenic gene expression and increasing AMPK activity and glucose intake (Lenhard et al. 1997; Huypens et al. 2005; Alexandre et al. 2008; Fischer et al. 2010). Metformin effects on human adipocytes are likely to mediate through organic cationic transporter 1, which is induced during adipocyte differentiation (Moreno-Navarrete et al. 2011).

3.5.3 Other Players in the Regulation of Adipogenesis

3.5.3.1 Epigenetic Factors in Adipogenesis

Epigenetic regulation plays a critical role in several differentiation processes and possibly in adipocyte differentiation (D'Alessio et al. 2007). Recently, differentiation of 3T3-L1 cells was demonstrated to be associated with genome-wide epigenetic changes, as evidenced by the ratio of demethylation/methylation and furthermore maintenance of a static demethylated/methylated state, both of which depend on differentiation phase (Sakamoto et al. 2008). DNA methylation might be associated with the course of determination phase.

In addition, the study of 3T3-L1 cells using microarray-based integrated method clarified that adipogenesis is regulated by a ras homologue guanine nucleotide exchange factor (RhoGEF, WGEF) expression through DNA methylation change (Horii et al. 2009). Furthermore, like DNA demethylation, the methylation of histone H3 lysine 4 was related to transcriptional activation. In order to detect the change of histone methylation, 3T3-L1 fibroblast cells were treated with low dose of the methyltransferase inhibitor methylthioadenosine, which eliminates this epigenetic sign from the promoters, and generates a significant decreased adipogenesis, therefore, suggesting the crucial role of this histone modification in the regulation of adipocyte differentiation (Musri et al. 2006). The transcription factors and co-regulators involved in preserving appropriate levels of histone methylation and modification at the late adipogenic genes remain unknown. Above all, the role of DNA and histone modification in adipogenesis is very important, and some functions remain unknown.

3.5.3.2 The Role of miRNAs in Adipogenesis

MicroRNAs (miRNAs) are small non-coding RNAs that bind to regulatory sites of target mRNA and modify their expression, either by translational repression or target mRNA degradation, resulting in decreased protein production. MiR-143 was the first miRNA associated with regulation of adipocyte differentiation. Its expression increases in differentiating adipocytes, and antisense oligonucleotides against miR-143 inhibit human-cultured adipocyte differentiation and lead to a decrease in triglyceride accumulation and the downregulation of PPAR-γ2, adipocyte fatty acid binding protein and GLUT-4. Several miRNAs (including miR-103, miR-107 and miR-143) are induced during adipogenesis, which may play a role in accelerating adipocyte differentiation, and then be downregulated in the obese state. Conversely, miR-222 and miR-221 are decreased during adipogenesis but upregulated in obese adipocytes. Forced miR-103 and miR-143 expression accelerate the rate of 3T3-L1 differentiation, increasing triglyceride accumulation and the expression of many adipocyte important genes at early stages of adipogenesis (Xie et al. 2009).

miRNA378/378 is highly expressed during adipocyte differentiation. Overexpression of miRNA378/378 during adipogenesis also increased triglyceride

triacylglycerol accumulation, and lipogenic genes, PPAR-γ2 and GLUT-4 expression. In addition, in the presence of microRNA378/378, C/EBP-α and C/EBP-β activity on the GLUT-4 promoter was increased (Gerin et al. 2010).

The miRNA expression profile has been recently demonstrated to change during adipocyte differentiation (Ortega et al. 2010). These authors found a differential expression of 70 miRNAs during adipocyte differentiation. In addition. The miRNA expression profile of visceral and subcutaneous adipose tissue is different in obese and non-obese subjects (Ortega et al. 2010; Klöting et al. 2009). A genome-wide miRNA profiling study of 723 human miRNAs have disclosed the expression of 40 (in preadipocytes) and 31 (in adipocytes) mature miRNAs that significantly differed according to obese status. The expression pattern of 22 miRNAs in human subcutaneous adipose tissue was also associated with parameters of adipose tissue physiology, glucose metabolism and obesity status. This study revealed that miRNAs may constitute biomarkers for obesity and obesity-related complications. For example, some miRNAs (miR-221, miR-125b, miR-34a and miR-100) were upregulated in fat depots from obese subjects and downregulated during adipocyte differentiation. On the contrary, miR-185 was upregulated in mature adipocytes while downregulated in obese men. Others, as 130b and miR-210, were both downregulated during adipocyte differentiation and in fat depots from obese subjects. Only miR-34a was found to be positively upregulated during adipogenesis and associated positively with BMI (Ortega et al. 2010).

3.5.3.3 Chronobiology in Adipogenesis

Some clock genes, especially Bmal1 and Rev-Erba, may play a part in adipocyte differentiation and lipogenesis. It has also been shown that clock genes can oscillate accurately and independently of the central nervous system in human AT explants and that this intrinsic oscillatory mechanism may participate in regulating the timing of other clock-controlled gene such as PPAR-γ and GC metabolism genes. Moreover, these circadian patterns differ between visceral and subcutaneous AT depots (Gómez-Santos et al. 2009; Hernández-Morante et al. 2009).

A number of adipocyte-specific factors show rhythmic expression. Some examples are leptin, adipsin, resistin, adiponectin and visfatin, all of them showing circadian rhythmicity, For example, adiponectin shows both ultradian pulsatility and a diurnal variation (Gómez-Abellan et al. 2010). Recently, nocturnin, a circadian-regulated gene, has been demonstrated to promote adipogenesis by stimulating PPAR-γ nuclear translocation and enhancing its transcriptional activity (Kawai et al. 2010).

3.6 Future Perspectives

This review provides a brief overview on various adipocyte cell lines that could be used in appropriate experiments to gain insight in the molecular mechanisms that underlie adipocyte differentiation. The selection and use of an in vitro system must consider all known levels of regulation of proliferation, differentiation and function to ensure relevant results.

The information summarized here concerning intracellular pathways and nuclear and extranuclear modulators of adipocyte differentiation is continuously expanding. Further research is necessary to gain insight in the molecular processes that are involved in adipocyte differentiation, connecting extranuclear and nuclear mediators. New areas, as epigenetic, microRNAs and circadian clock, also need to be more investigated. An in-depth knowledge of adipocyte differentiation is absolutely essential to gain insight in the treatment of important metabolic diseases associated with obesity and adipose tissue expandability, such as type 2 diabetes, atherosclerosis, cardiovascular disease and cancer.

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Chapter 4
Brown Adipose Tissue

Martin Klingenspor, Andrea Bast, Florian Bolze, Yongguo Li, Stefanie Maurer, Sabine Schweizer, Monja Willershäuser, and Tobias Fromme

Abstract A constant body temperature can only be maintained when the rate of heat dissipation equals the rate of heat loss. Thermoregulatory heat production mechanisms compensating heat loss are classically categorized as shivering and non-shivering thermogenesis. Non-shivering thermogenesis occurs in brown adipose tissue, a unique heat producing organ only found in mammals. In brown adipose tissue mitochondria, the proton motive force across the inner membrane is dissipated as heat rather than converted to ATP. This tightly regulated process is catalyzed by the uncoupling protein 1. Non-shivering thermogenesis is elicited by the sympathetic innervation from hypothalamic and brain stem control regions which are activated by cold sensation. In a cold environment up to half of the metabolic rate of rodents can be attributed to non-shivering thermogenesis in brown adipose tissue. The high thermogenic capacity of brown adipose tissue recruited in the defense of normothermia may also play a role in the regulation of energy balance in the face of hypercaloric nutrition. In this light, the discovery of significant amounts of metabolically active brown adipose tissue in healthy adult humans reintroduces an old player in human energy balance research and may enable new strategies to prevent excess body fat accumulation in man.

Keywords Uncoupling protein 1 • UCP1 • Brown adipose tissue • White adipose tissue • Adipocyte • Progenitor • Mitochondria • ATP synthesis • Non-shivering thermogenesis • Progenitor • Proliferation • Differentiation

M. Klingenspor (✉) • A. Bast • F. Bolze • Y. Li • S. Maurer • S. Schweizer
M. Willershäuser • T. Fromme
Else Kröner-Fresenius Center for Nutritional Medicine, Technical University of Munich,
Gregor-Mendel-Str. 2, 85356 Freising-Weihenstephan, Germany
e-mail: mk@tum.de

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4.1 Biological Significance of Brown Adipose Tissue

A simple thermophysiological principle states that body temperature can only be maintained at a constant level when the rate of heat dissipation equals the rate of heat loss. The rate of heat loss depends on two variables, the body's thermal conductance (reciprocal of insulation) and the difference between body and ambient temperatures. In the thermoneutral zone, heat released by basal metabolic rate is sufficient to maintain body temperature. In this zone, which is around 30 °C for mice and men, physical adjustments of thermal conductance suffice and the rate of heat loss is rather low. When moving to colder environments, however, increased heat loss will inevitably result in a drop in body temperature. Two strategies are regularly employed to prevent sustained and life threatening hypothermia in the cold. The thermal conductance of the body is decreased to a minimum, and chemical thermoregulatory mechanisms are activated (Scholander et al. 1950a, b). The underlying thermogenic mechanisms are classically categorized as shivering and non-shivering thermogenesis. Shivering involves episodic or sustained vigorous contractions of antagonistic muscle fibers without efficient work output which cause an increased turnover of the myofibrilar ATP pool and thus heat dissipation. Non-shivering thermogenesis occurs in brown adipose tissue, a unique heat producing organ only found in mammals.

We here provide a condensed overview of brown adipose tissue biology covering the anatomical distribution and principle physiological function of brown adipose tissue for non-shivering thermogenesis in the cold, the evidence for a role of brown adipose tissue thermogenesis in energy balance and possible mechanisms of regulation, the new discoveries on the developmental origin of brown adipocytes, the presence of brown adipocyte-like cells in classical white adipose tissue depots, the evolution of brown adipose tissue and UCP1 in vertebrates and finally highlight the most recent discoveries of metabolically active brown adipose tissue in adult healthy humans.

4.2 Anatomy and Innervation

Brown adipose tissue is found in several distinct anatomical locations including subcutaneous, intraperitoneal and intrathoracic sites. The subcutaneous depots are found in the interscapular and subscapular, dorsal-cervical, suprasternal and axillary regions. Together they surround the upper part of the body like a 'heating jacket' worn underneath the fur (cf. Heldmaier et al. 2013, see page 121). Intraperitoneal depots are mainly found around the kidneys and adrenals (perirenal and suprarenal), and the main intrathoracic depots surround the large mediastinal blood vessels, heart, trachea, esophagus, and descending aorta.

In most studies the subcutaneous interscapular brown adipose tissue depot has been investigated. It is organized into two lobes and displays the prototypical

morphology, being densely capillarized to ensure sufficient supply of oxygen and substrates and is drained by a large blood vessel (Sulzer's vein) to rapidly redistribute locally produced heat into the body. Upon activation of thermogenesis, blood flow through interscapular brown adipose tissue is massively increased by more than ten-fold and together with all brown adipose tissue depots can engross an incredible fraction of more than 25% of total cardiac output (Foster and Frydman 1979; Puchalski et al. 1987). Conversion of tissue oxygen consumption rates (mL O_2/min) measured in vivo into energy units (assuming 20 J/mL O_2) reveals that the maximal rates of heat dissipation range from 160–190 to 330–480 mW/g of brown adipose tissue in warm and cold acclimated rodents, respectively (Table 4.1) (Foster and Frydman 1978; Puchalski et al. 1987; Thurlby and Trayhurn 1980). With this impressive thermogenic capacity brown adipose tissue can contribute up to nearly 50% of the total oxygen consumption of a rat in the cold (Foster and Frydman 1979). Activation is conveyed by postganglionic sympathetic nerves which independently (unilaterally) innervate the two interscapular brown adipose tissue lobes. These nerves form a dense network of unmyelinated fibers within the tissue and can thereby reach virtually every cell by release of their transmitter norepinephrine through varicosities (Bargmann et al. 1968; De et al. 1998). Surgical denervation studies on interscapular brown adipose tissue have clearly demonstrated the indispensible role of this innervation in the control of the thermogenic function of brown adipose tissue. A parasympathetic innervation of brown adipose tissue is largely absent, with the exception of pericardial and mediastinal brown adipose tissue (Giordano et al. 2004; Schafer et al. 1998).

Table 4.1 Mass-specific metabolic rates in brown adipose tissue (brown adipose tissue) of warm- and cold-acclimated rodents

Species	Acclimation temperature (°C)	Oxygen consumption (mL O_2/g min)	Heat production (mW/g brown adipose tissue)	Reference
Rat	28	0.57	160	Foster and Frydman, (1979) Can J Physiol Pharmacol 57(3):257–70
	6	1.44	330	
Mouse	23[a]	1.26	420	Thurlby and Trayhurn, (1980) Pflugers Arch 385(3):193–201.
Djungarian hamster	23[a]	0.48	190	Puchalski et al. (1987) J Exp Zool 242(3):263–71
	−2 to +12[b]	1.05	480	

Oxygen consumption values were calculated from measurements of blood flow and arteriovenous O_2 differences across the interscapular brown adipose tissue depot and converted into heat production assuming 20 J/mL O_2

[a]The thermoneutral zone of mice is around 30 °C, whereas it is about 23–25° in Djungarian hamsters. At room temperature (23 °C) mice are cold acclimated, whereas Djungarian hamster are not

[b]Djungarian hamsters were kept outdoors in this study

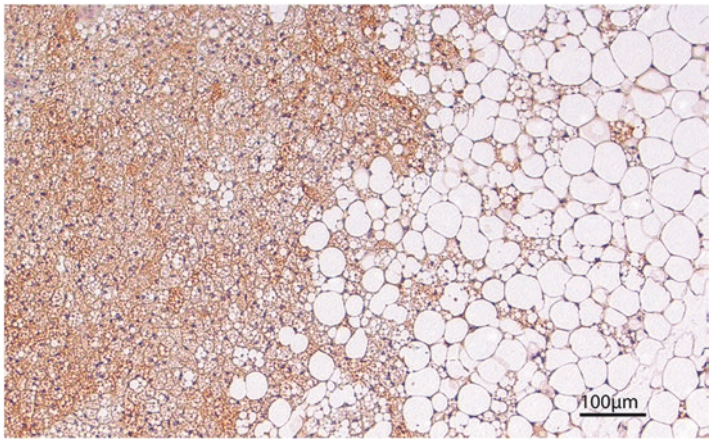

Fig. 4.1 Histology of brown and white adipose tissue—A section of paraffin-embedded interscapular brown adipose tissue was treated with haematoxylin to stain nuclei in *blue*. UCP1 was immunodetected and is indicated by a *brownish* color. Typical multilocular brown adipocytes positive for UCP1 can be seen in the *left half* of the picture, while unilocular white adipocytes devoid of UCP1 dominate the *right half* (image: David Lasar)

Brown adipocytes are characterized by an abundance of small lipid droplets (multilocular) in contrast to white adipocytes which typically feature a single large lipid droplet (unilocular). They furthermore contain an unusually high amount of mitochondria which confer the eponymous brown color to the tissue. The interscapular brown adipose tissue lobes are surrounded by adhering white adipose tissue which allows direct comparison of brown and white adipocyte morphologies in histological sections (Fig. 4.1). In such cross-sections the adipose tissue type often gradually fades from brown to white, i.e. the number of brown adipocytes per white adipocyte steadily decreases. It is difficult to draw a clear border between both tissue types by visual inspection in this classical brown adipose tissue depot. In other adipose tissue depots, the categorization is even more complicated. Some depots usually regarded as white adipose tissue contain some interspersed brown adipocytes, and vice versa, within classical brown adipose tissue depots white adipocytes are found. Furthermore, the fraction these cells constitute is not stable and can be altered by the ambient temperature or during developmental processes. For instance, in mice the retroperitoneal fat depot changes its appearance from classically white to completely brown and backwards to white adipose tissue during the first weeks of life (Xue et al. 2007). In the light of this difficult distinction between white and brown adipose tissue depots both are sometimes described as two aspects of a single ‘adipose organ’ (Cinti 2005). However, novel insight into the origin of brown and white adipocytes questions this view, as we will highlight later.

Unknowingly anticipating the current debate on the origin of brown adipocytes (Sect. 6.1), Conrad Gesner already made a proposition of his own in the first written

account of brown adipose tissue in 1551. He wrote about the marmot: *"They have a lot of fat on their back, although the other parts of the body are lean. In truth it can be called neither fat nor flesh, but similar to the bovine mammary gland, it is something in between"*[1] (Gesner 1551, page 842). More than 400 years after this first anatomical description the thermogenic function of brown adipose tissue was recognized (Smith 1961). Elegant blood flow studies conducted in warm- and cold-acclimated rats revealed that a large fraction of cold-induced thermogenesis (60%) is contributed by heat dissipated from brown adipose tissue (Foster and Frydman 1979). In addition to the role of brown adipose tissue in cold defence, a role of brown adipose tissue in energy balance regulation was suggested (Rothwell and Stock 1979). Since publication of the first evidence for a thermogenic function of brown adipose tissue almost 50 years ago several labs have made important contributions to unravel the underlying biochemical mechanism of heat dissipation in the mitochondria of brown adipocytes [reviewed by (Cannon and Nedergaard 2004; Nicholls 2001)].

4.3 Molecular Mechanism of Thermogenesis in Brown Adipose Tissue

4.3.1 Mitochondrial Bioenergetics

Bioenergetic studies on mitochondria isolated from brown adipose tissue demonstrated a complete lack of the regular control of mitochondrial respiration (Smith et al. 1966). In the absence of ADP mitochondria normally consume oxygen at a low rate (state 2), but strongly increase oxygen consumption in the presence of ADP (state 3). Once ADP is completely converted to ATP, respiration returns to the initial low rate (state 4). Freshly isolated brown adipose tissue mitochondria, however, always respire at their maximal rate and are devoid of respiratory control. Initially, this was a puzzling finding because in all other tissues, the central function of mitochondria is to convert the energy contained in nutrient and storage macromolecules (carbohydrates, fat and proteins) into the universal cellular energy currency ATP which can then be utilized by all energy demanding enzymatic processes in the cell. The bulk of ATP is produced at complex V of the respiratory chain. Complex V, the ATP synthase, is located within the mitochondrial inner membrane and driven by a flux of protons from the intermembrane space through transmembrane subunits of the complex into the mitochondrial matrix. The energy driving this flux and being chemically fixed in ATP is called proton motive force and stems from an unequal distribution of protons across the inner membrane. This proton gradient is constantly maintained by the proton pumps of the respiratory chain which are powered

[1] Dorsum præpingue habent, quũ cæteræ corporis partes sint macræ. Quandȝ hæc vere nec pinguitudo nec caro dici potest: sed ut mamillarũ caro in bubus, inter eas est. medium quidda.

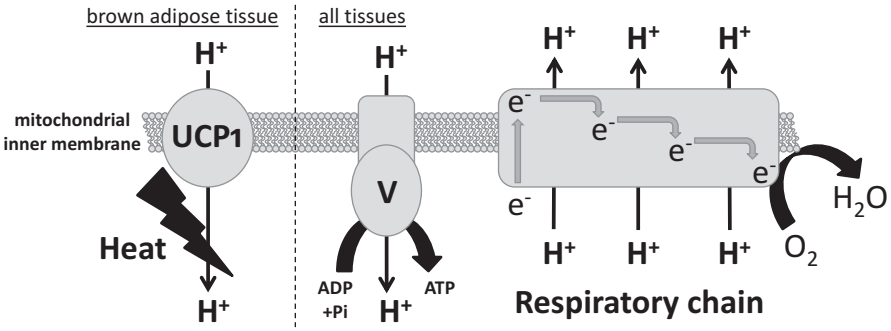

Fig. 4.2 Uncoupling the respiratory chain—the respiratory chain generates a proton gradient across the mitochondrial inner membrane by translocation of protons from the matrix into the intermembrane space. This process is driven by energy-rich electrons from nutrient macromolecules which stepwise release their energy to proton pumps and are afterwards discarded by reaction with oxygen to water. Protons can re-enter the matrix by complex V or by proton leak. The latter is catalyzed by UCP1. Proton motive force is either chemically fixed in the form of ATP at complex V or dissipated as heat energy at UCP1

by energy-rich[2] electrons delivered from reduction equivalents out of TCA cycle and β-oxidation. After discharging their energy[3] to the proton pumps and thus to the proton gradient across the mitochondrial inner membrane, electrons are discarded by reaction with oxygen to water (Fig. 4.2).

Whenever protons re-enter the matrix without concomitant ATP synthesis the energy formerly stored in the proton gradient is released as heat. The diminished proton gradient has to be restored by electron driven proton pumping leading to oxygen consumption. Therefore, protons leaking through the membrane cause an 'uncoupling' of oxygen consumption from ATP production (Fig. 4.2). Uncoupling (= proton leak) is a constant process in all mitochondria and accounts for more than 20% of total oxygen consumption in mammals (Rolfe and Brand 1996). Brown adipose tissue mitochondria are an exception to this rule. They are able to dissipate up to 100% of their proton motive force by a regulated leak mechanism driving non-shivering thermogenesis in this heater organ (Nicholls and Locke 1984).

4.3.2 UCP1 as a Catalyst of Uncoupled Respiration in Brown Adipocytes

Today it is well established that the thermogenic proton leak in brown adipocyte mitochondria is catalysed by the uncoupling protein 1 (UCP1), a member of the mitochondrial transporter family. UCP1 was discovered as a purine nucleotide

[2] i.e. electrons provided by redox half-reactions with high, negative redox potential.

[3] i.e. following an electron transport chain along redox half-reactions with increasing redox potential towards a positive potential.

binding protein inserted into the inner mitochondrial membrane with an apparent molecular weight of 32 kDa (Heaton et al. 1978; Ricquier and Kader 1976). In reference to the size and the preferential binding of GDP it was initially termed 32 kDa protein or GDP-binding protein. First insight into the regulation of UCP1 activity by fatty acids and purine nucleotides was gained by two experimental conditions: (1) removal of endogenous fatty acids by stimulation of mitochondrial beta-oxidation (Hittelman et al. 1969) as well as (2) addition of GDP or GTP (Rafael et al. 1969). In both conditions respiration in isolated brown adipose tissue mitochondria could be measured in a coupled state. It took 10 more years until the primary structure of UCP1 was determined on the cDNA and protein level, respectively (Aquila et al. 1985; Bouillaud et al. 1986). This has triggered a lot of ongoing efforts to scrutinize the molecular details of the unique heat dissipation mechanism. The final proof of concept that UCP1 is indeed essential for the thermogenic function of brown adipose tissue was delivered by the discovery that UCP1 knockout mice are cold-sensitive (Enerback et al. 1997).

UCP1 is exclusively found in brown adipocytes although recent evidence suggests low level expression in thymocytes (Carroll et al. 2004). Biochemical purification of UCP1 from brown adipose tissue of cold-acclimated golden hamsters demonstrated that UCP1 constitutes 5–8% of mitochondrial protein and even 15–20% of the extractable membrane protein fraction (Lin and Klingenberg 1980). A similar high abundance of UCP1 was found in other cold acclimated rodents (Stuart et al. 2001). In the activated state UCP1 increases proton leak by facilitating proton translocation into the matrix and thus collapses the proton motive force at the inner mitochondrial membrane. This completely uncoupled state in turn leads to maximal activity of the respiratory chain with all the food energy conserved in the proton motive force being dissipated as heat. It is by this mechanism that brown adipose tissue can serve as a central heater organ of mammals.

4.3.3 Mode of UCP1 Action

In brown adipocytes, in vivo UCP1 activity is under tight control and dissipates proton motive force in response to appropriate stimuli. It is not finally settled whether UCP1 is completely inactive or may still exhibit some basal leak activity (Parker et al. 2009; Shabalina et al. 2010). Strong inhibitors of UCP1 uncoupling activity are the Mg^{2+}-free di- and triphosphate forms of purine nucleotides (i.e. ADP, ATP, GDP and GTP). They interact with a nucleotide binding site located on a matrix loop of UCP1 that is accessible from the cytosolic side probably due to its steric position close to the transport channel (Ledesma et al. 2002). The apparent binding affinity (K_D) of UCP1 for GDP is ~1 μM (K_D increases with pH) (Nicholls 1976; Rafael et al. 1994), and accordingly, the GDP concentration required for half-maximal inhibition of proton conductance across the inner mitochondrial membrane is 10 μM (Nicholls 1974). As the total purine nucleotide concentration in a cell is usually in the millimolar range (Traut 1994), a complete block of UCP1

activity appears to be the default setting. The affinity of binding, however, is largely reduced in the presence of Mg^{2+} cations, which chelate the di- and tri-phosphate moiety of purine nucleotides in the cell. A much larger cytosolic concentration of ~1 mM Mg-ATP is required to fully inhibit UCP1 (Nicholls and Locke 1984). It remains to be elucidated to which extent modulations of the free purine nucleotide concentration contributes to changes in UCP1 activity. Canonical, positive regulators are free fatty acid anions that in the nanomolar range already partially overcome UCP1 inhibition by endogenous purine nucleotide concentrations (Nicholls and Locke 1984). The exact molecular mechanism of fatty acid induced uncoupling by UCP1 is unresolved and is tightly linked to the question how UCP1 actually creates a proton leak. Possibly, UCP1 acts as a direct translocase which forms a channel with negatively charged amino acid residues passing protons from the intermembrane space to the matrix. In such a model fatty acids could act as a cofactor with their carboxyl terminus serving to close a gap in the transport chain of residues (Winkler and Klingenberg 1994). Patch-clamp measurements of UCP1 currents indeed indicate a type of symport of proton and fatty acid, in which the latter is unable to leave the carrier (Fedorenko et al. 2012). In an otherwise similar model UCP1 is able to translocate protons without a cofactor but is prevented from doing so by bound inhibitory nucleotides. Fatty acids compete for an overlapping binding site and can thereby overcome inhibition (i.e. activate) without being part of the actual transport process (Huang 2003; Shabalina et al. 2004). In a third hypothesis fatty acid anions are themselves the transported substrate. Free fatty acid anions can be protonated into their neutral form when they encounter high proton concentrations as is the case in the mitochondrial intermembrane space. Neutral fatty acids can cross biological membranes uncatalyzed by a so-called flip-flop-mechanism and could thereby enter the mitochondrial matrix. In this environment of low proton concentration, the carboxyl group would release its proton and thereby generate a net proton flux across the inner membrane. UCP1 closes the circuit by exporting fatty acid anions out of the mitochondrial matrix and perhaps additionally catalyzes flip-flop events in its vicinity (Garlid et al. 1996; Skulachev 1991). All models are compatible with the observable increase in UCP1-mediated uncoupled respiration upon liberation of free fatty acid anions in a brown adipocyte.

4.4 Activation of Brown Adipose Tissue

When a mouse or a rat is transferred from room temperature to a cold environment (5 °C) thermoregulatory mechanisms need to be enhanced in order to maintain a constant body temperature. These involve an acute vasoconstriction to reduce thermal conductance, activation of shivering by muscle contraction as well as non-shivering thermogenesis by brown adipose tissue. Concerning the cold response in BAT three components can be distinguished. First, in an acute phase presently available UCP1 is activated resulting in rapid heat production by uncoupled respiration. Second, a long lasting cold stimulus further induces a rise in non-shivering

thermogenesis capacity by increased UCP1 gene expression and mitochondrial biogenesis within the existing brown adipocytes. Third, this remodeling of the quality of brown adipocytes is accompanied by an increase in their quantity. Brown preadipocyte proliferation is induced to increase brown adipose tissue mass and thus to further increase its maximal thermogenic capacity. But how is the environmental information on a lowering of ambient temperature sensed and processed by the animal and immediately translated into an appropriate thermogenic response in brown adipocytes? To answer this question, we need to examine how neuronal and endocrine communication along the brain-brown adipose tissue-axis controls non-shivering thermogenesis. During the past decade considerable progress has been made in this research area by the application of electrophysiology and neuroanatomical tracing techniques.

4.4.1 The Central POA-DMH-rPAa Axis Controls Acute Brown Adipose Tissue Thermogenesis

Upon cold exposure thermal afferent signals elicited by thermoceptors are transmitted to the brain and result in efferent stimulation of vasoconstriction in peripheral blood vessels, non-shivering thermogenesis in brown adipose tissue and shivering in skeletal muscle. A current model of this thermal somatosensory reflex suggests that cold- and warm-sensitive thermoreceptors distributed in the skin, core of the body, the spine and the brain itself transmit temperature sensation through afferent neuronal projections to a command centre in the brain, the preoptic area (POA) in the anterior hypothalamus (Fig. 4.3) (Morrison et al. 2008). Primary somatosensory neurons deliver cold- and warm-sensitive temperature sensation to the dorsal horn (DH) of the spinal cord, from where secondary neuronal afferent fibers relay information to the lateral parabrachial nucleus (LPB) in the midbrain. Projections from LPB neurons reach the POA and converge finally on GABAergic BAT-regulating interneurons to integrate signals from the cold- and warm-sensitive axes. BAT-regulating POA neurons control sympathoexitatory neurons in the dorsomedial hypothalamus (DMH) which regulate sympathetic outflow from the rostral raphe pallidus (rRPa) to BAT (Nakamura and Morrison 2008).

At warm ambient temperatures (e.g. thermoneutrality), POA interneurons inhibit DMH activity by the release of GABA, thus, blocking sympathetic nerve activity in the rRPa. During skin cooling, the activation of the cold-sensitive axis inhibits the release of GABAergic from POA interneurons on sympathoexitatory DMH neurons. This disinhibition of DMH interneurons excites sympathetic outflow from the rostral raphe pallidus (rRPa) causing an activation of BAT thermogenesis (Nakamura and Morrison 2008).

Several studies, however, suggest that independent of the POA-DMH-rPAa neuronal pathway, the rRPa also receives direct sensoric input from peripheral thermoceptors and can directly increase sympathetic outflow to BAT (Bartness et al. 2010; Nautiyal et al. 2008).

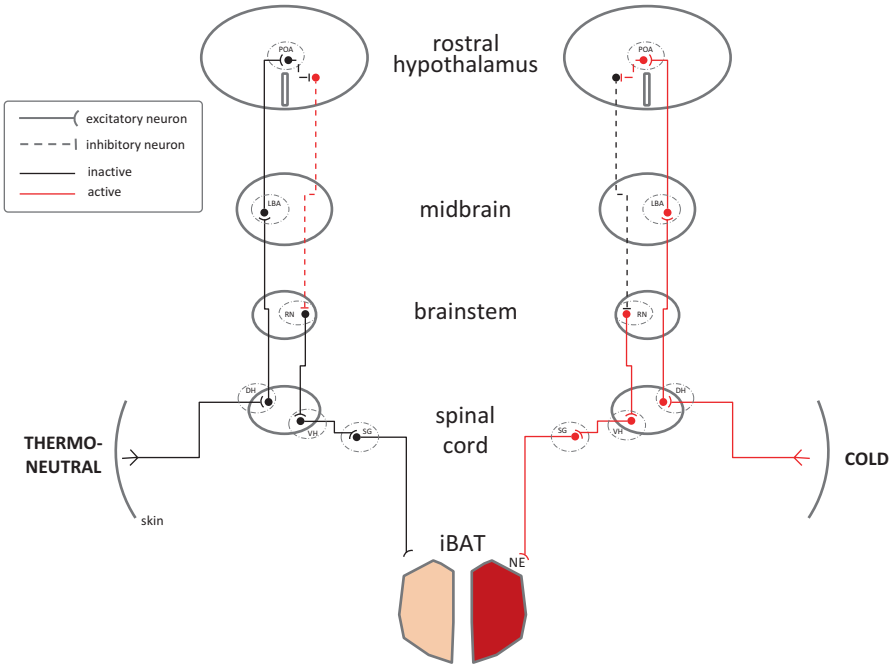

Fig. 4.3 Schematic illustration of the thermal somatosensory reflex—Thermal afferent signals elicited by thermoceptors in the skin are transmitted to the brain and activate inhibitory GABAergic interneurons in the POA of the rostral hypothalamus. In the activated state these interneurons block the activity of efferent inhibitory neuronal projections to the brain stem. Target neurons in the medullary raphe nuclei of the brain stem upon disinhibition convey increased sympathetic outflow to BAT (see text for further details). *DH* dorsal horn, *VH* ventral horn, *RN* medullary raphe nuclei, *LBA* lateral parabrachial nucleus, *POA* preoptic area, *SG* stellate ganglion, *NE* norepinephrine, *iBAT* interscapular brown adipose tissue

Of particular note, BAT not only receives sympathetic efferent signals from the central sites described above, but also exhibits afferent-sensory neurons projecting towards the brain (Ryu et al. 2015). Interestingly, most brain areas receiving sensory input from BAT are also part of the sympathetic outflow network. This overlap of the sympathetic and sensory system suggests a feedback circuit crucial for coordinated control of BAT function. Indeed, the specific denervation of sensory BAT neurons disturbs the thermogenic response during acute cold exposure (Vaughan and Bartness 2012).

4.4.2 Neuroendocrine Stimulation of Diet-Induced BAT Thermogenesis

Several lines of evidence suggest that BAT not only serves in the defense of body temperature but may also dissipate food energy in states of positive energy balance (Himms-Hagen 1979; Nedergaard and Cannon 2010). The peripheral signals and

central mechanisms and pathways involved to elicit diet-induced thermogenesis in BAT are only partially understood. Many peripheral signals are involved in the regulation of energy intake and expenditure and it has been suggested that BAT is the effector organ for the catabolic action of some of these signals (Spiegelman and Flier 2001). In the same manner as the thermoregulatory heat production described above, diet-induced non-shivering thermogenesis is thought to be activated by the excitation of sympathetic neurons innervating brown adipose tissue.

The nuclei of the sympathetic circuit regulating BAT activity were mainly identified by experiments using pseudorabies virus as retrograde tracer in rodents. Within the hypothalamus, the previously mentioned POA and DMH, but also the paraventricular hypothalamus (PVN), lateral hypothalamus (LH) and the arcuate nucleus (ARC) were determined as key areas for the control of BAT but also for the control WAT activity. These nuclei are also responsible for the integration of peripheral signals (e.g. from adipose tissue and the gastrointestinal tract) to regulate energy homeostasis. One major signal is the adipocyte derived hormone leptin which communicates peripheral fat storage to central sites including the hypothalamus. Leptin binds its cognate receptor and regulates different neuronal subpopulations that either produce anorexigenic or orexigenic neuropeptides (Morton et al. 2006). During states of energy deprivation (e.g. dieting), endogenous fat stores are expended and as a consequence blood leptin levels drop. In such hypoleptinemic states, appetite is increased whereas energy expenditure is depressed to lessen the energy deficit—a response also named adaptive thermogenesis (Rosenbaum et al. 2005; Rosenbaum and Leibel 2014). Low leptin promotes the secretion of the neuropeptides agouti-related protein (AGRP) and neuropeptide Y (NPY) from orexigenic neurons but inhibit the release of α-melanocyte stimulating hormone (MSH) derived from anorexigenic neurons expressing the α-MSH precursor pro-opiomelanocotin (POMC). NPY is one major driver for the metabolic response during energy deprivation. Besides promoting appetite, NPY inhibits the sympathetic outflow to BAT, hence, lowering whole body energy expenditure (Shi et al. 2013).

Activation of the melanocortin-4-receptor (MC4R) in different nuclei including the PVN by its agonist α-MSH promotes satiety. Furthermore, the activation of MC4R signaling in PVN and DMH neurons stimulates BAT activity (Enriori et al. 2007; Song et al. 2008). A special feature of the melanocortin pathway is AGRP which acts as an antagonist and inverse agonist at the MC4R. Consequential, AGRP binding to MC4R blocks its activation and thereby promotes food intake and prevents BAT thermogenesis (Enriori et al. 2011; Ollmann et al. 1997). In addition to its reciprocal regulation of POMC and AGRP/NPY neurons in the ARC, leptin can directly activate sympathoexcitatory neurons in the DMH to increase sympathetic tone towards BAT (Enriori et al. 2011; Rezai-Zadeh et al. 2014). In hyperleptinemic diet-induced obese mice, BAT temperature is higher than in mice on a regular diet (Enriori et al. 2011) further supporting the existence of BAT mediated diet-induced thermogenesis in states of energy excess.

Studies in mice and humans have demonstrated that the adaptive thermogenic response during energy scarcity can be overcome by restoring hypoleptinemia through recombinant leptin administration (Doring et al. 1998; Rosenbaum et al. 2005).

Although the contribution of BAT was not addressed in these studies, the normalization of the sympathetic tone and thyroid function during leptin treatment suggest that BAT activity might contribute to the abolishment of adaptive thermogenesis.

The involvement of the ventromedial hypothalamus (VMH) has also been demonstrated by several studies. Systemic hyperthyroidism and central triiodothyronine (T3) injections into the VMH stimulate sympathetic nerve activity in BAT (Lopez et al. 2010). Moreover, activation of glucagon-like peptide-1 receptor (GLP-1R) signaling by intracerebroventricular injections of agonist compounds elevates BAT thermogenesis (Lockie et al. 2012). Subsequent experiments suggest that the mechanism regulating this action is located in VMH (Beiroa et al. 2014). The route by which the VMH influences BAT activity is unclear, since retrograde tracing revealed the VMH was not a major nuclei involved in sympathetic BAT innervation (Bamshad et al. 1999). There is clear evidence that besides the thermoregulatory POA-DMH-rPAa pathway other neuronal networks mainly located in the hypothalamus control sympathetic output to BAT. Conditions of energy excess and energy scarcity both affect BAT thermogenesis. Further studies are needed to better understand the interaction between thermal and metabolic efferent pathways.

4.4.3 Acute Activation of Uncoupled Respiration in Brown Adipocytes

Within minutes after cold exposure the sympathetic outflow from the brainstem to brown adipose tissue leads to the acute activation of non-shivering thermogenesis in brown adipocytes by the release of norepinephrine from postganglionic sympathetic neurons. Parenchymal varicosities and axon terminals of sympathetic nerve fibers have been described in close proximity to brown adipocytes (Bargmann et al. 1968). Upon cold exposure norepinephrine release activates adrenoreceptors situated in their plasma membrane (Fig. 4.4). Adrenoreceptors (AR) of all three known β-subtypes (β1-, β2- and β3-AR) as well as the two α-subtypes (α1- and α2-AR) are expressed in brown adipocytes. The β3-AR seems to be most relevant for the acute activation of thermogenesis (Lafontan and Berlan 1993). This Gs-protein coupled receptor activates adenylyl cyclase, an enzyme that converts ATP to the second messenger molecule cyclic AMP (cAMP). Signal transduction proceeds to the protein kinase A (PKA) complex that releases its catalytic subunit to phosphorylate target proteins in response to increased cytosolic cAMP levels (Fig. 4.4).

Activation of PKA in the β-adrenergic signaling cascade leads to an increase in lipolytic activity at the surface of lipid droplets in the brown adipocyte. Remarkably, the first and crucial step in the breakdown of triglycerides catalyzed by adipose triglyceride lipase (ATGL) is adrenergically activated by a different and so far unresolved pathway (Zimmermann et al. 2004). However, the lipid droplet coating protein perilipin and hormone sensitive lipase (HSL) are direct PKA targets. Their phosphorylation leads to an increased rate of lipolysis and emergence of cytosolic

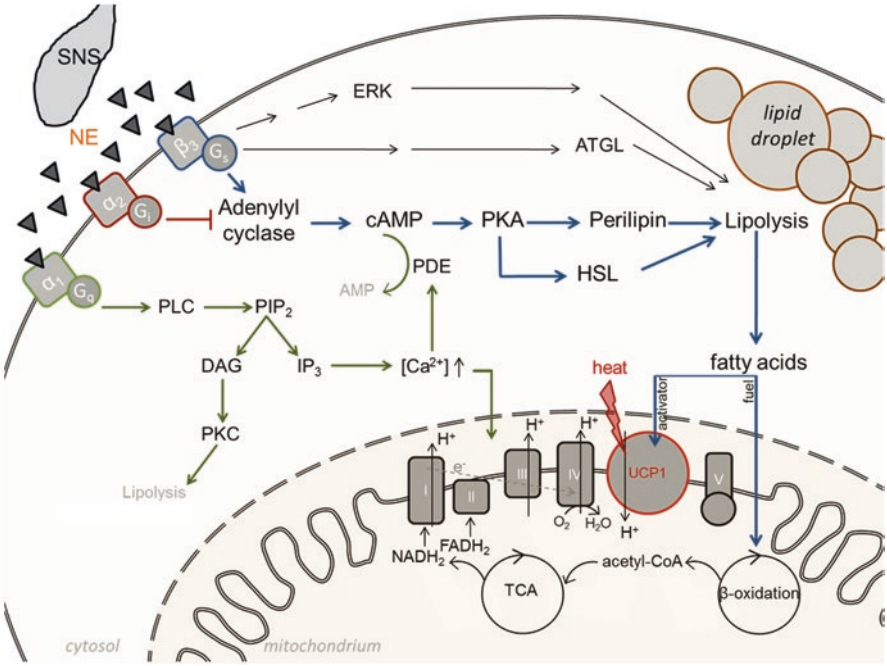

Fig. 4.4 Signaling pathways in brown adipocytes involved in the regulation of non-shivering thermogenesis—Release of norepinephrine (NE) at the plasma membrane of brown adipocytes leads to activation of G-protein coupled adrenoreceptors (AR). β3-AR mediated signaling via PKA activation results in the lipolytic release of fatty acids from lipid droplets. As fuel and as UCP1 activators these fatty acids are the final effectors inducing non-shivering thermogenesis. α1- and α2-AR dependent signaling via inhibition of adenylyl cyclase, increase of cytosolic calcium (Ca^{2+}) and protein kinase C (PKC) activation might further modulate this process. Please refer to the main text for a detailed description of all depicted processes. *Double arrows* indicate pathway segments with interconnections unknown or deliberately left out

free fatty acid anions (Holm 2003). Beyond this well characterized mechanism, the β3-receptor can additionally activate a second, extracellular signal regulated kinase (ERK) mediated signaling cascade to reach a maximal lipolytic activity in the presence of high ligand concentrations. The proteins involved and targeted in this process are still in debate, but ERK signaling further augments maximal PKA-mediated lipolysis by ~20% (Robidoux et al. 2006). Brown adipocytes abundantly express both heart-type and adipose tissue-type fatty acid binding proteins, to shuttle lipophilic fatty acids through the cytosolic compartment (Daikoku et al. 1997). These fatty acids serve a dual purpose in brown adipocyte thermogenesis. Esterified to coenzyme A, fatty acids are shuttled as metabolic fuel into the mitochondrial beta-oxidation pathway, while in their unbound non-esterified anion form they act as potent activators of UCP1 (Sect. 3.3). At this point the complex regulatory pathway from cold sensation to acute heat generation by uncoupled respiration in brown

adipose tissue is complete. The increased cytosolic free fatty acid concentration is the final effector directly leading to increased UCP1 activity and concomitant heat production to compensate an increased heat loss by non-shivering thermogenesis.

This process can however be further modulated by norepinephrine mediated stimulation of α1- and α2-AR that occurs in parallel to β3-AR activation. Upon activation, α2-AR couple to Gi-proteins resulting in the inhibition of adenylyl cyclase activation by β3-AR. Significantly stronger β3-mediated increases in cAMP levels in the presence of an α2-AR antagonist in mature brown adipocytes are indicative for this (Bronnikov et al. 1999). α1-AR, that are highly expressed in brown adipose tissue, activate Gq-proteins when stimulated by norepinephrine. Subsequent phospholipase C (PLC) activation leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG).

The formation of IP3 in turn mediates an increase in cytosolic calcium levels $[Ca^{2+}]i$. This seems to be achieved by the release of Ca^{2+} from intra- and extracellular stores in a process referred to as store operated calcium entry (SOCE) (Leaver and Pappone 2002; Lee et al. 1993; Lewis 2007; Wilcke and Nedergaard 1989). The second product of PIP2 breakdown, DAG, leads to the activation of protein kinase C (PKC).

Although the direct effect of α1-adrenergic signaling on BAT thermogenesis is considered to be rather low, potential modulatory effects on the β3-mediated thermogenesis process should not be neglected. Early studies applying α1-adrenergic agonists and antagonists in hamster brown adipocytes are supportive for this view (Mohell et al. 1983, 1984). Even though underlying mechanisms are not yet completely clarified in brown adipocytes, there are hints from other tissues showing that increased Ca^{2+} concentrations are required for full activity of glycerol-3-phosphate dehydrogenase and other intra-mitochondrial substrate dehydrogenases (McCormack et al. 1990). α1-adrenergically mediated increases in cytosolic Ca^{2+} levels and subsequently elevated uptake of Ca^{2+} into mitochondria might thus to some extent exert control over activity of the OXPHOS machinery and thus thermogenesis. In that line, α1-adrenergic stimulation via $[Ca^{2+}]i$ increase is able to potentiate forskolin-induced respiration elucidating an α1/β3-AR synergism in thermogenesis of hamster brown adipocytes (Zhao et al. 1997). Apparently contrasting this thermogenesis promoting effect, α1-AR stimulation via $[Ca^{2+}]i$ elevation and subsequent phosphodiesterase (PDE) activation is also reported to induce degradation of high cAMP concentrations, explaining the bell shaped dose response curve for norepinephrine stimulated cAMP formation. However, since thermogenesis is fully activated already at submaximal cAMP concentrations, this cAMP degrading effect of α1-adrenergic signaling is unlikely to affect thermogenesis under physiological conditions (Bronnikov et al. 1999).

Besides, there are hints from white adipose tissue and 3T3-L1 adipocytes that PKC could be involved in lipolysis, further supporting α1-adrenergic signaling dependent regulation of BAT thermogenesis (Carmen and Victor 2006; Flechtner-Mors et al. 2002; Fricke et al. 2004; Galvin-Parton et al. 1997).

4.5 In Vivo Function and Fuels for Thermogenesis

Maintenance of BAT function requires fuel supply, which includes oxygen, glucose and fatty acids. These substrates originate either from breakdown of intracellular stores or are taken up from circulation.

New technologies enable visualization of BAT *in vivo* (Izzi-Engbeaya et al. 2015; Bauwens et al. 2014). Computed tomography (CT) allows imaging and quantification of BAT (Lubura et al. 2012) but has the disadvantage that it uses radiation, provides only a snapshot and does not allow functional analysis of BAT. Magnetic resonance imaging (MRI) is a radiation-free method that uses strong magnetic fields. First tests with MRI for morphological imaging of BAT in rats were already performed around 1990 (Osculati et al. 1989; Sbarbati et al. 1991). It is possible to distinguish between BAT and WAT because WAT has a higher fat and lower water content compared to the BAT as shown by chemical shift and IDEAL-MRI (Lunati et al. 1999; Hu et al. 2010). Because of this the magnetic resonance signal of WAT derives mainly from fat protons, whereas in BAT fat and water protons contribute to the signal (Sbarbati et al. 1997) resulting in a different contrast. Beside morphological imaging MRI also allows one to analyse changes in the fat-signal fraction (lipid content) reflecting the functional status of BAT after acclimation to different ambient temperatures (Smith et al. 2013; Grimpo et al. 2014) as well as in the perfusion (hemodynamic changes) of BAT after pharmacological stimulation with adrenaline using contrast enhanced MRI (Sbarbati et al. 2006) or the β_3-adrenoreceptor agonist CL-316,243 using functional MRI (Chen et al. 2012). BOLD MRI is used to monitor BAT activity by detecting changes in the blood oxygenation after activation of BAT by norepinephrine (Khanna and Branca 2012). Another approach to measure BAT activity is hyperpolarised ^{13}C imaging where non-radioactive pre-polarized [1-^{13}C] pyruvate that is taken up and converted into ^{13}C bicarbonate and [1-^{13}C] lactate with increased conversion after norepinephrine injection (Lau et al. 2014). In one study hyperpolarized xenon was used to image BAT and its thermogenic capacity (Branca et al. 2014).

4.5.1 Oxygen

In situations of active non-shivering thermogenesis, BAT becomes the major oxygen consuming organ (Cannon and Nedergaard 2004). In small mammals, injection of norepinephrine, which activates maximal thermogenesis in BAT, leads to a 3–4 fold increase in oxygen consumption although BAT accounts for only 1–4% of total body mass (Meyer et al. 2010). The high oxidative capacity is facilitated by mitochondrial abundance, a high content of oxidative enzymes and a dense capillary network which continuously delivers oxygen, since oxygen cannot be efficiently stored within the cell (Li et al. 2014b; Orava et al. 2011).

The thermogenic capacity of BAT can be determined by measuring the oxygen consumption and carbon dioxide production of the animal with indirect calorimetry

in a state of fully activated BAT (Tschop et al. 2011; Even and Nadkarni 2012; Speakman 2013). The maximum capacity of the BAT for non-shivering thermogenesis can be activated by injection of β_3-adrenoreceptor agonists, like norepinephrine or CL-316,243 (Meyer et al. 2010; Cannon and Nedergaard 2011; Virtue and Vidal-Puig 2013). The increase in oxygen consumption after injection of the agonist represents the thermogenic capacity of the BAT, but also includes minor effects of the sympathomimetics on metabolic rate in other organs.

A direct method to determine the thermogenic activity of BAT employs the simultaneous measurement of blood flow with labelled plastic microspheres and arteriovenous difference in blood oxygenation across BAT (Foster and Frydman 1978, 1979; Puchalski et al. 1987).This invasive method requires sophisticated surgical interventions but only delivers one endpoint read-out of heat production in BAT. Alternatively, the clearance of infused radioactive labelled Xenon (^{133}Xenon) was also used to measure the blood flow (Astrup et al. 1984b). Although this method uses radioactivity, needs animal surgery and is only usable when the blood flow is not too high it has the advantage of allowing continuous measurement of the blood flow without killing the animal. Near-infrared fluorescence imaging was also used for quantification of the BAT perfusion using the indocyanine IR-768 in vivo (Nakayama et al. 2003). Today new non-invasive methods to analyse the blood flow by contrast ultrasound (Baron et al. 2012; Clerte et al. 2013; Ernande et al. 2016) or high-resolution laser-Doppler imaging (Abreu-Vieira et al. 2015) are available. These studies showed that the blood flow to the BAT increases upon norepinephrine treatment, suggesting that blood flow could be a used to estimate the rate of heat production in BAT. This assumption has been challenged recently as the adrenergic stimulation of blood flow was similar in wildtype and Ucp1KO mice, despite loss of thermogenic BAT function in the latter (Abreu-Vieira et al. 2015).

4.5.2 Glucose

The amount of glucose taken up by BAT is relatively high and is markedly increased under cold exposure (Shibata et al. 1989; Vallerand et al. 1990). Glucose functions in BAT either as energy substrate or as carbon source. It is used for fatty acid synthesis and after transformation into G3P, it facilitates FA esterification and TAG synthesis. (Ma and Foster 1986). Besides, BAT stores significant amounts of glucose as glycogen (Farkas et al. 1999). In the uncoupled state glucose serves as energy substrate to glycolytically produce cytosolic ATP. Furthermore, glucose derived pyruvate can either be converted into oxaloacetate by pyruvate carboxylase and thereby increase the capacity of citric acid cycle or it can be decarboxylated to acetyl-CoA, enter the citric acid cycle and serve as direct oxidative substrate of thermogenesis. It has been estimated that glucose fuels 2–12% of the thermogenesis (Ma and Foster 1986).

In BAT glucose uptake can be triggered in two opposite metabolic states. During non-shivering thermogenesis, glucose uptake is stimulated by norepinephrine, where as in an anabolic state, when energy stores are replenished, glucose uptake is

triggered by insulin through activation of GLUT4 translocation (Cannon and Nedergaard 2004; Leto and Saltiel 2012).

Adrenergically induced glucose uptake is mediated via cAMP, PI3K and mTORC2-signaling (Albert et al. 2016; Hutchinson et al. 2005; Dallner et al. 2006; Chernogubova et al. 2004, 2005; Olsen et al. 2014). *In vitro* data suggest that glucose uptake in brown adipocytes is completely UCP1 independent (Hutchinson et al. 2005). In contrast, *in vivo*, at least in mice, this process seems to be dependent on UCP1 (Inokuma et al. 2005; Jeanguillaume et al. 2013).

The glucose uptake can be used to visualize BAT with positron-emission tomography (PET) often in combination with computed tomography (CT) for a more precise anatomical location of the metabolic active tissue. Radiation exposure is limiting the application of PET-CT in clinical trials with longitudinal study designs. For the PET studies the glucose analogue 2-deoxy-2-^{18}F-fluoro-D-glucose (^{18}F-FDG) is used. ^{18}F-FDG is taken up by the BAT after stimulation by cold or β_3-adrenoreceptor activating agonists or reduced after pharmacological inhibition (Tatsumi et al. 2004; Mirbolooki et al. 2011; Wu et al. 2011) but is not metabolized in the cell after phosphorylation by the enzyme hexokinase leading to the accumulation of ^{18}F-FDG that can be detected by PET. Using ^{18}F-FDG as tracer the glucose uptake in BAT of rats during acute cold exposure (10°C) for 6 hours was estimated to be ~33 nmol/min and chronic cold exposure for 21 days increased the uptake to 153 nmol/min (Labbe et al., 2015). Beside the measurement of the metabolic activity the method also allows for the volume measurement of the iBAT (Mirbolooki et al. 2011). ^{18}F-FDG was also used for in vivo BAT imaging after activation by cold or norepinephrine by Cerenkov luminescence imaging (CLI) using an optical imaging system instead of PET (Zhang et al. 2013). Before in vivo imaging methods of BAT activity like PET were inaugurated the glucose uptake in BAT was analysed with radioactive labelled 2-deoxyglucose (2-[1-^{14}C]-DG or 2-[^{3}H]-DG) after sympathetic stimulation by norepinephrine (Cooney et al. 1985; Inokuma et al. 2005). In these animal studies 2-DG was injected in vivo and the 2-DG uptake was quantified post-mortem in the dissected BAT, thus providing a final endpoint measurement.

4.5.3 Fatty Acids

The primary fuel for active BAT are fatty acids (Ma and Foster 1986; Nedergaard and Lindberg 1982; Nicholls and Locke 1984). They can either derive from lipolysis of intracellular lipid stores, the main energy source for thermogenesis, or from circulating chylomicron- and lipoprotein-bound triglycerides. The first are degraded when β-adrenergic signaling increases cAMP release to activate PKA and thereby stimulates lipolysis (described in Sect. 4.3). The latter are hydrolyzed by endothelial lipoprotein lipase (LPL). LPL activity of BAT is stimulated by norepinephrine (Carneheim et al. 1984; Radomski and Orme 1971; Labbe et al. 2015). Together with an increase in the transmembrane FA transporter CD36, a pronounced LPL

activity qualify BAT as a major plasma lipid clearing organ in cold exposed rodents (Bartelt et al. 2011; Festuccia et al. 2011).

Since fatty acids are the primary fuel in BAT thermogenesis their transport and metabolism have also been addressed in PET studies. The fatty acid tracer ^{18}F-fluoro-thia-heptadecanoic acid (^{18}FTHA) has been employed to analyze changes in fatty acid uptake rate after cold exposure and/or pharmacological activation of BAT with CL-316,243 (Labbe et al. 2015, 2016). Acute cold exposure (10°C) for 6 hours lead to an uptake of ~12.5 nmol/min NEFA in BAT of rats and a chronic cold exposure for 21 days increased the uptake to 25 nmol/min NEFA (Labbe et al., 2015). Alternatively, optical imaging was applied to monitor the uptake of a long-chain fatty acid probe conjugated to the reporter molecule luciferin in BAT after activation (Henkin et al. 2012). Changes in the lipid content of BAT can be detected by CT (Baba et al. 2010; Lubura et al. 2012).

4.5.4 Further Methods and Tracers to Image BAT

Further PET tracers are ^{11}C-Acetate as a marker for oxidative metabolism of BAT (Labbe et al. 2015, 2016) and the norepinephrine analogue ^{11}C-meta-hydroxyephedrine (^{11}C-MHED) to image the sympathetic nervous system activity of the BAT (Quarta et al. 2013). ^{99m}Tc-Methoxyisobutylisonitrile (^{99m}Tc-MIBI) is used in single-photon emission computed tomography (SPECT) to analyse BAT perfusion and thereby the blood flow after activation (Cypess et al. 2013).

A new approach to image BAT is the development of ligand guided fluorescent probes where selective peptides are detected by near-infrared fluorescence imaging (Azhdarinia et al. 2013). Curcumin analogues (Zhang et al. 2015) and the deep red SRFluor680 (Rice et al. 2015) are also applicable as fluorescent probes for in vivo BAT imaging by near-infrared fluorescence imaging.

Finally, monitoring of skin surface temperatures is a simple and noninvasive method to analyze BAT thermogenesis in vivo, for example using a hand-held infrared camera. BAT temperature increases above 41 °C upon activation by the β_3-adrenergic agonist which results in a rise of the skin surface temperature in the interscapular region and can be measured by infrared thermography (Crane et al. 2014).

4.6 Recruitment of Non-shivering Thermogenesis Capacity

4.6.1 Magnitude of Cold-Induced Increase in Heat Production Capacity

A “normal black 6 (B6)” mouse, that are typically used for rodent studies (weight ~23 g), when subjected to a cold challenge test in which ambient temperature is rapidly lowered from 30 to 5 °C must develop a thermogenic power of ~850 mW

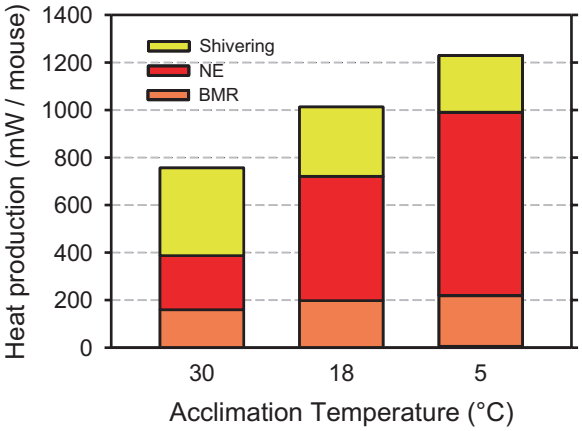

Fig. 4.5 Maximal cold-induced heat production—Contribution of basal metabolic rate and NE-induced thermogenesis to maximal cold-induced heat production. Wildtype B6 mice were acclimated to 30, 18 and 5 °C for several weeks. Basal metabolic rate was measured during 3–4 h at 30 °C, NE-induced thermogenesis in response to a single injection of 1 mg/kg NE and maximal cold induced heat production by stepwise lowering of ambient temperature until the cold limit was attained (Meyer et al. 2010)

to prevent life-threatening hypothermia. When previously housed at thermoneutrality, however, the mouse only has a maximal heat production capacity (HPmax) of 750 mW. Despite this substantial 4.7-fold increase in the power of heat dissipation above basal metabolic rate it is insufficient to survive at 5 °C (Meyer et al. 2010) (Fig. 4.5). Acclimation of the mouse to moderate cold conditions (18 °C) for >3 weeks causes an increase of HPmax to ~1000 mW and enables the mouse to pass the acute cold challenge test without problems. A mouse cold-acclimated at 5 °C will further increase HPmax to ~1200 mW. This recruitment of additional capacity for heat production with decreasing acclimation temperature has been reported in many small rodents (Heldmaier et al. 1990). It is due to a large rise in non-shivering thermogenesis capacity in brown adipose tissue and a comparatively small increment in basal metabolic rate. This is also true for the laboratory mouse. The maximal capacity for non-shivering thermogenesis is determined by measuring the thermogenic response to a single subcutaneous injection of norepinephrine (Heldmaier 1971). Comparing B6 mice acclimated to 30, 18 and 5 °C a three to four-fold increase in norepinephrine-inducible non-shivering thermogenesis capacity can be observed (Fig. 4.5). In spite of vigorous shivering and maximal activation of uncoupled respiration in brown adipose tissue, the thermoneutral-acclimated mouse cannot generate 850 mW for survival at 5 °C. A mouse acclimated to moderate cold (18 °C) has to utilize the maximal capacity for non-shivering thermogenesis, but also needs some shivering to survive. In the cold-acclimated mouse already submaximal activation of non-shivering thermogenic capacity compensates for heat loss in the cold with no additional need for shivering thermogenesis (Fig. 4.5). This is why it is often stated that during cold acclimation non-shivering thermogenesis replaces shivering thermogenesis. It should be noted, however, that

a further lowering of ambient temperature will also cause the activation of shivering thermogenesis in cold-acclimated mice once the capacity for non-shivering thermogenesis approaches maximal power. The limit of cold-acclimated mice is reached at approximately −18 °C (Meyer et al. 2010).

Translated to a wildlife scenario small rodents benefit from the large increase in the capacity for non-shivering thermogenesis during cold-acclimation in two ways. They can move around in cold environments more freely when foraging for food or in the escape from predators, and they have a better chance of survival in extreme cold bouts. The large increase in non-shivering thermogenesis is due to adaptive remodeling of brown and white adipose tissue.

4.6.2 Beta Adrenergic Control of Cold Induced Adaptations in Brown Adipose Tissue

Noradrenergic stimulation of brown adipose tissue leads to immediate UCP1 activation by means of increased lipolysis. At the same time brown adipocytes increase their capacity for heat production by expression of genes encoding components of the thermogenic machinery, prominently among them UCP1. Transcriptional control by transcription factor binding sites in the UCP1 promoter and an essential upstream enhancer region are well studied and can serve as an example to illustrate this process. Both the posttranslational and the transcriptional responses to norepinephrine share a common signaling pathway up to the point at which PKA is activated by cAMP (compare Figs. 4.4 and 4.6). A direct target of PKA is the transcription factor *cAMP response element binding protein* (CREB) which is activated upon phosphorylation and binds to response elements in the promoter region of many genes including UCP1 (Rim et al. 2004). In parallel to PKA, a fraction of CREB phosphorylation is mediated by an independent, less well characterized pathway emanating from α1-adrenoreceptors and involving protein kinase C (Thonberg et al. 2002).

CREB is a central transcription factor in the regulation of gene expression following noradrenergic stimulation of brown adipose tissue but certainly not the only one (Fig. 4.6). The *mitogen activated pathway kinase* (MAPK) p38 is activated downstream of PKA although p38 is not itself a direct target of PKA (Cao et al. 2004). This MAP kinase phosphorylates the *activating transcription factor 2* (ATF-2) which binds and transactivates the UCP1 enhancer and the promoter of the *PPARγ coactivator 1α* (PGC1α) gene. PGC1α is not only positively regulated by p38 by ATF-2 on the transcriptional level but also posttranslationally activated as a direct target of p38 phosphorylation. Activated PGC1α in turn is a strong coactivator of UCP1 transcription and of many genes involved in mitochondrial biogenesis.

This interwoven, self-amplifying network of transcriptional control processes is typical for the noradrenergically induced gene expression cascade in brown adipocytes in response to cold. We find a further example in peripheral thyroid hormone

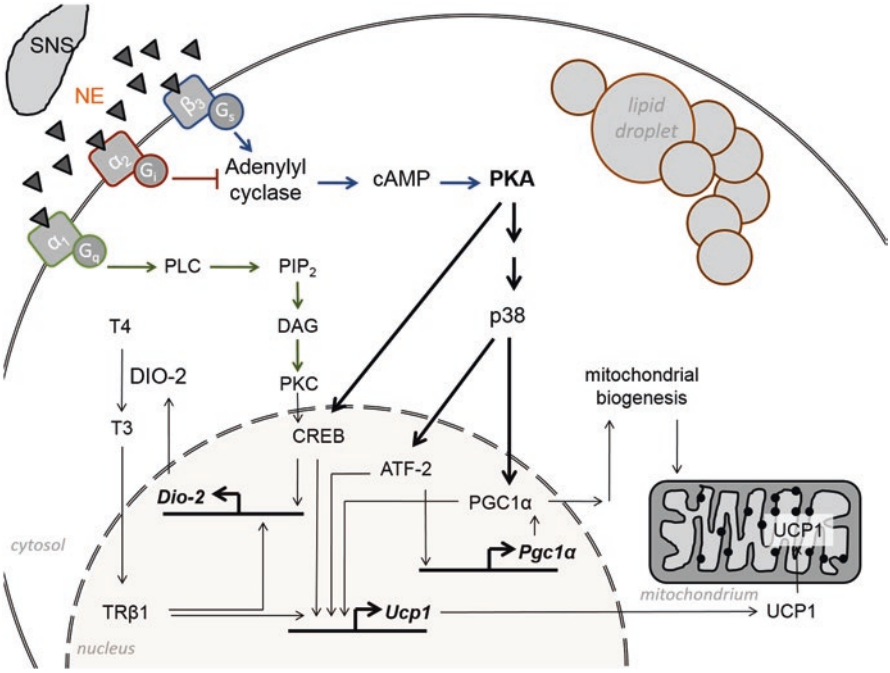

Fig. 4.6 Signaling pathways in brown adipocytes involved in the recruitment of thermogenic capacity—The recruitment of thermogenic capacity in response to norepinephrine is mediated by α- and β-AR, activating an interwoven, self-amplifying network of transcriptional control processes. Within this network adaptive gene expression is mainly initiated by the transcription (co-) factors CREB, ATF-2 and PGC1α. Please refer to the main text for a detailed description of all depicted processes. *Double arrows* indicate pathway segments with interconnections unknown or deliberately left out

actions beyond the centrally mediated effects on brown adipose tissue already discussed above (see Sect. 4.2). One of the CREB target genes is the thyroid hormone converting enzyme *deiodinase 2* (DIO-2) (Canettieri et al. 2000). DIO-2 converts the transport form of thyroid hormone thyroxine (T4) to the bioactive form T3. As a ligand of thyroid hormone receptors, T3 transactivates these transcription factors. In brown adipose tissue the T3 receptor β1 isoform is a positive (albeit permissive) regulator of UCP1 gene transcription (Golozoubova et al. 2004). DIO-2 is also targeted by this T3 receptor thus forming a positive feedback loop (Martinez de et al. 2010).

The goal of cold induced transcriptional changes in brown adipose tissue is to increase the capacity for heat generation and thus for oxidative metabolism. Accordingly, the protein amounts of virtually all components of energy metabolism including fatty acid oxidation and transport, citrate cycle, respiratory chain and many more are increased (Watanabe et al. 2008; Forner et al. 2009). The key organelle implicated in these processes is the mitochondrion. Mitochondrial biogenesis is strongly activated in the cold and results in a more than three-fold increase in the

amount of mitochondrial protein per brown adipocyte (Rafael et al. 1985; Klingenspor et al. 1996). The transcriptional coactivator PGC1α is regarded the master regulator of this adaptation, because overexpression in several cell types including white adipocytes and muscle cells leads to strong elevation of mRNA levels for both nuclear- (COX4, β-F_1-ATPase) and mitochondrial- (Cox2) encoded subunits of the respiratory chain as well as mitochondrial copy number (Lowell and Spiegelman 2000; Wu et al. 1999). PGC1α enforces expression of the *nuclear respiration factors 1 and 2* (NRF-1, NRF-2) which bind to response elements in many genes coding mitochondrial proteins. Given the prominent role of PGC1α in the regulation of the UCP1 gene and its strong norepinephrine-induced expression and activation in brown adipose tissue, it seems clear that mitochondrial biogenesis in response to cold is also under the control of PGC1α and NRF1/2. In addition, the efficiency of the mitochondrial translation machinery in brown adipocytes contributes to the cold-induced mitochondrial biogenesis (Klingenspor et al. 1996).

4.6.3 Non-sympathetic Regulation of BAT Thermogenesis and Recruitment

Beyond the described canonical adrenergic induction of acute thermogenesis and recruitment of thermogenic capacity, a number of alternative factors capable of activating BAT have been identified. Among them, adenosine, atrial natriuretic peptide (ANP), bile acids and FGF21 can be named as examples. High expression levels of their respective target receptors in brown adipocytes support their involvement in BAT regulation.

Adenosine, released as purinergic co-transmitter from sympathetic nerves as well as from brown adipocytes themselves, increases norepinephrine induced lipolysis and expression of thermogenic marker genes in murine and human brown adipocytes via the A_{2A} receptor. Reduced BAT derived thermogenesis in A_{2A}-deficient new-born mice confirms the role of adenosine in BAT function also in vivo (Gnad et al. 2014).

ANP, a natriuretic peptide of cardiac origin, is reported to induce lipolysis and thermogenesis in brown adipocytes by a pathway involving intracellular cyclic guanosine monophosphate-dependent protein kinase (cGMP) and p38 MAPK (Bordicchia et al. 2012). Notably, the lipolytic action is observable only in primate fat cells, but not in rodents, rabbits and dogs (Sengenes et al. 2000). In rodents, high expression levels of the clearance receptor NPRC, encoded by the Npr3 gene, removes natriuretic peptides from the circulation and thereby suppresses their endocrine activities (Sengenes et al. 2002).

Brown adipocytes further express the G-protein coupled receptor TGR5, which upon binding of bile acids mediates increase of BAT thermogenic capacity via induction of thyroid hormone signaling. Mice on high fat diet supplemented with cholic acid reveal elevated UCP1, PGC-1α and DIO-2 expression in BAT

accompanied by increased energy expenditure and resistance to diet induced obesity (Watanabe et al. 2006).

Released from liver and brown adipocytes, FGF21 presents a further non-sympathetic factor regulating BAT activity in both, endocrine and autocrine manner. It acts through the FGF receptor and its specific co-receptor β-Klotho to elicit a thermogenic gene expression program, including UCP1 and PGC-1α (Fisher et al. 2012; Hondares et al. 2010).

These and other alternative factors are of central interest regarding pharmacological activation of BAT in the attempt to treat obesity. In contrast to sympathomimetics they could provide the possibility to regulate BAT function bypassing severe cardiovascular side effects.

By all these means brown fat cells enhance their capacity for heat production. This improvement in the *quality* of brown adipocytes is complemented by increasing their *quantity* during adaptive thermogenesis. In laboratory mice and rats cold exposure elicits growth of brown adipose tissue and a large increase of ^{3}H-thymidine incorporation can be observed during the first days of cold acclimation, indicating proliferation mainly of preadipocytes (Rehnmark and Nedergaard 1989). The mass of the interscapular depot increases three- to four-fold when warm-acclimated rats are cold-acclimated for several weeks (Bukowiecki et al. 1982). The larger tissue mass not only reflects the increased number of brown adipocytes, but in part also lipid storage in the newly differentiated cells, mitochondrial biogenesis and notably angiogenesis. The formation of new capillaries under adrenergic control (Asano et al. 1997) highlights that these processes all contribute to adaptive hyperplasia of the entire brown adipose tissue organ in response to cold exposure.

4.7 Molecular Control of Brown Adipogenesis

At the cellular level, similar to white adipocytes, brown adipocyte differentiation is generally described as a two-step process: a commitment step, wherein committed brown adipocyte progenitors (or preadipocytes) are generated from multipotent mesenchymal stem cells (MSCs), and a differentiation step, wherein pre-adipocytes acquire the features of mature, functional brown adipocytes (Fig. 4.7).

4.7.1 Brown Lineage Commitment

For decades, researchers aimed to answer the question whether white and brown adipocytes share a common preadipocyte-type cell that can be triggered to differentiate into the white or the brown lineage, or whether there are two distinct sets of preadipocytes, brown and white. Recently, major advances have shed some light on the identity of brown adipocyte precursors and revealed an unexpected relationship. The pattern of expressed genes in primary cells derived from brown adipose tissue

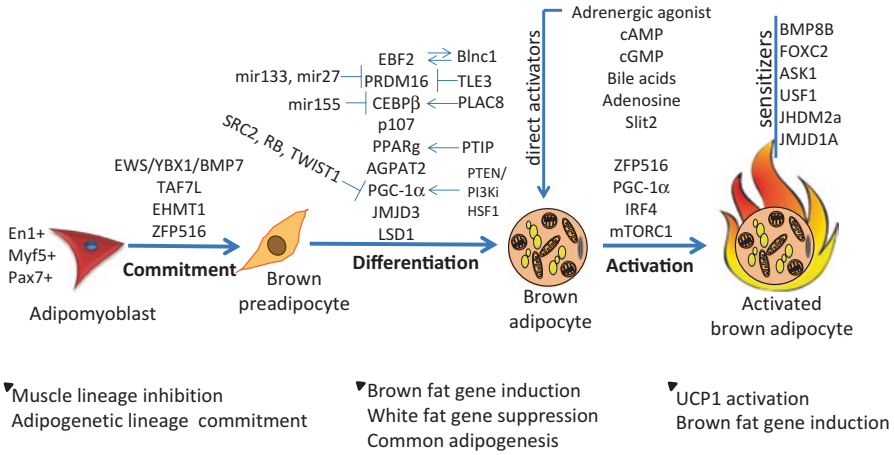

Fig. 4.7 Brown adipogenesis—Schematic representation of the commitment, differentiation and activation of brown adipocytes. Classical brown adipocytes arise from myogenic progenitors that express En1+/Myf5+/Pax7+, here referred to as adipomyoblasts. These progenitors express a commitment gene program as they differentiate into committed precursor cells—brown preadipocytes. The commitment phase probably contains two steps, muscle lineage inhibition and adipogenetic lineage induction. Following commitment to the adipocyte lineage, committed precursor cells can further differentiate into mature brown adipocytes by activating a gene network that shares common features with white adipogenesis. Importantly, built on top of common adipogenesis, brown fat–specific genes are induced and white-selective genes are repressed by the brown fat–specific differentiation gene program, which includes transcriptional factors, coactivators, microRNAs, long noncoding RNAs and epigenetic modifiers. Finally, mature adipocytes that express UCP1 can be further activated by adrenergic or non-adrenergic activators. During activation, thermogenic genes can be further induced. Notably, the activation signaling pathway can be manipulated by diverse gene programs to sensitize adipocytes to stimulation. For more details, please refer to the text

is much more similar to that of skeletal muscle than of white adipose tissue cells (Timmons et al. 2007). This is also true for the mitochondrial proteome of mouse adipose tissues (Forner et al. 2009). Initially interpreted as the activation of a muscle gene set in adipocytes, it has now become clear that skeletal muscle myotubes and brown adipocytes indeed share a common progenitor that is distinct from white preadipocytes. In lineage tracing experiments, all cells of an organism that have ever expressed a certain gene at any timepoint during ontogenesis are labeled by reporter gene expression. Such studies demonstrated that both brown adipocytes and skeletal muscle myotubes derive from progenitor cells expressing Engrailed-1 (En1), myogenic factor 5 (Myf5) and paired box 7 (Pax7) (Seale et al. 2008; Atit et al. 2006; Lepper and Fan 2010). White adipocytes, in contrast, had never expressed these factors. These genes are almost certainly expressed at the earliest stages of BAT development, even before adipogenic commitment factors, such as PPARγ, are detectable. The black-(Myf5+)-and-white (Myf5−) model of brown and white adipocyte origins is elegant in its simplicity; however, recent studies indicate that the

situation is more complex. It has been demonstrated that Myf5^{+} precursors also give rise to white adipocytes in retroperitoneal WAT (Sanchez-Gurmaches et al. 2012). From this study, it is clear that a lineage marker unique to only brown adipocyte precursors remains elusive.

The divergence between myoblast and BAT precursors occurs between stage E9.5 and 11.5 in mice (Lepper and Fan 2010). BAT becomes visible in mouse embryos at stage E14.5 (Atit et al. 2006). It is believed that the commitment is triggered by induction cues in the stem cell niche, which is found in proximal spatial relationship to the vascular network (Jones and Wagers 2008). Upon commitment to brown fat lineage, the myogenic gene program in the common precursors (adipomyoblast) will be repressed, thereby repressing muscle formation. This action prepares the adipomyoblast for entering the brown adipocyte differentiation program. However, very little is known regarding the molecular pathways that are involved in this process. Most notably, bone morphogenetic protein 7 (BMP7) seems responsible for triggering commitment of progenitor cells specifically towards the brown adipogenic lineage (Tseng et al. 2008). BMP7 pre-treatment of either mesenchymal progenitor cells (non-committed C3H10T1/2 cells) or brown preadipocytes during the proliferation phase (before hormonal induction of adipogenesis) promotes the differentiation of brown adipocytes, and BMP7 KO animals display a severe defect in BAT development (Tseng et al. 2008). Consistent with the critical role of BMP7 in the commitment of early mesenchymal progenitors to brown fat, loss of BMP7 regulating gene Ewing sarcoma (Ews) in mice led to a near-complete loss of BAT formation (Park et al. 2013). Mechanistically, EWS interacts with its binding partner Y-box binding protein 1 (YBX1) to induce the transcriptional activation and production of BMP7. Treatment of EWS-null cells with recombinant BMP7 leads to a full rescue of brown adipogenic differentiation capacity. Of note, EWS knockout causes ectopic expression of muscle-specific genes in both residual BAT and isolated brown preadipocytes, suggesting that EWS controls the cell fate choice between skeletal muscle and BAT development (Park et al. 2013). So far neither the source of BMP7 signal in vivo nor the information it conveys are known and thus remains to be investigated in much more details.

Another recently reported molecular switch between brown fat and muscle lineages is TATA-binding protein-associated factor 7L (TAF7L). TAF7LKO mice have decreased brown fat tissue and increased muscle mass. Mechanistically, TAF7L interacts with PPARγ and serves as tissue-specific subunit of TFIID, involved in the coordination of long-range chromatin interactions to specify the brown fat lineage (Zhou et al. 2014). This study highlights a role of chromatin interactions during BAT lineage specification.

Euchromatic histone-lysine *N*-methyltransferase 1 (EHMT1) is a histone lysine methyltransferase, which forms a complex with PRD1-BF-1-RIZ1 homologous domain containing protein-16 (PRDM16) (Ohno et al. 2013). Mice lacking the EHMT1 in Myf5+ lineage display a profound deficit in brown fat development. The residual EHMT1-deficient BAT ectopically expresses muscle-specific transcripts. At the molecular level, EHMT1 promotes BAT formation by demethylation of histone 3 lysine 9 (H3K9me2 and 3) in muscle-selective gene promoters, thereby

repressing muscle lineage marker expression. Ectopically expressed, EHMT1 does not stimulate brown adipogenesis in mouse embryonic fibroblasts which lack endogenous PRDM16 expression, indicating that the lineage specification action of EHMT1 requires other cooperators, notably PRDM16. In this scenario, the commitment phase probably comprises two steps, muscle lineage inhibition (EHMT1) and adipogenetic lineage induction (PRDM16) (Fig. 4.7).

Lastly, in an unbiased search for transcription factors that activate the *Ucp1* promoter, zinc-finger protein 516 (ZFP516), a cold-inducible transcription factor enriched in BAT compared to WAT, is found to play a critical role in both the commitment and the activation of brown adipocytes (Dempersmier et al. 2015). Embryos lacking Zfp516 at E20.5 show a striking defect in BAT development. On the molecular level, the mechanism of Zfp516 action is unresolved.

4.7.2 Brown Fat Differentiation

Following commitment to the adipocyte lineage, differentiation into mature adipocytes occurs. Despite all differences between white and brown adipocytes, they share several key transcription factors involved in adipogenic differentiation (Rosen and MacDougald 2006), including PPARγ and members of the CCAAT/enhancer binding protein family (C/EBP). Although they are essential for brown adipocyte differentiation and the maintenance of other adipogenesis-induced genes, the expression of PPARγ or C/EBPα in stem cells leads to formation of white and not brown fat cells (Kim et al. 2005; Wu et al. 1995), demonstrating they are not sufficient to drive brown fat programming of adipocytes. Based on the current understanding, brown fat differentiation is built on top of a general adipogenesis program to induce brown fat–specific genes and in parallel to repress white-selective genes (Fig. 4.7).

In this scenario, the essential role of PPARγ in brown adipogenesis is not unexpected, since PPARγ is the master regulator of adipocyte differentiation (Rosen et al. 1999). Consistently, activation of PPARγ activity by synthetic agonists promotes brown adipocyte differentiation (Tai et al. 1996). Similarly, mice ablated for 1-Acylglycerol-3-phosphate-O-acyltransferase 2 (AGPAT2), an enzyme required for triacylglycerol synthesis during adipocyte differentiation, lose both the white and brown adipose tissue (Cortes et al. 2009).

On top of the general adipogenesis program, a pivotal regulator of brown adipogenesis is early B-cell factor 2 (EBF2), which acts as a pioneer factor in brown fat cells to “mark” genes for the later recruitment of PPARγ and transcriptional activation (Rajakumari et al. 2013). BAT from EBF2 KO mice displays a near-complete loss of brown fat–specific characteristics and increased expression of white fat–selective gene but no signs of impaired adipogenic potential, demonstrating that EBF2 is not required for general adipogenic process but specially regulates BAT-specific gene expression and represses white-selective genes. Downstream of EBF2, it seems likely that activation PRDM16 homologous domain containing protein-16

(PRDM16) is partially critical for the action of EBF2 in brown fat differentiation (Rajakumari et al. 2013). While studies in cultured cells had shown that classical brown fat cells require PRDM16 to develop and maintain a thermogenic gene program, ablation of PRDM16 from all fat cells in vivo was not sufficient to significantly alter the function of the classical brown fat, indicating possible compensatory mechanisms or additional redundancy pathways that support embryonic BAT development (Seale et al. 2007, 2008; Harms et al. 2014). Mechanistically, PRDM16 is a master transcriptional coregulator in brown adipocytes. It forms complexes with various transcriptional cofactors in a promoter-dependent context, acting bifunctionally to promote expression of brown fat-selective genes and repress white-selective genes (Chi and Cohen 2016). For instance, PRDM16 forms a transcriptional complex with CCAAT/enhancer binding protein b (C/EBPβ) to initiate brown fat development (Kajimura et al. 2009). In addition, PRDM16 also complexes with C-terminal binding proteins 1, 2 (CTBP1,2) to repress particular white-specific genes, such as resistin and angiotensinogen, by acting directly on their promoters (Kajimura et al. 2008). Nevertheless, since a specific deletion of PRDM16 in early myogenic progenitors using Myf5-Cre show normal embryonic BAT development, other regulators of brown adipogenesis must exist.

Within the PRDM16 pathway, the Rb family member p107 has recently been shown to be a downstream target (De Sousa et al. 2014). PRDM16 directly suppressed p107 transcription via promoter binding. Notably, the sustained expression of p107 blocked the ability of Prdm16 to induce brown fat genes. Nevertheless, it is not clear how p107 functions at molecular level.

Another key regulator of brown adipogenesis is PPARγ co-activator 1α (PGC1α), which was identified in a screen for proteins that bind PPARγ in brown fat and highly induced in response to cold exposure (Puigserver et al. 1998). Although PGC1α is regarded a master regulator that orchestrates thermogenic gene expression and mitochondrial biogenesis in mature brown adipocytes in response to cold/β-adrenergic agonists, as mentioned previously, it is not required for brown fat cell differentiation per se (Uldry et al. 2006). Nevertheless, in gain-of-function analysis, forced expression of PGC-1α in white adipocytes induces mitochondrial biogenesis and expression of UCP1, demonstrating PGC-1α can promote brown adipogenesis (Tiraby et al. 2003). In fact, many factors known to be involved in brown adipocyte differentiation are working at least partially through impacting PGC1α expression and/or activity (see below).

In addition to these key factors listed above, not surprisingly, factors that modulate either gene expression or activity of these key factors have also been found to be capable of modulating brown fat development. For example, Blnc1 (brown fat lncRNA 1) is a BAT-enriched long noncoding RNA, which promotes brown adipocyte differentiation through forming a ribonucleoprotein complex with EBF2 (Zhao et al. 2014). Groucho family member TLE3 is a white fat–selective PPARγ cofactor that antagonizes the function of PRDM16 by blocking the interaction of PRDM16 with PPARγ and thus suppresses brown fat differentiation (Villanueva et al. 2013). Several microRNAs such as *miR-133* and *miR-27* also negatively regulate brown fat development through targeting PRDM16 (Trajkovski et al. 2012; Sun and Trajkovski

2014). Plac8 is an upstream activator of C/EBPβ transcription and induces brown fat differentiation (Jimenez-Preitner et al. 2011). In contrast, miR-155 serves as a negative regulator for brown fat differentiation via direct targeting of C/EBPβ (Chen et al. 2013). PPARγ levels are also regulated by epigenetic regulation. PAX transactivation domain-interacting protein (PTIP; also known as PAXIP1), a histone methylation regulator, increases the activating histone mark H3K4me3 at the PPARγ promoters (Aguilo et al. 2010). Deletion of PTIP inhibits differentiation of both white and brown preadipocytes. Nevertheless, PTIP-null mice have defects in BAT formation but normal WAT development (Cho et al. 2009). Examples of regulators controlling brown fat development through modulation of either gene expression or activity of PGC-1α include SRC2/TIF2/GRIP1, a member of the steroid receptor coactivator (SRC) family that represses PGC-1α transcriptional activity (Picard et al. 2002). SRC2 deletion leads to increases in adaptive thermogenesis and energy expenditure in vivo. The pocket protein family member, retinoblastoma (Rb) protein negatively regulates PGC-1α gene expression. Adipocytes derived from Rb-deficient fibroblasts have high mitochondrial content and elevated expression of UCP1, PGC-1α, and mitochondrial genes. Rb deletion results in the expansion of interscapular BAT in vivo (Calo et al. 2010). Lastly, TWIST1 is also a negative regulator of PGC-1α function in brown fat. Heterozygous TWIST1 KO mice exhibit an induction of brown fat–selective genes, whereas transgenic mice overexpressing TWIST1 repress these genes in a PGC-1α-dependent manner (Pan et al. 2009). Phosphatase and tensin homolog (PTEN) positively regulates a BAT-selective thermogenic program by blocking the phosphatidylinositol 3-kinase type I (PI3K) pathway, which also seems to operate through Pgc1α. PTEN counteracts the activity of PI3K, a major kinase targeted by signaling downstream of insulin, insulin-like growth factors, and other molecules generally involved in cellular growth, metabolism, survival, and proliferation. Activation of PI3K is followed by the activation of Akt, which in turn triggers a complex cascade of events that leads to inhibition of Foxo transcription factors Foxo1 and Pgc1α. In contrast, blocking of the PI3K pathway leads to upregulation of Pgc1α. Consistently, pharmacological PI3K inhibitors increase BAT thermogenesis and whole-body energy expenditure (Ortega-Molina et al. 2012). Lastly, heat shock factor 1 (HSF1) regulates energy expenditure through activation of a PGC1α-dependent metabolic program. HSF1 occupies the heat shock element present in the PGC1α promoter. HSF1 ablation in mice downregulates mitochondrial and brown fat gene programs in brown and inguinal fat tissues and impairs thermogenic function and energy expenditure. Pharmacological activation of HSF1 via celastrol has the opposite effect (Ma et al. 2015).

Similar to the commitment phase, chromatin dynamics states and epigenetic enzymes are also involved during differentiation. For example, BAT-selective genes such as Ucp1, Cidea and Prdm16 are selectively marked by histone H3 lysine 27 trimethylation (H3K27me3) in brown preadipocytes compared to mature adipocytes; whereas common fat genes such as Fabp4, Adiponectin and Pparg are devoid of this mark. H3K27me3 features silenced promoters (Pan et al., 2015). Notably, during differentiation the histone demethylase JMJD3 catalyzes H3K27me3 removal, which is required for BAT-selective genes expression but not for adipogen-

esis per se, indicating fundamental roles of epigenetic marks and enzymes in specifying diverse gene expression programs during brown differentiation. In addition, the epigenetic enzyme lysine-specific demethylase 1 (LSD1) has been recently identified as a new and important player in the regulation of brown adipogenesis. BAT-selective LSD1 ablation induces a downregulation of BAT-specific and upregulation of WAT-selective gene expression. While the effect of LSD1 ablation on brown adipocyte whitening is undisputed, the exact mechanism of how LSD1 functions is not yet fully resolved. On one hand, it has been reported that LSD1 demethylates H3K4 at genomic loci near WAT-specific genes by interacting with PRDM16 (Zeng et al. 2016) and interacts with ZFP516 to activate BAT-specific genes by demethylation of H3K9 in brown adipocytes (Sambeat et al. 2016). On the other hand, Duteil et al. (2016) report that there are no direct interactions between LSD1 and PRDM16, neither between LSD1 and ZFP521. Instead the authors demonstrate that LSD1 activates BAT-selective gene expression in concert with the transcription factor NRF1 and represses WAT-selective genes through recruitment of the CoREST complex. The reasons for these discrepancies are unknown. Nevertheless, LSD1 apparently is a central hub that is capable of interacting with various transcription factors and orchestrating histone demethylation during brown adipogenesis.

4.7.3 Brown Fat Activation

Since UCP1 is inherently not leaky without stimulation, mature brown adipocytes without stimulation are not thermogenic (Li et al. 2014a; Shabalina et al. 2010). Activation of lipolysis by adrenergic stimulation represents the canonical pathway to stimulate thermogenesis in mature brown adipocytes. In addition to the activation of UCP1, it is worth noting that adrenergic stimulation also induces the thermogenic gene expression program. This catecholamine-linked activation of gene expression occurring in mature brown fat cells seems to be largely independent from molecular mechanisms responsible for the embryonic development of brown adipocytes early in life. For example, there is little evidence that the brown fat–specific differentiation-linked factors like PRDM16, EBF2, and EWS/YBX1 are directly involved in the β-agonist–induced activated gene program. Instead, PPARγ coactivator-1α (PGC-1α) is now appreciated to be the key factor that orchestrates thermogenic gene expression in response to cold/*β*-adrenergic agonists (Puigserver et al. 1998). Loss of PGC-1α does not affect brown adipocytes differentiation but reduces the capacity for cold-induced thermogenesis in vivo and the response to cAMP signaling in cultured brown fat cells (Uldry et al. 2006). Upstream of PGC-1α, it has been demonstrated that PGC-1α is phosphorylated and directly activated by p38 mitogen-activated protein kinase following *β*-adrenergic stimulation of brown fat cells (Cao et al. 2004); while downstream of PGC-1α, interferon regulatory factor 4 (IRF4) has been shown recently to interact with PGC1α to mediate thermogenesis. In the absence of IRF4, PGC-1α is not able to activate thermogenic genes, suggesting that IRF4 recruits PGC-1α to thermogenic genes (Kong et al. 2014).

Zinc-finger protein 516 (ZFP516) is an exception. It has been demonstrated to play critical roles in both the differentiation and the activation of brown fat cells (Dempersmier et al. 2015). As described previously, ZFP516 is a cold-inducible transcriptional factor that binds directly to the proximal region of the Ucp1 promoter, indicating an involvement in the acute induction of thermogenic genes. Nevertheless, since ablation of ZFP516 is embryonic lethal, mature adipocyte specific knockout of this factor will be essential to differentiate functional roles during development, recruitment and activation.

Recent studies revealed that the mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) is essential for brown fat activation (Labbe et al., 2016; Liu et al., 2016). β-adrenergic stimulation and cold exposure activate mTORC1 signaling in BAT (Labbe et al., 2016; Liu et al., 2016). Adipose-specific loss of Raptor, an essential component of mTORC1, completely blocks cold-induced BAT thermogenic gene expression, reduces mitochondrial biogenesis and severely impairs BAT thermogenesis (Labbe et al., 2016). These effects are recapitulated by pharmacological inhibition of mTORC1 with rapamycin. These results clearly establish mTORC1 as an important regulator of BAT activation, nevertheless the overall translational program is poorly defined. Interestingly, the mTORC2 signaling axis is also activated in brown adipocytes upon β-adrenergic stimulation and cold exposure, which has a role in adrenergically induced glucose uptake (described in Sect.4.5.2). Therefore, these mTOR complexes may play complementary roles to insure optimal heat production: on the one hand, mTORC2 promotes glucose uptake, an event required to support lipogenesis and thermogenesis; on the other hand, mTORC1 promotes BAT thermogenic gene expression, mitochondrial biogenesis and thermogenesis, which are all needed to sustain heat production (Labbe et al., 2016).

It is possible to directly manipulate the adrenergic signaling pathway to sensitize adipocytes to adrenergic stimulation. For example, secreted protein bone morphogenetic protein-8b (BMP8b), which is induced by nutritional and thermogenic factors in mature BAT, acts directly on BAT to enhance the response to adrenergic stimulation. Furthermore, it also acts within discrete nuclei of the hypothalamus to modulate the sympathetic outflow and activation of BAT (Whittle et al. 2012a). The transcription factor FOXC2 sensitizes adipocytes to adrenergic stimulation by modulating the expression and activity of adrenergic signaling molecules (Cederberg et al. 2001). Apoptosis signal-regulating kinase 1 (ASK1) links the PKA-p38 MAPK axis. Loss of ASK1 in brown adipocytes leads to downregulation of thermogenic genes and repression of cellular thermogenesis when treated with βAR agonists (Hattori et al. 2016). USF1 (upstream stimulatory factor 1) is a transcription factor associated with familial combined hyperlipidemia and coronary artery disease in humans. A direct effect of USF1 on BAT activation was demonstrated by an amplified adrenergic response in brown adipocytes after Usf1 silencing, and by augmented norepinephrine-induced thermogenesis in mice lacking USF1. Notably, Mice lacking Usf1 displayed increased BAT-facilitated, diet-induced thermogenesis with up-regulation of mitochondrial respiratory chain complexes, as well as increased BAT activity even at thermoneutrality and after BAT sympathectomy

(Laurila et al. 2016). A role for the H3K9-specific histone demethylase 2a (Jhdm2a) in regulation of brown fat activation has been shown in mice (Tateishi et al. 2009). Loss of Jhdm2a function results in obesity and hyperlipidemia and defective adaptive thermogenesis in mice. Jhdm2a expression is induced by *β*-adrenergic stimulation. Upon induction, Jhdm2a binds to the PPAR responsive element (PPRE) of the Ucp1 gene and reduces H3K9me2 (dimethylation of lysine 9 of histone H3) at this response element, thereby facilitating the recruitment of Pparγ and RXRα and their co-activators Pgc1α, CBP/p300 and Src1 (Ncoa1) to the PPRE of Ucp1. This results in an increased lipolysis and thermogenesis in brown fat.

Similarly, H3K9 demethylase JMJD1A has recently also been identified as a cAMP-sensing epigenetic determinant that couples DNA long-range looping and transcription of thermogenic genes (Abe et al., 2015). Activated PKA phosphorylates JMJD1A. Phosphorylated JMJD1A forms a transcriptional complex with the SWI/SNF and PPARγ. The phosphorylated JMJD1A–SWI/SNF–PPARγ complex induces enhancer–promoter proximity through forming a three-dimensional long-range chromatin loop and activates the transcription of thermogenic genes (Abe et al., 2015).

In addition to the canonical adrenergic pathway, several alternate pathways or such as ANP mediated cGMP-PKG pathway (Bordicchia et al. 2012) factors capable of activating BAT thermogenesis have been described (Fig. 4.7). such as bile acids (Broeders et al., 2015), adenosine (Gnad et al. 2014) and slit2 (Svensson et al., 2016) Most of these new activators work through the PKA-cAMP pathway. It should be noted that there are around 230 G-protein-coupled-receptors (GPCRs) expressed in mature brown adipocytes (Klepac et al. 2016). It is conceivable that more novel brown fat activators will be identified soon.

Apart from intracellular factors, external stimuli have been identified that either promote the commitment of a precursor to the brown adipocyte lineage or induce terminal differentiation of a committed brown preadipocyte. An endogenous factor of paramount importance in this context is certainly norepinephrine. This sympathetic neurotransmitter not only represents a potent activator of non-shivering thermogenesis but also promotes the proliferation of cells in BAT. A recent study indicates that *in vivo*, norepinephrine interacts with the β1-adrenoreceptor causing proliferation of precursor cells (Lee et al. 2014). This mechanism likely translates the effect of cold-exposure on *de novo* brown adipogenesis from PDGFRα-positive progenitors and localizes the dorsal rim of interscapular BAT in adult mice as depot-specific niche with high proliferative potential. Physiological expansion of BAT requires concomitant angiogenesis to attend to the metabolic demands of new adipocytes. This neo-vascularization appears to be regulated by the brown (pre)adipocyte itself as this cell abundantly expresses the vascular endothelial growth factor A (VEGF-A). This protein acts in bi-functional manner (Mahdaviani et al. 2016; Bagchi et al. 2013; Sun et al. 2014): it mediates the survival and proliferation of brown preadipocytes and promotes angiogenesis in BAT. The net result is an increase in BAT mass and an enhanced transport of nutrients and oxygen within the tissue to supplement the thermogenic machinery, consequently affecting heat production in cold-exposed mice.

Among the class of non-adrenergic effectors, several members of the transforming growth factor β (TGFβ) superfamily with adipogenic potential have been identified. As discussed above, BMP7 is one of them, whose cellular actions cumulate *in vivo* in an essential function for embryonic BAT development (Tseng et al. 2008). Recently, BMP6 has been described as a further molecule of this family with high potential to determine the brown adipocyte identity of a myogenic precursor (Sharma et al. 2014). Exposure of C2C12 myoblasts to BMP6 prior to the hormonal induction results in adipogenic differentiation essentially characterized by the induction and repression of thermogenic and myogenic genes, respectively, and a pronounced effect on basal, maximal and fatty acid-induced cellular respiration. Interestingly, these effects appear to depend on optineurin and cyclooxygenase-2 but not PRDM16 expression, of which the latter has been shown to drive the brown adipocyte lineage commitment in C2C12 cells (Seale et al. 2008). In the same study, the adipogenic potential of BMP6 is higher than that of BMP7, whereas it is completely absent for BMP8 (Sharma et al. 2014). In line with the latter observation, the BAT-enriched protein BMP8B primes the ability of BAT to thoroughly respond to thermogenic stimulation *in vivo* rather than affecting the maturation of functional brown adipocytes (Whittle et al. 2012b). Another member of the TGFβ superfamily, growth differentiation factor 5 (GDF5), stimulates UCP1 expression during the differentiation of brown preadipocytes and BAT-derived stromal vascular cells (Hinoi et al. 2014c). GDF5 signaling in committed preadipocytes is likely initiated via the Alk2/3 receptor and transduced via PI3K, Akt and Smad5 (Hinoi et al. 2014a, c). In fact, expression of GDF5 is upregulated in BAT of HFD-fed and ob/ob mice as well as in cultured brown preadipocytes in response to inflammatory cytokines and saturated fatty acids (Hinoi et al. 2014b, c). Autocrine and/or paracrine, GDF5-mediated stimulation of brown adipogenesis may thus be relevant *in vivo* to modulate energy expenditure during obesity, representing a state of increased lipid turnover and chronic low-grade inflammation. Besides these positive effector molecules, myostatin, another member of the TGFβ superfamily, represents a negative regulator of brown adipogenic differentiation (Kim et al. 2012). In line with this, ablation of myostatin and inhibition of the myostatin/activin receptor IIB pathway has been shown to promote brown adipogenesis *in vitro* and *in vivo* (Fournier et al. 2012; Braga et al. 2013). This molecular function may be exerted by the secreted protein follistatin under physiological conditions. Exogenous follistatin is capable of enhancing thermogenic protein expression during the differentiation of immortalized brown preadipocytes and mouse embryonic fibroblasts, a phenotype that is ablated in cells derived from follistatin null embryos (Braga et al. 2014). Given its function as myostatin binding protein, follistatin is hypothesized to antagonize components of the myostatin/TGFβ signaling pathways consequently affecting brown adipogenic differentiation (Singh et al. 2014). As follistatin knockout pups are not viable and follistatin is a cold-regulated protein of BAT (Braga et al. 2014), such mechanism may be relevant during embryonic BAT development and/or to adjust the capacity for non-shivering thermogenesis at later stages of life, respectively.

An endocrine aspect in the control of brown adipogenesis has recently emerged by the identification of chemerin, apelin and orexin as novel modulators of this process. Chemerin, a novel adipokine, has been shown to induce adipogenic gene expression and Ucp1 when administered to differentiating C2C12 cells (Li et al. 2015). Concomitantly, myogenic protein expression is repressed, indicating this hormone to favor the development of a myoblast towards a thermogenic adipocyte. The information conveyed by this mechanism and thus the physiological relevance, however, remains to be assessed. Another member of the adipokine family, apelin, may not only initiate brown adipocyte differentiation via an endocrine mechanism, but also enhance terminal, thermogenic maturation in an autocrine/paracrine feedback loop while differentiation progresses (Than et al. 2015). As expression of apelin is increased in white adipose tissue and plasma during obesity, this mechanism may attenuate inflammation-induced impairment of brown adipogenesis under this pathological condition. Another endocrine effector with a crucial role in embryonic BAT development was recently identified. Orexin is a hypothalamic neuropeptide capable of affecting the cellular fate of several cell lines (C3H10T1/2, mouse embryonic fibroblasts, primary mouse brown adipocytes, HIB1b) towards brown adipogenesis when added during the proliferative phase (Sellayah et al. 2011). This transition of a stem cell towards a brown fat cell involves orexin receptor 1-mediated activation of PLC and p38MAPK as well as a downstream-crosstalk with the BMP receptor 1a/Smad pathway (Sellayah et al. 2011; Sellayah and Sikder 2013). *In vivo*, orexin and orexin receptor 1 null mice exhibit severely blunted brown adipocyte lipid droplet incorporation in neonate and adult state along with reduced UCP1, impaired heat production and the inability to properly defend core body temperature at 4 °C (Sellayah et al. 2011; Sellayah and Sikder 2012). The development of functional BAT may be triggered at the embryonic stage by placental orexin production via direct, local interaction with mesenchymal target cells (Sellayah et al. 2011). Moreover, orexin administration attenuates age-induced dysfunction of BAT in mice thus implying a therapeutic potential (Sellayah and Sikder 2014). In addition to these endocrine peptides, acetate, a short-chain fatty acid derived from the colonic metabolization of nutrients by the intestinal microbiota, is capable of affecting the maturation of differentiating, immortalized brown fat cells (Hu et al. 2016). In doing so, acetate binds the G-protein-coupled-receptor 43 subsequently stimulating mitochondrial biogenesis and Ucp1 expression, resulting in an increase in maximal, uncoupled respiration. The physiological relevance remains, however, questionable as there is a major discrepancy between circulating acetate levels *in vivo* (150 μM) and the concentration used to induce *in vitro* effects (10 mM).

Besides these endogenous factors, several herbal agents with brown adipogenic potential were recently identified. These include extracts from *Platycodon grandiflorum* (also known as the common balloon flower; PGE), Rubi fructus (an Asian type of red raspberry; RFE) as well as mulberries (ME) and mulberry wine (MWE). Among them, PGE and RFE extract were shown to affect mitochondrial abundance, PGC1α and Ucp1 expression in a dose-dependent manner when added to primary brown adipocytes during the differentiation phase (Kim et al. 2015; Jeong et al. 2014). Similarly, thermogenic gene expression and mitochondrial genome copy

number are upregulated during the adipogenic differentiation of C3H10T1/2 cells in the presence of ME and MWE (You et al. 2015). All of these extracts exert anti-obesity effects hypothesized to originate at least in part from an increase in energy expenditure due to an effect on brown adipocyte differentiation.

Recent evidence demonstrates that brown adipocytes are not the only type of thermogenic fat cells found in mammalian species (Li et al. 2014a). In fact, inducible brown fat cells termed "brite" (brown-in-white) (Petrovic et al. 2010) or "beige" (Ishibashi and Seale 2010) are occasionally present in white adipose tissues. Similar to brown adipocytes in BAT, their abundance can be increased by adrenergic stimulation (Galmozzi et al. 2014) and cold exposure (Maurer et al. 2015). It is therefore not surprising that some of the aforementioned effectors of brown adipogenesis (BMP7, GDF5, apelin, VEGF-A, Acetate) are, besides a considerable number of other effectors, as well capable of influencing brite adipogenesis (Elsen et al. 2014; Schulz et al. 2011; Boon et al. 2013; Okla et al. 2014; Hinoi et al. 2014c; Than et al. 2015; Sun et al. 2012; Sahuri-Arisoylu et al. 2016; During et al. 2015). As brite adipocytes are a promising target to combat the current obesity pandemic and its associated disorders, the induction of "WAT browning" and its therapeutic potential are currently under intense investigation (for a recent review, see (Giordano et al. 2016)).

4.8 Emergence of Brown Adipose Tissue in Mammals

Beyond the ontogenic origin of brown adipocytes their evolutionary history has been subject of investigation. Based on the study of a small subset of investigated species it was often stated that brown adipose tissue can be found in all Eutherian mammals (>5000 species) and is indeed a monophyletic trait of this vertebrate subclass. This view is supported by the identification of brown adipose tissue in the rock elephant shrew (*Elephantulus myurus*) and the lesser hedgehog tenrec (*Echinops telfairi*), both species belonging to the Afrotheria, a group of mammals that evolved exclusively in Africa and is thought to be at the base of the Eutherian radiation (Mzilikazi et al. 2007; Oelkrug et al. 2013). The observation of brown adipose tissue in the tenrec is of particular interest, as this species preferably spends most of the time in an ectothermic-like mode of thermoregulation, but exhibits periodic bouts of endogenous heat production during the nocturnal phase (Lovegrove and Genin 2008). These phases of endothermy are extended during the breeding season (Poppitt et al. 1994). In the tenrec, unlike other mammalian species, 60–70% of total brown adipose tissue mass was found intra-abdominally in proximity to the reproductive organs (Oelkrug et al. 2013). It was therefore suggested that heat production by brown adipose tissue is of particular importance for reproduction in the tenrec. In mammalian evolution this may have increased reproductive fitness even before the establishment of sustained endothermy (Oelkrug et al. 2015).

The actual origin of brown adipose tissue may date back to the common ancestors of all mammals (Theria) as UCP1 expression is induced in response to cold

exposure in the interscapular adipose tissue depot of a small Australian marsupial, the fat-tailed dunnart *Sminthopsis crassicaudata* (Jastroch et al. 2008). A first study, however, failed to identify recruitment of UCP1-mediated capacity for nonshivering thermogenic in response to cold acclimation in the fat-tailed dunnart (Polymeropoulos et al. 2012). Further physiological studies are needed to verify the capacity and the mechanisms for adaptive thermogenesis in evolutionary ancient mammalian species.

In the subset of species in which brown adipose tissue was studied some anatomical and functional differences have been identified. The relative mass of brown adipose tissue decreases with increasing body mass which is related to the decreased need for thermoregulatory heat production with increasing body mass. Larger mammals rather rely on improved insulation. The relative amount of brown adipose tissue in relation to body mass also varies between species. Comparing 16 non-hibernators and 8 hibernators in a body mass range of <10 g—5 kg it was observed that hibernators have about twice the amount of brown adipose tissue (Heldmaier 1971) which most likely represents a genetic adaptation to the increased need for non-shivering thermogenesis in periodic arousals during the hibernation season. Pertaining to the anatomical distribution of brown adipose tissue the interscapular depot typically found in rodents is regularly found only in neonate and juvenile humans but is lost during adolescence (Drubach et al. 2011; Heaton 1972).

The recruitment of thermogenic capacity in response to cold acclimation is a hallmark of brown adipose tissue in rodents. The mechanisms involved in recruitment, however, differ even between closely related species. In mice and rats the capacity is increased by hyperplasia as described above. In contrast in the Djungarian hamster only a small increase in the cellularity of brown adipose tissue occurs (Klingenspor et al. 1996). In fact, brown adipose tissue wet weight in this species decreases during cold acclimation due to a reduction in the cellular lipid content. In spite of decreased brown adipose tissue mass cold acclimated Djungarian hamsters in winter develop a maximal non-shivering thermogenesis capacity of 1600 mW as compared to 1000 mW in warm acclimated hamsters in summer (Heldmaier et al. 1990). This is accomplished by a nearly three-fold increase of mitochondrial protein content in brown adipose tissue when expressed on a per animal basis (Rafael et al. 1985).

In the light of the recent discovery of metabolically active brown adipose tissue in healthy adult humans (see Sect. 4.8.2) interspecific differences in the recruitment mechanisms should be kept in mind.

4.8.1 The Invention of Uncoupling Protein 1

The previous assumption that UCP1 has emerged ~150 million years ago with the evolution of eutherian mammals, has been disproved. Based on molecular phylogeny and conserved synteny orthologs of mammalian UCP1 were found in fish, amphibians and non-eutherian mammals (Fig. 4.8) (Jastroch et al. 2005, 2008). Not

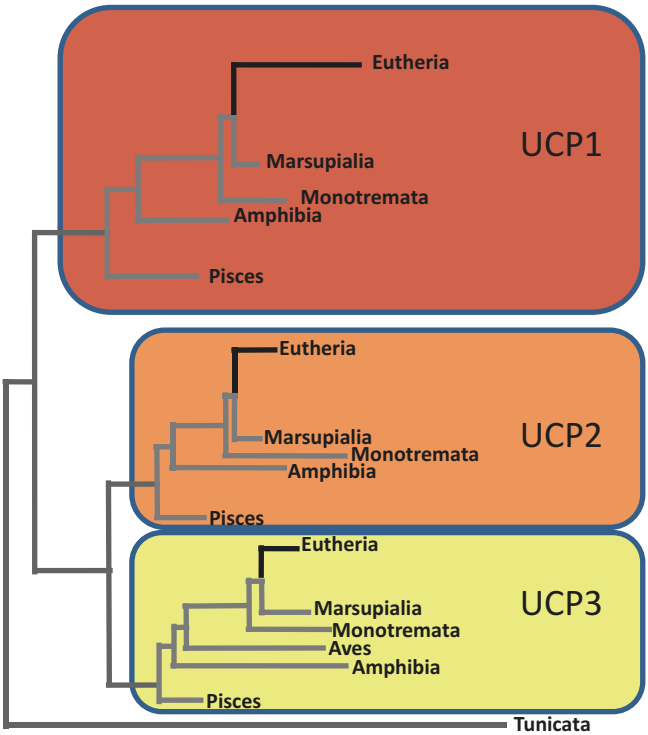

Fig. 4.8 Schematic representation of a UCP species tree—The molecular phylogeny of the UCP family was analysed using 79 UCP sequences from vertebrates. Branch lengths represent the number of substitutions; the Eutherian branches are highlighted in black (for details see Hughes et al. 2009)

only UCP1, but also the paralogs UCP2 and UCP3 have already been present 420 million years ago in the common ancestors of ray-finned and lobe-finned vertebrates. In contrast to mammalian UCP1 in brown adipose tissue, fish UCP1 is mainly expressed in the liver and downregulated in response to cold exposure. GDP-sensitive uncoupled respiration in the presence of palmitate was found in liver mitochondria isolated from warm acclimated fish, but absent after cold acclimation (Jastroch et al. 2007). Thus, the biochemical properties of fish UCP1 may resemble mammalian UCP1. Regarding the molecular phylogeny of UCP1 in vertebrates a striking observation was made. The branch length between UCP1 in marsupials and eutherian mammals is more than twice the length between marsupials and amphibians (Fig. 4.8) (Jastroch et al. 2008). In evolutionary time, marsupials are much closer related to eutherians than to amphibians. This demonstrates that UCP1 underwent an accelerated rate of sequence changes during the evolution of eutherian mammals which is most likely explained by relaxed constraints (Hughes et al. 2009), not positive selection (Saito et al. 2008). A possible evolutionary scenario assumes that all three UCPs initially had a common physiological function.

This functional redundancy as well as the ubiquitous expression of Ucp2 enabled relaxed constraints for the evolution of UCP1. At some point in time the UCP1 gene gained exclusive expression in mammalian adipocytes of the multilocular type. In this specialized cell type the original function of UCP1 was no longer essential and allowed for structural and functional changes (Hughes et al. 2009). It is of interest to identify the functional residues critical for uncoupling activity and fatty acid induced activation of UCP1 (Klingenspor 2003; Klingenspor et al. 2008; Rial and Zardoya 2009). Notably, the UCP1 gene is non-functional in several breeds of domestic pigs and in wild boars, clearly demonstrating a loss of brown adipose tissue function in some Eutherian species (Berg et al. 2006).

Recently, the loss of Ucp1 gene function was identified by analysis of genomes in the public databases for several other mammalian species, including dolphins, orcas, armadillos, sloths, and hyraxes (McGaugh and Schwartz, 2017).

4.8.2 Brown Adipose Tissue in Humans

In a review published in 2007 the unexpected presence of brown adipose tissue in adult humans was brought to the broader attention of physiologists and physicians interested in thermoregulation and energy balance (Nedergaard et al. 2007). The key observations had initially been made by radiologists applying fluordeoxyglucose positron emission tomography (FDG PET) combined with computerized tomography (CT) in tumor diagnosis. Using this metabolic imaging technique, regions with high glucose uptake identified in the upper part of the body were found to be due to adipose tissue rather than musculature (Hany et al. 2002). These metabolically active adipose tissues can be visualized by FDG PET in different anatomical regions, namely the neck and supraclavicular, para-aortic, paravertebral and suprarenal regions and were reported to cause false-positive results in tumor diagnosis (Fig. 4.9). Less successful attempts to reduce the intensity of these adipose FDG PET signals, including the pretreament of patients with benzodiazepines and other sedating drugs, had been made until two treatments were found to be efficient. Prior to FDG infusion patients were either subjected to a core warming maneuver (Christensen et al. 2006) or treated with the β-adrenoceptor antagonist propranolol (Soderlund et al. 2007). Both treatments diminished the adipose FDG PET signals. These two independent observations strongly supported the view that adipose FDG PET signals were due to metabolically active brown adipose tissue.

From what we learned in animal studies brown adipose tissue thermogenesis is turned on in the cold by increased sympathetic nerve activity which increases glucose uptake into brown adipocytes (Cannon and Nedergaard 2004). The observed inhibition of glucose uptake into brown adipose tissue in the warmth and in response to propranolol is therefore exactly what we would anticipate in brown adipose tissue. This motivated several labs worldwide to conduct follow up studies which clearly confirmed the presence of brown adipose tissue in adult humans.

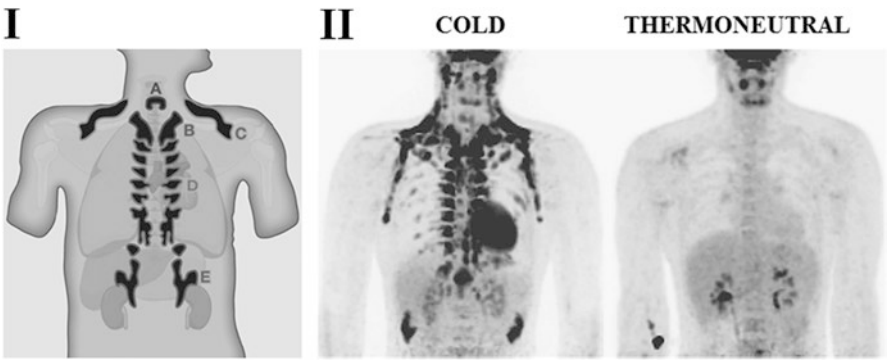

Fig. 4.9 Brown adipose tissue in humans—**I**: In humans, brown adipose tissue depots are found in the tracheal (*A*), mediastinal (*B*), supraclavicular (*C*), paravertebral (*D*) and supra-/perirenal (*E*) areas (depicted from Enerback 2010). **II**: In a comparison of FDG PET scans taken after cold exposure and in thermoneutral conditions the localization of active brown adipose tissue is evident (depicted from van Marken Lichtenbelt et al. 2009)

Some of these studies performed FDG PET scans on a small number of healthy adult volunteers (Saito et al. 2009; van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009) whereas others analysed a large number of clinical scans which had been performed on patients with different indications (Cypess et al. 2009; Au-Yong et al. 2009; Gerngroß et al. 2017). All studies identified substantial depots of brown adipose tissue in the cervical and thoracal region. The retrospective analyses of clinical scans reported rather low prevalence of FDG visualization in brown adipose tissue with more positive scans in females (~7%) than in males (~3%) (Cypess et al. 2009; Au-Yong et al. 2009; Gerngroß et al. 2017). Notably, the prevalence of brown adipose tissue positive patients was largely altered by season. The percentage of brown adipose tissue positive scans increased four-fold from summer to winter, and also the number of FDG positive depots increased in the winter season (Au-Yong et al. 2009; Gerngroß et al. 2017). Naturally these scans were all performed under routine conditions in the hospital with no experimental control of ambient temperature before and during the scan. In contrast the experimental studies investigating healthy adult volunteers repeated FDG PET scans under warm and cold conditions. In the warm condition the detection of brown adipose tissue was negligible whereas in the cold condition subjects showed FDG uptake in several brown adipose tissue depots (Virtanen et al. 2009; Saito et al. 2009; van Marken Lichtenbelt et al. 2009). Cold induced FDG uptake in brown adipose tissue was detected in 16 out of 31, 23 out of 24 and 19 out of 27 subjects, respectively (Saito et al. 2009; van Marken Lichtenbelt et al. 2009). These results demonstrate cold induced glucose uptake in brown adipose tissue and suggest that the prevalence of brown adipose tissue is much higher than estimated from clinical scans.

Despite the presence of brown adipose tissue, the activity of the tissue under routine clinical conditions is mostly low. This view is supported by the reanalysis of a set of FDG PET scans which demonstrated a poor reproducibility of brown

adipose tissue detection in repeated scans of the same individuals (Lee et al. 2010; Gerngroß et al. 2017). Only 13% of the patients which had been positive for brown adipose tissue in their first scan were also positive in the second. Correcting for this high rate of false negative observations the prevalence of brown adipose tissue was estimated at 64% which is in line with the reported high prevalence in healthy adult subjects (Saito et al. 2009; van Marken Lichtenbelt et al. 2009). Clearly cold exposure stimulates human brown adipose tissue activity and notably, the seasonal increase in brown adipose tissue positive scans in winter is similarly associated with the change in photoperiod as with ambient temperature (Au-Yong et al. 2009; Gerngroß et al. 2017). Photoperiod may therefore represent an important environmental signal in the control of human brown adipose tissue activity. In seasonal rodents it is well known that a short winter-like photoperiod stimulates mitochondrial biogenesis and UCP1 expression to increase the thermogenic capacity of brown adipose tissue (Heldmaier and Klingenspor 2002), but it remains to be investigated by FDG PET or other means whether brown adipose tissue activity in vivo is also increased in this winter-acclimatized state.

The conclusion that FDG positive adipose tissue depots are indeed brown adipose tissue was confirmed by the analysis of biopsy specimen. FDG PET detection was combined with CT for precise anatomical localization of the depots and enabled sampling of biopsies for immunohistological inspection and Western blot analysis. The presence of UCP1 was confirmed in several studies (Saito et al. 2009; van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009; Cypess et al. 2009; Zingaretti et al. 2009).

Taken together the above studies found unexpected amounts of brown adipose tissue in healthy adult humans, which could be metabolically activated by acute cold exposure of the subjects. Furthermore, several studies observed a high prevalence in young adults which decreased with age and body mass index, and low fasting glucose levels were associated with the presence of brown adipose tissue. Obviously, the urging question is whether energy expenditure due to the metabolic activity of brown adipose tissue depots significantly alters energy balance regulation in humans. This is not a new question, and has been a matter of heated debates in the past. The presence and anatomical distribution of brown adipose tissue in humans was described decades ago (Heaton 1972) and had been suggested to lower the susceptibility to obesity in man (Himms-Hagen 1979; Rothwell and Stock 1979). Based on the heating power of brown fat in rodents (Table 4.1), Rothwell and Stock estimated that human brown fat could dissipate ~20% of the daily energy intake, thus accounting for at least 2000 kJ/day (Rothwell and Stock 1983). In the following several critical papers have dismissed this possibility (Astrup et al. 1984a; Cunningham et al. 1985). In the light of the present FDG PET findings, however, previous negative outcomes were mainly due to the underestimation of brown adipose tissue mass in adult humans. The new studies estimate brown adipose tissue mass to account for 0.05–0.1% of body mass in humans, thus in the range of 35–70 g in a 70 kg individual. The metabolic activity of this brown adipose tissue mass has been calculated based on a combination of different

methods. Taking into account that only 10% of the oxygen consumption in brown adipose tissue is fueled by glucose the estimated metabolic rate, as determined by ^{18}FDG-PET uptake rates (8 μmol/min), this could allow us to burn an excess of >320 kJ every day, roughly corresponding to 120 MJ/year, or 4 kg of adipose tissue per year. This amount of energy is less than ~3% of the annual energy budget of a 70 kg individual but in the long run may effectively partition excess food energy towards catabolism in brown adipose tissue and thereby prevent this energy to be stored as triglyceride in adipocytes. To achieve this anti-obesity effect human brown adipose tissue would have to continuously dissipate heat at a power of ~50 mW/g of tissue which is below the values reported for brown adipose tissue in rodents (Table 4.1). Based on such theoretical assumptions, it seems feasible to expect a significant contribution of brown adipose tissue to the defence of energy balance in humans, even more so since human brown fat mass may have been underestimated in the past (Gerngroß et al. 2017). Pertaining to the research efforts in the prevention of obesity and impaired glucose homeostasis it appears worthwhile to search for treatments effectively slowing down the loss of brown adipose tissue with age and also increasing the proportion of brown adipocytes in white adipose tissue.

The actual thermogenic activity of brown adipose tissue in humans remains to be quantified, which will be a technically challenging task. Although human brown adipose tissue may be relevant for long term energy balance regulation, a significant contribution to thermoregulatory heat production at ambient temperatures below the thermoneutral zone must be questioned. Using Kleiber's equation an individual with 70 kg body mass at thermoneutrality would dissipiate 82 W for basal metabolic rate (Kleiber 1967). In the cold (5 °C) heat production is increased by 3–4-fold BMR, thus corresponding to ~300 W (Scholander et al. 1958). Assuming the estimated heating capacity of 50 mW/g, the contribution of human brown adipose tissue to cold induced increment of heat production would be negligible (1.7%). Only if human brown adipose tissue could attain the maximal thermogenic power measured in brown adipose tissue of cold acclimated rats (480 mW/g) it could be of marginal significance. One important prerequisite to achieve such a high thermogenic activity is that the abundance of UCP1 in the mitochondria of human brown adipocytes should be of comparable high level as in rodents (5–8% of the mitochondrial protein). The presence of UCP1 in human brown adipose tissue depots has been demonstrated unequivocally but whether the abundance of the protein in the inner mitochondrial membrane is sufficient to support a high power of heat dissipation is not known, so far.

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Chapter 5
White Adipose Tissue

Stephane Gesta and C. Ronald Kahn

Abstract White adipose tissue (WAT) is one of the most abundant tissues in mammals, exhibiting numerous complex functions. The primary purpose of WAT is to store excess energy in the form of fat for future use by other cells of the organism during periods of energy deprivation. In order to do this, white adipocytes acquire the expression of specific enzymes during their differentiation, which enable both the accumulation and mobilization of fat. Fat accumulation is achieved by de novo synthesis of fatty acids (lipogenesis) as well as fatty acid uptake, while fat mobilization is accomplished during lipolysis. Both processes are regulated by various hormones including insulin and catecholamines. In addition, WAT secretes various factors, known as adipokines, which can act locally or distally on other tissues. These adipokines, which include leptin, adiponectin, RBP4, and others, are involved in the regulation of whole body energy homeostasis. In mammals, WAT is distributed throughout the body in two main depots, located subcutaneously and intra-abdominally. In obesity, intra-abdominal fat accumulation is strongly associated with the development of related diseases, including type 2 diabetes, while accumulation of subcutaneous fat exhibits no correlation. This phenomenon is the result of differences in anatomical location and developmental intrinsic properties of subcutaneous and intra-abdominal white adipose depots. In this chapter, we discuss how the developmental origins of fat may play a role in the heterogeneity of WAT distribution and function and the impact of fat distribution on obesity-related diseases.

Keywords White adipose tissue • Anatomy • Metabolism • Adipokines

5.1 Introduction

Every organism must have the ability to acquire and use energy to live. While simple organisms, like bacteria, acquire energy only in response to their immediate needs and are therefore highly dependent on the constant presence of energy sources in their ecosystem for survival, higher organisms have developed mechanisms to

S. Gesta • C.R. Kahn (✉)
Joslin Diabetes Center, Harvard Medical School, One Joslin Place, Boston, MA 02215, USA
e-mail: c.ronald.kahn@joslin.harvard.edu

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store excess energy which can be used as fuel when external energy sources are limited. For this, in virtually all animal species, from *Caenorhabditis elegans* to *Homo sapiens*, the major form of energy storage is fat. In most higher animal species, this is done in a specialized tissue – adipose tissue.

In mammals, adipose tissue exists in two forms, white adipose tissue (WAT) and brown adipose tissue (BAT), each performing different functions. The primary role of BAT is to store only small amounts of fat that can be used, when needed, to produce heat and maintain body temperature (Nicholls and Locke 1984). WAT, on the other hand, is designed to store large amounts of excess energy in the form of triglycerides for use during periods of food deprivation. This requires the process of lipogenesis as well as triglyceride uptake for accumulation of fat, and the mobilization of this energy for use by other cells of the organism through the process of lipolysis. In addition, WAT has an endocrine function which contributes to the regulation of whole body energy homeostasis through the secretion of various adipose-derived hormones or adipokines.

WAT is the most abundant tissue in mammals, and its bodily distribution varies greatly among species, as well as between individuals from the same species. Generally, WAT is considered to exist in two main depots: the subcutaneous adipose tissue located beneath the skin and the intra-abdominal adipose tissue, which is present surrounding the intestine, kidneys, and in rodents, the gonads. These depots harbor major differences in their properties and function. When excessive fat accumulation occurs in obesity, whether it is deposited in the subcutaneous or intra-abdominal depots has a very different impact on the development of obesity-related diseases.

5.2 Development of WAT: Adipocyte Differentiation

The major lipid storage cell in WAT is the white adipocyte which conducts the primary functions of WAT, e.g., lipid and glucose transport, fatty acid synthesis and mobilization, regulation of insulin sensitivity, and endocrine function. These cells are derived from undifferentiated preadipocytes, which undergo terminal differentiation through a complex process orchestrated by a transcriptional cascade involving the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) and members of the CCAAT/enhancer-binding protein (C/EBP) family (Farmer 2006). Over the past 3 decades, these transcriptional events have been extensively studied using 3T3-L1 and 3T3-F442A preadipocyte cell lines (Rosen and Spiegelman 2000; Rosen and MacDougald 2006). In these cultured preadipocytes, induction of adipocyte differentiation is under the control of hormonal stimuli including glucocorticoids, cyclic adenosine monophosphate (cAMP), and the insulin/IGF-1 pathways. In culture, this induction occurs during the first 2 days of differentiation and involves a sequential transciptional cascade beginning with a transient high expression of C/EBPβ and C/EBPδ, which in turn promotes the expression of the transcription factors involved in terminal adipocyte differentiation, C/EBPα and PPARγ. These two

latter transcription factors cooperate to induce terminal differentiation by increasing the expression of genes involved in the acquisition of adipocyte function, such as the glucose transporter (GLUT) 4, the fatty acid transporter aP2, the insulin receptor, and the enzymes involved in triglyceride synthesis (e.g., fatty acid synthase [FAS]) and lipolysis (e.g., hormone sensitive lipase [HSL]) (Rosen and Spiegelman 2000; Farmer 2006). A similar transcriptional cascade and pattern of differentiation is observed with brown preadipocytes in culture (Tseng et al. 2008).

In order to understand the relative importance of these transcription factors in controlling adipocyte differentiation, the role played by PPARγ and the C/EBPs has been carefully dissected using gain and loss of function studies both in vitro and in vivo. PPARγ plays a critical role in the control of adipogenesis and has been demonstrated to be necessary and sufficient for adipocyte differentiation. Indeed, forced expression of PPARγ is sufficient to induce adipocyte differentiation of non-adipogenic fibroblastic cells (Tontonoz et al. 1994b). Conversely, loss of function of PPARγ reduces or eliminates adipogenesis in vivo and in vitro (Barak et al. 1999; Rosen et al. 1999; Kubota et al. 1999). PPARγ also appears to be required for maintenance of the terminal differentiated state of adipocytes, and expression of a dominant negative PPARγ in differentiated 3T3-L1 cells induces dedifferentiation with loss of lipid accumulation and decreased expression of adipocytes markers (Tamori et al. 2002). Likewise, an inducible knockout of PPARγ in mature adipocytes in vivo leads to death of both brown and white adipocytes followed by generation of new adipocytes (Imai et al. 2004). However, mice with adipocyte-specific inactivation of the *Pparg* gene still develop some WAT, suggesting some mechanism of escape from this genetic manipulation (He et al. 2003).

There are two isoforms of PPARγ, PPARγ1 and PPARγ2, that are generated by alternative splicing and alternative promoter usage of the *Pparg* gene (Fajas et al. 1997; Tontonoz et al. 1994a). While both are expressed in the adipocyte, PPARγ2 is more specific to white and brown adipocytes and has been regarded as a specific marker of these cell types (Tontonoz et al. 1994a). However, mice with germline knockout of PPARγ2 still have some WAT, suggesting that PPARγ1 has the ability to compensate for many of the adipogenic functions of PPARγ2 (Zhang et al. 2004; Medina-Gomez et al. 2005). Interestingly, mice with PPARγ2 knockout develop whole body insulin resistance, suggesting a specific role for PPARγ2 in the control of insulin sensitivity, independent of its effects on adipogenesis (Medina-Gomez et al. 2005). Together, these studies have led to the now commonly used characterization of PPARγ as the "master regulator" of adipogenesis.

However, it is important to note that PPARγ expression during adipocyte differentiation is partly under the control of the C/EBP transcription factors. Indeed, the transient expression of C/EBPβ and C/EBPδ during early adipocyte differentiation has been shown to promote the expression of C/EBPα and PPARγ (Farmer 2006). Indeed, forced expression of C/EBPβ in 3T3-L1 cells can promote adipocyte differentiation even in the absence of the required hormonal inducers (Yeh et al. 1995). Overexpression of C/EBPδ, on the other hand, accelerates the process of differentiation after it is triggered by these agents (Yeh et al. 1995). Although expression of C/EBPβ and C/EBPδ appears earlier than PPARγ during the progression of adipocyte

differentiation, it seems that these two factors are not absolutely required for WAT development. Mice deficient in the *Cebpb* gene have a reduced WAT mass; however, mesenchymal embryonic fibroblasts (MEFs) derived from these mice are still able to differentiate into adipocytes in vitro, albeit with reduced efficiency (Tanaka et al. 1997). Furthermore, mice with deletion of both C/EBPβ and C/EBPδ still develop some WAT (Tanaka et al. 1997).

The transcription factor C/EBPα, like PPARγ, appears to be essential for adipocyte differentiation in vitro. MEFs derived from C/EBPα deficient mice lose their capacity to differentiate into adipocytes. Interestingly, although forced expression of PPARγ in these cells restores their adipogenic capacity, these cells present several defects in triglyceride storage and insulin-stimulated glucose transport capacitiy. In addition, while forced overexpression of PPARγ in *C/ebpa*$^{-/-}$ MEFs can restore adipocyte differentiation, forced expression of C/EBPα in *Pparg*$^{-/-}$ MEFs is unable to restore the adipocyte differentiation capacity of these cells, suggesting that PPARγ is a dominant factor controlling adipocyte differentiation. In vivo, C/EBPα has been shown to be essential for the development of only certain adipose depots. Although germline deletion of the *C/ebpa* gene is postnatal lethal due to the critical role played by C/EBPα in the control of gluconeogenesis in liver (Wang et al. 1995), re-expression of *C/ebpa* in the liver rescues these mice from death and these animals present with an absence of subcutaneous, perirenal, and epididymal WAT, but near normal WAT in the mammary gland (Linhart et al. 2001). Furthermore, in these mice, BAT is actually somewhat hypertrophied. These observations indicate a depot-specific importance played by C/EBPα in the development of adipose tissue, as well as intrinsic developmental differences which exist in the formation of the various adipose depots in mice.

5.3 Functions of WAT

5.3.1 Metabolic Function of WAT

One of the main characteristics of the energy metabolism in mammals is that energy utilization by cells is continuous, whereas energy intake is discontinuous. Therefore, to maintain energy balance and address the needs of all cells, the organism must be able to store and quickly mobilize excess energy sources. This requires two closely related energetic compartments. The first is a circulating compartment, i.e., the blood, which continuously provides energy to the cells. The second is the WAT, which constitutes a storage compartment constantly exchanging substrates with the circulating compartment. Only glucose and fatty acids, which are the two principal energy sources of the organism, can be stored in WAT in the form of triglycerides. For this, WAT can take up and transform the glucose into fatty acids, through the process of lipogenesis. Following this, intracellular glycerol is esterified with fatty acids derived from the circulation or lipogenesis, to form triglycerides (Fig. 5.1).

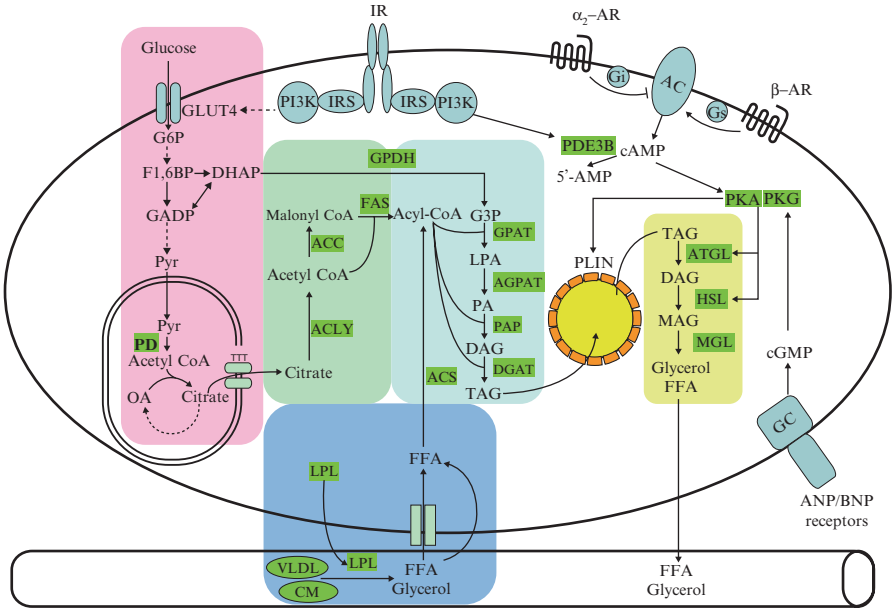

Fig. 5.1 Metabolic functions of WAT. The main metabolic functions of WAT are the storage of energy in the form of triglycerides and the mobilization of this energy when it is required by the body. In WAT, triglycerides can be synthesized (*turquoise box*) following the uptake and metabolism of glucose (*pink box*) by the process of de novo lipogenesis (*green box*) and/or after the uptake of free fatty acids from the circulation (*blue box*). The triglycerides stored in the adipocyte can be hydrolyzed by the process of lipolysis (*yellow box*), which delivers free fatty acids to the circulation. These processes are regulated by the insulin pathway, the adrenergic pathway and the atrial natriuetic hormone pathways. *α2-AR* α2-adrenergic receptor; *β-AR* β-adrenergic receptor; *5′-AMP 5′-adenosine monophosphate*; *AC* adenylate cyclase; *ACC* acetyl-CoA carboxylase; *ACLY* ATP citrate lyase; *ACS* acyl-CoA synthetase; *AGPAT* 1-acylglycerol-3-phosphate *O*-acyltransferase; *ANP* atrial natriuretic peptide; *ATGL* adipose triglyceride lipase; *BNP* brain natriuretic peptide; *cAMP* cyclic adenosine monophosphate; *cGMP* cyclic guanosine monophosphate; *CM* chylomicron; *DAG* diacylglycerol; *DGAT* diacylglycerol acyltransferase; *DHAP* dihydroxyacetone phosphate; *F1,6BP* fructose 1,6 bisphosphate; *FAS* fatty acid synthase; *FFA* free fatty acid; *G3P* glycerol-3-phosphate; *G6P* glucose 6 phosphate; *GADH* glyceraldehyde 3-phosphate; *GC* guanylate cyclase; *Gi* Gαi protein; *GLUT4* glucose transporter 4; *GPAT* glycerol 3-phosphate acyltransferase; *GPDH* glycerol-3-phosphate dehydrogenase; *Gs* Gαs protein; *HSL* hormone sensitive lipase; *IR* insulin receptor; *IRS* insulin receptor substrate; *LPA* lysophosphatidic acid; *LPL* lipoprotein lipase; *MAG* monoacylglycerol; *MGL* monoacylglycerol lipase; *OA* oxaloacetate; *PA* phosphatidic acid; *PAP* phosphatidic acid phosphatase; *PDE3B* phosphodiesterase 3B; *PI3K* phosphatidylinositol 3-kinase; *PKA* cAMP-dependent protein kinase; *PKG* cGMP-dependent protein kinase; *PLIN* perilipin; *Pyr* pyruvate; *PD* pyruvate dehydrogenase; *TAG* triacylglycerol; *TTT* tripartite tricarboxylate transporter; *VLDL* very low density lipoprotein

5.3.1.1 Adipose Tissue Lipogenesis

Lipogenesis ensures the de novo synthesis of fatty acids from glucose for storage. This occurs in WAT and liver. While lipogenesis in rodents is considered to generate an important amount of the triglycerides stored in WAT, lipogenesis only minimally contributes to the total body lipid storage in humans (Hellerstein

1999). De novo fatty acid synthesis requires the production of cytoplasmic acetyl-coenzyme A (CoA) from metabolism of glucose. For this, glucose enters the cells through specific GLUTs and is then metabolized to pyruvate via glycolysis. Under aerobic conditions, pyruvate enters the mitochondria and is transformed by pyruvate dehydrogenase into acetyl-CoA which then enters the tricarboxylic acid cycle to be condensed with oxaloacetate to form citrate. Citrate is then able to leave the mitochondria and enter the cytoplasm, through the mitochondrial tricarboxylate transporter (Kaplan et al. 1993). This cytoplasmic citrate is then broken down by citrate lyase to give cytoplasmic acetyl-CoA, which is the mainstay of de novo fatty acid synthesis.

Fatty acid synthesis is carried out by the sequential action of two cytosolic enzymatic systems: acetyl-CoA carboxylase (ACC), which mediates the formation of malonyl-CoA from acetyl-CoA, and the multi-enzyme complex referred to as FAS, which mediates elongation of malonyl-CoA to acyl-CoAs with various carbon chain lengths by the successive addition of acetyl-CoA molecules.

Key enzymes of de novo fatty acid synthesis can be controlled by hormones, especially insulin, or metabolites. Thus, glucose uptake in adipocytes is increased after insulin stimulation by its transfer across the plasma membrane by GLUT4 (James et al. 1988). This process is reduced by high intracellular levels of ATP (Begum et al. 1993). Pyruvate dehydrogenase is also activated by insulin through dephosphorylation of its alpha subunit (Macaulay and Jarett 1985), and can be inactivated when the ratio of ATP/ADP, NADH/NAD+ or acetyl-CoA/CoA are increased (Pettit et al. 1975). FAS and ACC gene expression have both been shown to be upregulated by insulin, but this regulation is dependent on the presence of glucose, as insulin alone has no effect on these genes. Thus, insulin indirectly increases the gene expression of FAS and ACC by stimulating glucose metabolism through the regulation of glucose transport (Foufelle et al. 1992). In addition, insulin activates ACC, via activation of protein phosphatases which dephosphorylate the enzyme (Witters et al. 1988).

Deletion of the insulin receptor in adipose tissue of mice (the fat insulin receptor knockout (FIRKO)) leads to a 90% decrease in insulin-stimulated glucose uptake and a corresponding decrease in insulin-stimulated incorporation of glucose into triglycerides, lactate, and carbon dioxide (Bluher et al. 2002). These mice have a ~50% reduction in WAT mass and are protected against diet-induced obesity. Unexpectedly, histological examination of FIRKO fat tissue also reveals that a small subset of adipocytes (~45%) are protected from excessive triglyceride load, whereas a second subset maintains normal triglyceride storage capacity, despite a 90% decrease in insulin-stimulated lipogenesis. This adipocyte knockout unveils intrinsic differences of adipocytes within a given WAT depot.

5.3.1.2 Fatty Acid Uptake

As noted above, de novo lipogenesis in adipose tissue makes only a minor contribution to total lipid storage in humans. In fact, in humans, most de novo lipogenesis and triglyceride synthesis occurs in the liver, after which triglycerides are

transported in the circulation by very low density lipoproteins (VLDL) to peripheral tissues including WAT. The main source of fat accumulation in human WAT, therefore, comes from the uptake of circulating triglycerides and fatty acids from VLDL produced in the liver and chylomicrons produced by absorption of fat in the small intestine. In order for fatty acids to be stored in the WAT, triglycerides from chylomicrons and VLDL must first be processed in the extracellular space by the enzyme lipoprotein lipase (LPL). This enzyme is produced by various tissues, including WATs and BATs, skeletal muscle, heart, mammary gland, brain, and macrophages (Camps et al. 1990; Goldberg et al. 1989; Khoo et al. 1981). Low levels of LPL activity can also be found in liver, spleen, and lung, where it is found in Kupffer cells and infiltrating macrophages (Camps et al. 1991; Neuger et al. 2004).

In adipose tissue, LPL is secreted by the adipocytes and released into the lumen of capillaries where it becomes anchored to endothelial cells. Here, this enzyme interacts with chylomicrons and VLDL to liberate fatty acids and monoacylglycerol (MAG), facilitating their uptake (Seo et al. 2000). LPL activity depends on its interaction with the co-factor apoC-II (Kinnunen et al. 1977) and in adipocytes apoC-II expression and activity is increased by insulin (Semenkovich et al. 1989). Interestingly, this regulation appears to be both depot and gender dependent. In humans, regulation of LPL expression and activity by insulin is observed in subcutaneous, but not omental, adipose tissue (Fried et al. 1993). However, after stimulation by glucocorticoids, both depots show an increase in LPL expression and activity in response to insulin, but this is still more marked in subcutaneous adipose tissue of women (Fried et al. 1993). Although LPL plays an important role in the fatty acid uptake by adipose tissue, mice with LPL deficiency in adipose tissue are able to maintain normal fat mass by increasing de novo lipogenesis in adipose tissue (Weinstock et al. 1997). In addition, patients with LPL deficiency also exhibit normal fat mass (Ullrich et al. 2001). However, there is a change in the fatty acid composition of their adipose tissue with an increase in 16:1 and decrease in 18:0, 18:2, and 18:3 fatty acids. The reduction in essential fatty acids, which cannot be synthesized by cells, associated with the increase in non-essential fatty acids, which can be synthesized, suggests that fat mass is maintained in these subjects primarily through an increase in adipocyte de novo lipogenesis (Ullrich et al. 2001).

The fatty acids generated by the action of LPL on lipoproteins are rapidly taken up by the adipocytes. The mechanisms for fatty acid uptake are still a subject of debate and may include passive diffusion across the membrane and active transport facilitated by a membrane transporter (Kampf and Kleinfeld 2007). Fatty acids can diffuse passively across the membrane through a mechanism called "flip-flop." This mechanism was first tested using an in vitro model membrane (small unilamellar vesicle [SUV]) by measuring pH gradients across a protein-free phospholipid membrane bilayer in response to free fatty acid (FFA) (Kamp and Hamilton 1992). Addition of long-chain fatty acids to this model membrane causes their absorption within the outer leaflet of SUV, and 50% of these absorbed fatty acids are then ionized. Unionized fatty acids "flip" from the outer to the inner leaflet of the SUV. This is associated with a release of protons creating a proton gradient which is then slowly dissipated. On the other hand, addition of albumin to the external buffer extracts fatty acids from the external leaflet. Unionized fatty acids "flop" from the

inner to outer leaflet of the SUV rapidly to restore the concentration equilibrium in the bilayer. This theory has been proven using isolated adipocytes incubated with FFAs or treated with a lipolytic agent, which cause a rapid intracellular acidification that can be reversed by addition of albumin (Civelek et al. 1996). Conversely, stimulation with insulin, which promotes fatty acid esterification, leads to alkalization of the cells (Civelek et al. 1996). However, this passive diffusion of fatty acids across the phospholipid bilayer can be accelerated by certain membrane proteins, including fatty acid translocase (CD36/FAT) (Abumrad et al. 1993), caveolin (Trigatti et al. 1999), fatty acid transport protein (FATP) (Schaffer and Lodish 1994), and fatty acid binding protein plasma membrane (FABPpm) (Schwieterman et al. 1988), implicating these proteins in facilitated fatty acid uptake by adipocytes.

The fatty acid translocase CD36/FAT is highly expressed in adipose tissue (Abumrad et al. 1993), where its role in regulating fatty acid uptake has been clearly demonstrated (Harmon and Abumrad 1993; Baillie et al. 1996). Mice with a whole body deletion of CD36/FAT have higher levels of circulating FFAs and triglycerides (Febbraio et al. 1999). Injection of labeled fatty acid analogs in these mice revealed a 60–70% reduction in the uptake of these analogs by adipose tissues (Coburn et al. 2000). Isolated adipocytes of CD36/FAT null mice exhibit a 60% reduction in ^{3}H-labeled palmitate (Coburn et al. 2000) and oleate (Febbraio et al. 1999) uptake, consistent with the in vivo observations. This impairment in fatty acid uptake results in a decrease in triglyceride accumulation in adipose tissue of CD36/FAT null mice (Coburn et al. 2000). In 3T3-L1 adipocytes, CD36/FAT is located in lipid rafts, along with caveolin-1 (Pohl et al. 2004a). Disruption of these lipid rafts by beta-cyclodextrin reduces the uptake of ^{3}H-labeled oleate in these cells (Pohl et al. 2004a). Furthermore, the presence of caveolin-1 appears to be required for FAT/CD36 localization and function at the plasma membrane (Ring et al. 2006).

The FATP family is comprised of six members, FATP1–6, of which two, FATP1 and FATP4, are present in adipose tissue (Pohl et al. 2004b). The transport activity of FATPs appears to be specific for long-chain fatty acids (Schaffer and Lodish 1994; Stahl et al. 1999); however, no specific binding sites have yet been identified. In fact, these FATPs appear to differ from other fatty acid binding proteins, in that they possess an acyl-CoA synthetase activity conveyed by an AMP-binding motif (DiRusso et al. 2005). This acyl-CoA synthetase activity has been reported for FATP1, and there is strong evidence suggesting that the uptake of fatty acids by FATP1 requires the conversion of fatty acids to fatty acyl-CoA within the intracellular leaflet of the plasma membrane (Schaffer and Lodish 1994). In addition, a constitutive interaction between FATP1 and acyl CoA synthetase 1 contributes to the efficient cellular uptake of long-chain fatty acids in adipocytes through vectorial acylation (Richards et al. 2006). The expression of FABP1 and FABP4 is induced during adipocyte differentiation of 3T3-L1 cells, and a peroxisome proliferator-activated receptor response element has been described in the promoter of the murine FATP1 gene (Frohnert et al. 1999). In addition, positive regulation of the expression of these transporters has been observed in response to activators of PPARα and PPARγ (Martin et al. 1997).

FATP1 and FATP4 appear to have a distinct and complementary role in the regulation of long-chain fatty acid uptake by adipocytes (Lobo et al. 2007). FATP4 has been shown to be involved in fatty acid re-uptake and re-esterification after stimulation of lipolysis (Stahl et al. 2002), whereas FATP1 appears to play a major role in the uptake of fatty acids in response to insulin, which induces its translocation from an intracellular perinuclear compartment to the plasma membrane (Stahl et al. 2002; Lobo et al. 2007). This critical role of FATP1 in fatty acid uptake regulated by insulin has also been demonstrated in vivo. Indeed, mice with inactivation of the FATP1 gene are protected against long-term high fat diet (HFD) induced-obesity, and their fatty acid uptake in response to insulin is completely abolished in isolated adipocytes (Wu et al. 2006b). However, when exposed to a short-term HFD or lipid infusion, these mice have no alteration of whole body adiposity but exhibit a decrease in intramuscular accumulation of fatty acyl-CoA associated with improved insulin sensitivity in skeletal muscle (Kim et al. 2004). Interestingly, these mice also fail to maintain their body temperature under cold exposure, indicating a critical role of FATP1 in BAT in the regulation of non-shivering thermogenesis (Wu et al. 2006a). While there is strong evidence for a role of CD36/FAT, caveolin 1, fatty FATPs and FABPpm in the regulation of fatty acid influx and efflux in adipocytes, in preadipocytes, a different and still unknown membrane protein pump has been proposed to regulate fatty acid uptake (Kampf et al. 2007).

5.3.1.3 Triglyceride Synthesis

In adipocytes, fatty acid esterification with CoA followed by acylation of the glycerol backbone represent the last steps in the formation of triglycerides (Coleman and Lee 2004). This requires the formation of glycerol 3-phosphate from glycolysis. For this, fructose 1,6-bisphosphate is broken down to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate by the fructose bisphosphate aldolase. In adipocytes, the dihydroxyacetone phosphate is then reduced into glycerol 3-phosphate by the glycerol-3-phosphate dehydrogenase (Schlossman and Bell 1976). As mentioned above, esterification of fatty acid with CoA can occur through the acyl-CoA synthetase activity of the FATPs during fatty acid uptake, but also through a long-chain fatty acyl-CoA synthetase which acts in synergy with FATPs (Gargiulo et al. 1999). Subsequently, the acylation of the glycerol backbone occurs by action of glycerol 3-phosphate acyltransferase (GPAT) which catalyzes the addition of acyl-CoA on position 1 of glycerol 3-phosphate to give 1-acyl-*sn*-glycerol-3-phosphate, also known as lysophosphatidic acid or LPA. Two different GPAT isoforms have been characterized based on their subcellular localization and biochemical properties (Saggerson et al. 1980). In adipocytes, the major isoform is microsomal and is the product of two separate genes, *Gpat3* and *Gpat4* (previously named *Agpat6*) (Cao et al. 2006; Shan et al. 2010). Expression of these two genes is regulated during adipocyte differentiation, but only GPAT3 knockdown leads to profound inhibition of triglyceride accumulation, suggesting a critical role of this gene in triglyceride synthesis in adipocytes (Shan et al. 2010). Interestingly, in vivo studies have

suggested that GPAT4 has an important role in triglyceride accumulation in certain fat depots, as GPAT4/AGPAT6-deficient mice have been reported to exhibit a mild decrease in intra-abdominal epididymal fat and subcutaneous inguinal fat mass, but an almost complete absence of subdermal adipose tissue (Vergnes et al. 2006).

The addition of a second fatty acid on position 2 of LPA occurs through the action of the 1-acylglycerol-3-phosphate *O*-acyltransferase (AGPAT) (also called lysophosphatidate acyltransferase) which produces the 1,2-diacyl-sn-glycerol 3-phosphate (also called phosphatidic acid or PA). To date, ten different AGPATs have been reported (AGPAT1–10), but only AGPAT1 and AGPAT2 have been implicated in the regulation of triglyceride synthesis (Takeuchi and Reue 2009). Both enzymes are expressed in WAT, but AGPAT2 is the major isoform and is the only AGPAT which has been associated with a human disease. Several different mutations of *Agpat2* gene have been associated with congenital generalized lipodystrophy (Agarwal et al. 2002; Magre et al. 2003), and mice deficient in AGPAT2 have a generalized lipodystrophy demonstrating that this enzyme has a non-redundant function in adipose tissue triglyceride synthesis (Cortes et al. 2009). In vitro in 3T3-L1 adipocytes, overexpression of AGPAT1 increases oleate uptake and incorporation into triglycerides (Ruan and Pownall 2001). In addition, this overexpression leads to an increase in insulin-stimulated glucose transport and a suppression of FFA released during basal and stimulated lipolysis occurring without changes in glycerol release, suggesting a normal rate of lipolysis with increased re-esterification of FFAs (Ruan and Pownall 2001).

PA generated by the action of the AGPATs can serve as a precursor for the synthesis of acidic phospholipids, or be dephosphorylated by a phosphatidic acid phosphatase (PAP) to produce diacylglycerol (DAG), the last intermediate before the production of triglyceride. Two types of PAP enzyme have been described: PAP1 which is dependent of Mg^{2+} and PAP2 which is independent of Mg^{2+}. Only PAP1 appears to be involved in triglyceride synthesis (Coleman and Lee 2004). PAP1 activity in mammals is determined by the lipin family of proteins, lipin-1 (LPN1), lipin-2, and lipin-3, which have a distinct tissue expression pattern (Donkor et al. 2007). LPN1 accounts for all of the PAP1 activity in adipose tissue and was initially identified through the study of a spontaneous mouse mutation known as fatty liver dystrophy (*fld*) (Peterfy et al. 2001). In addition to other defects in lipid homeostasis, these mice have severe lipodystrophy indicating the critical role played by LPN1 in adipose tissue triglyceride synthesis. Interestingly, in addition to its PAP activity, LPN1 has been shown to act as a transcriptional co-activator for a number of transcription factors including PPARα, PPARδ, PPARγ, HNF 4α, and the glucocorticoid receptor (Finck et al. 2006). Thus, mouse embryonic fibroblasts from *fld* mice exhibit defects in the expression of key adipogenic genes PPARγ and C/EBPα suggesting that LPN1 plays a critical role in the regulation of adipocyte differentiation (Phan et al. 2004). More importantly, LPN1 is required for the maintenance of adipocytes and has been shown to be specifically recruited to the PPARγ-response elements of the phosphoenolpyruvate carboxykinase gene through a direct physical interaction with PPARγ (Koh et al. 2008).

Acylation of a third fatty acid on DAG to produce triglyceride can be catalyzed by several enzymatic activities, including those of the diacylglycerol acyltransferases (DGATs), which has been shown to be a critical step in vivo in transgenic mice. Two different DGAT enzymes have been characterized, encoded by two distinct genes, *Dgat1* and *Dgat2* (Cases et al. 1998, 2001; Lardizabal et al. 2001). Both enzymes are highly expressed in adipose tissue, and their levels increase with adipocyte differentiation, but they exhibit different biochemical properties and substrate selectivity (Yen et al. 2005). Mice lacking expression of DGAT1 have a normal body weight, enhanced insulin sensitivity, and are resistant to diet-induced obesity, due to increased energy expenditure and activity (Smith et al. 2000). Conversely, adipose tissue specific overexpression of DGAT1 in mice leads to an increase in adipose tissue mass when on a regular diet and a greater susceptibility to diet-induced obesity without impaired glucose tolerance (Chen et al. 2002). This last observation is consistent with results obtained in human adipose tissue, where DGAT1 expression has been reported to be strongly positively correlated with insulin sensitivity (Ranganathan et al. 2006). In addition, these studies demonstrate that DGAT1 is not essential for triglyceride synthesis in adipose tissue, but can be considered as a potential therapeutic target for obesity control. Unlike DGAT1, mice deficient in DGAT2 have a severe reduction of lipid in both blood and tissues and die shortly after birth due to a lack of sufficient substrates to maintain energy homeostasis. These studies demonstrate the fundamental role played by DGAT2 in mammalian triglyceride synthesis and the non-redundancy between DGAT1 and DGAT2 in vivo (Stone et al. 2004).

5.3.1.4 Lipolysis and Its Regulation

During lipolysis, the hydrolysis of triglycerides results in the efflux of non-esterified fatty acids (NEFA) and glycerol in the blood stream which can then be used as substrates by other tissues. For this, each fatty acid moiety is sequentially removed from triglyceride to produce successively DAG, MAG, and finally glycerol itself. In WAT, this lipolytic cascade is catalyzed by at least three different lipases, adipose triglyceride lipase (ATGL), HSL, and monoacylglycerol lipase (MGL), which have been proposed to act sequentially in the conversion of triglyceride to glycerol and three NEFAs (Jaworski et al. 2007). Since its cloning in 1988, HSL has been thought to be responsible for the first two steps of triglyceride hydrolysis (Holm et al. 1988). However, the characterization of mice deficient in this enzyme revealed a substantial residual triacylglycerol lipase activity in WAT (Osuga et al. 2000) which was associated with accumulation of DAG rather than triglyceride (Haemmerle et al. 2002), suggesting the presence of an additional unidentified triacylglycerol lipase. These unexpected observations led to the identification of a triacylglycerol lipase in adipose tissue by several groups, which has been called ATGL, desnutrin, TTS2.2, PNPLA2, or iPLA2ζ (Zimmermann et al. 2004; Villena et al. 2004; Jenkins et al. 2004). This enzyme, which is predominantly expressed in adipose tissue, exhibits high substrate specificity for triglyceride and is induced under conditions that favor

lipolysis, such as fasting. Mice deficient in ATGL have increased WAT mass and ectopic triglyceride storage in several tissues, including the heart, leading to heart failure and shortened lifespan (Haemmerle et al. 2006).

It is well accepted now that ATGL is responsible for the first step of the lipolytic cascade, hydrolyzing triglycerides to form DAG and releasing a NEFA. A second fatty acid is then removed by HSL to generate MGA. Finally, MGL hydrolyzes MGA, producing glycerol and a third NEFA. Recently, a hypothetical model for the regulation of basal and stimulated lipolysis has been proposed based on studies of the different components involved in the lipolytic cascade (Bezaire and Langin 2009). Under basal conditions, lipid droplets are coated with perilipin, a protein relatively specific to adipocytes (Greenberg et al. 1991). ATGL is found in the cytosol and on the surface of lipid droplets, associated with a co-factor named comparative gene identification 58 (CGI-58), which also interacts with perilipin (Subramanian et al. 2004; Yamaguchi et al. 2004). This complex has been shown to be required for ATGL activation (Lass et al. 2006; Schweiger et al. 2008). In this state, ATGL and CGI-58 facilitate the hydrolysis of triglyceride, delivering DAG to the cytosol. HSL, which is exclusively located in the cytosol under basal conditions (Egan et al. 1992), hydrolyzes the DAG produced by ATGL to give monoacylglycerol. Hormones which stimulate lipolysis, such as catecholamines, lead to the activation of protein kinase A (PKA), which phosphorylates perilipin (Miyoshi et al. 2007). This promotes the fragmentation of the lipid droplet and the release of CGI-58 and ATGL, which form a highly active complex around the small fragmented lipid droplets (Granneman et al. 2007). At the same time, phosphorylation of HSL by PKA increases its activity (Huttunen et al. 1970), promotes its association with fatty acid binding protein 4 (FABP4), and stimulates its translocation to the lipid droplet where it hydrolyzes the DAG produced by ATGL (Smith et al. 2004). In both basal and stimulated conditions, monoglycerol lipase completes this lipolytic cascade by hydrolyzing MAG and releasing a fatty acid and glycerol. FABP4 ensures the intracellular trafficking of NEFA from lipid droplets to the plasma membrane.

Regulation of intracellular cAMP levels in adipocytes allows rapid and precise regulation of PKA activity and subsequently lipolysis. Adenylyl cyclase is the enzyme responsible for the production of cAMP in adipocytes (Mendes et al. 1978). Its activity is tightly controlled by several membrane receptors including adrenergic receptors (Langin 2006). Catecholamines (epinephrine and norepinephrine) exert a bimodal regulation of lipolysis through their interaction with different adrenergic receptors (Lafontan et al. 1997). Binding of catecholamines to β-adrenergic receptors (β_1-, β-$_2$, and β_3-), acting through Gαs protein, stimulates adenylyl cyclase and induces cAMP production leading to the activation of lipolysis. This is counteracted by binding of catecholamines to the α_2-adrenergic receptor, which is coupled to the inhibitory Gαi protein, leading to inhibition of adenylyl cyclase and subsequently to inhibition of lipolysis. The regulation of lipolysis in response to catecholamine results in a balance between the affinity of α_2- and β-adrenergic receptors for catecholamines and their presence and number at the cell membrane. In humans, catecholamines have a higher affinity for the α_2-adrenergic receptor than for β-adrenergic receptors. More importantly, adipose tissue depots are heterogeneous

with regard to their response to catecholamine-stimulated lipolysis due to expression differences of these two receptors.

Although, catecholamines are able to stimulate lipolysis in both intra-abdominal and subcutaneous abdominal WAT in human, intra-abdominal WAT is more responsive to catecholamine-stimulated lipolysis than subcutaneous abdominal WAT, due to a greater presence of β-adrenergic receptors than α_2-adrenergic receptors on the cell membrane (Hellmer et al. 1992; Mauriege et al. 1987). By contrast, catecholamines have a very small lipolytic effect in gluteal subcutaneous WAT of normal and obese women and subcutaneous abdominal WAT of obese men, due to a concomitant increase in α_2-adrenergic and decrease in β-adrenergic responsiveness (Mauriege et al. 1991). Adipocyte hypertrophy strongly affects this functional balance between β- and α_2-adrenergic receptors (Arner et al. 1987). In addition to the role of G protein-coupled receptors in controlling adenylyl cyclase production of cAMP to regulate lipolysis, insulin can inhibit lipolysis through the activation of phosphodiesterase 3B which hydrolyzes cAMP and reduces PKA activity (Hagstrom-Toft et al. 1995). This regulation is critical in the postprandial state where insulin not only favors substrate uptake and storage but also limits hydrolysis of triglyceride in adipocytes. Interestingly, the anti-lipolytic effect of insulin is greater in subcutaneous than in visceral WAT, due to an increased insulin receptor autophosphorylation and signal transduction through the insulin-receptor substrate 1-associated phosphatidylinositol 3-kinase pathway in subcutaneous adipose tissue (Meek et al. 1999; Lafontan and Berlan 2003).

An alternative pathway in the regulation of lipolysis which does not involve PKA is starting to emerge. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), which are secreted by the heart, have been reported to stimulate lipolysis in human adipocytes through a cGMP/PKG-signaling pathway leading to the phosphorylation and activation of HSL (Sengenes et al. 2000, 2003). Although the physiological relevance for this regulation is still debated, it has been proposed that the secretion of ANP/BNP by the heart during and after strenuous endurance exercise contributes, in part, to the regulation of WAT lipolysis (Moro et al. 2004).

5.3.2 Endocrine Function of WAT

Classically, the role of WAT was viewed as limited to energy storage in the form of triglyceride. However, in 1953, Kennedy hypothesized that adipose tissue might make a circulating lipostatic factor that coordinated fat mass and food intake (Kennedy 1953). A decade later, LPL was the first protein characterized as being secreted by the adipocyte (Rodbell 1964). In 1994, the first adipocyte hormone was discovered with the cloning of leptin (Zhang et al. 1994). Since that time, the list of factors secreted from WAT, which influence metabolic homeostasis, has increased exponentially, leading to the notion of WAT as an endocrine organ (Mohamed-Ali et al. 1998). Indeed, WAT produces a large number of peptides (hormones, growth factors, cytokines, etc.), proteins (enzymes, extracellular matrix components), and

lipids (fatty acids and derived products) which affect metabolism. Many of these factors act locally within the WAT through autocrine/paracrine mechanisms, but others act systemically to influence the function of distant tissues like the brain, skeletal muscle, liver, pancreas, and heart.

More recently, proteomic screening approaches have been used to characterize the complete secretome of WAT. Using these approaches, over 250 proteins secreted by human visceral adipose tissue have been identified (Varez-Llamas et al. 2007). Using a similar technique, thus far only 84 proteins from isolated rat adipocytes have been identified (Chen et al. 2005). This may represent a species difference, but more likely indicates that many of the proteins secreted by the adipose tissue come from cells types others than adipocytes. Several studies have shown that macrophages contained in the stromovascular fraction of adipose tissue are responsible for many of the proteins secreted by WAT (Fain et al. 2004, 2006). Among the large number of factors secreted by the WAT, leptin, adiponectin, retinol binding protein 4 (RBP4), resistin, tumor necrosis factor α (TNFα), and interleukin-6 (IL-6) have been the most studied for their role and effect on metabolism homeostasis. Of these, leptin and adiponectin are the only two proteins recognized as being secreted almost exclusively by adipocytes (Fain et al. 2004), whereas the other proteins are also secreted by other tissues and other cell types within the fat pad. In addition, regional differences in the secretory capacity of the different adipose depots have been reported. Thus recently, a quantitative analysis of the secretomes comparing visceral and subcutaneous WAT showed that visceral WAT has a higher secretory capacity than subcutaneous WAT, and that this difference was an intrinsic feature of its cellular components (Hocking et al. 2010).

5.3.2.1 Leptin

In 1959, Hervey carried out a series of parabiotic experiments between rats which had hypothalamic lesions leading to hyperphagia-induced obesity and normal control rats (Hervey 1959). In these experiments, he noted that while the obese rats maintained their hyperphagia, the control rats stop eating, suggesting the presence of a circulating factor controlling food intake and coming from the obese rats. This hypothesis was further supported by the work of Coleman at the Jackson Laboratory in which mice carrying genetic lesions leading to obesity, the *ob/ob* and *db/db* mice, were subjected to parabiosis (Ingalls et al. 1950; Hummel et al. 1966). These experiments led to the conclusion that *ob/ob* mice are hyperphagic because they lack a satiety factor, and that *db/db* mice are hyperphagic because they are insensitive to this factor (Coleman 1973). Positional cloning revealed that the *ob* and *db* genes were leptin and its receptor, respectively (Zhang et al. 1994; Tartaglia et al. 1995).

Leptin is a 16 kDa hormone secreted by adipocytes which acts at the hypothalamus to control appetite and energy expenditure. The plasma concentration of leptin correlates with the size of the fat mass and nutritional state. Obesity is associated with an increase in the plasma levels of leptin, whereas subjects with lipodystrophies exhibit almost undetectable levels (Maffei et al. 1995). In addition, in the

postprandial state, plasma levels of leptin increase, at least in rodents (Saladin et al. 1995; Korbonits et al. 1997). In humans, high-fat meals also provoke a postprandial elevation of plasma leptin concentration (Poppitt et al. 2006). In both rodents and humans, fasting strongly decreases circulating leptin levels. In accordance with these results, insulin has been reported to regulate the expression or secretion of leptin in vitro and in vivo (Saladin et al. 1995; Kolaczynski et al. 1996; Rentsch and Chiesi 1996). Several other factors have been reported to regulate the expression and secretion of leptin including glucose, glucocorticoids (Kolaczynski et al. 1996), thiazolidinedione (De Vos et al. 1996; Kallen and Lazar 1996), TNFα (Zhang et al. 2000), fatty acids (Deng et al. 1997), estrogens (Shimizu et al. 1997), interleukin-1 (Janik et al. 1997), growth hormone (Isozaki et al. 1999), and several endotoxins (Grunfeld et al. 1996).

Leptin mediates its action through the activation of its transmembrane receptor termed ObR (Tartaglia et al. 1995). To date, six ObR isoforms have been identified (ObRa to ObRf) which are the result of alternative splicing of ObR messenger RNA (mRNA). These isoforms are categorized in three classes: long, short, and secreted (Myers 2004). Among these receptors, the long isoform ObRb, which contains an intracellular domain of 306 amino acids, is mainly expressed in the hypothalamus and is regarded as the signaling form of the receptor. Thus, *db/db* mice, which only lack the ObRb isoform (Lee et al. 1996), have a phenotype indistinguishable from that of mice lacking all isoforms of ObR (Lee et al. 1997; Cohen et al. 2001). The principal target of leptin in the hypothalamus is the arcuate nucleus which contains two populations of neurons, orexigenic, and anorexigenic that are involved in the control of energy homeostasis. Leptin inhibits orexigenic neuropeptide Y (NPY) and agouti-related protein (AgRP) neurons while it activates anorexigenic pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) neurons. ObRb is expressed in other tissues including cells of the immune system and pancreas. In the immune system, ObRb plays a critical role in regulating proliferation of naive and memory T lymphocytes (Lord et al. 1998), and its specific disruption in pancreas affects β-cell growth and function (Morioka et al. 2007).

Depot-specific variation in leptin secretion has been observed in WAT and appears to be determined by intrinsic factors. Thus, leptin expression and secretion are higher in subcutaneous than in visceral WAT in humans (Van Harmelen et al. 1998). In addition, β-adrenergic stimulation can inhibit leptin expression and production (Slieker et al. 1996; Gettys et al. 1996; Hardie et al. 1996). As noted above, the highest β-adrenergic responsiveness observed in visceral vs. subcutaneous WAT could explain these regional differences in leptin secretion.

5.3.2.2 Adiponectin

Adiponectin is a protein of 30 kDa (also called adipocyte complement-related protein of 30 kDa [ACRP30] or AdipoQ) which is specifically secreted by adipose tissue (Scherer et al. 1995; Maeda et al. 1996). However, the expression of adiponectin is reduced with obesity in rodents and humans (Hu et al. 1996). Adiponectin

is found in the plasma as a monomer, trimer, hexamer, and in higher molecular weight structures consisting of the assembly of up to six trimers (Pajvani et al. 2003; Tsao et al. 2003). In addition to these multimeric assemblies, a circulating globular form derived from the proteolysis of the C-terminal domain of adiponectin has been postulated to exist in vivo (Fruebis et al. 2001). Adiponectin exhibits insulin-sensitizing and anti-atherosclerotic properties (Fruebis et al. 2001; Yamauchi et al. 2001; Berg et al. 2001; Funahashi et al. 1999). Although mice deficient for adiponectin have normal body weight, these mice present all the characteristics of the metabolic syndrome including insulin resistance, glucose intolerance, hyperglycemia, and hypertension (Kubota et al. 2002; Maeda et al. 2002; Ouchi et al. 2003). Transgenic mice with overexpression of adiponectin show decreased weight gain and fat accumulation, due to inhibition of adipocyte differentiation, associated with an increase in life span and resistance to premature death induced by a high-calorie diet (Otabe et al. 2007; Bauche et al. 2007). Adiponectin mediates its effects through the activation of two unique seven transmembrane receptors, AdipoR1 and AdipoR2, which are ubiquitously expressed (Yamauchi et al. 2003). AdipoR1 has a high level of expression in skeletal muscle whereas AdipoR2 is most highly expressed in liver. These receptors have opposite functions in the control of metabolism. Mice deficient in AdipoR1 exhibit decreased energy expenditure and are obese and glucose intolerant, whereas AdipoR2 deficient mice are lean, exhibit increased energy expenditure, and do not become obese on a HFD (Bjursell et al. 2007).

In humans, a sexual dimorphism has been reported for the plasma concentration of adiponectin with higher levels being observed in women (Nishizawa et al. 2002). These differences are the consequence of a regulation of adiponectin by androgens. Indeed, while ovariectomy does not affect adiponectin plasma levels, castrated mice have higher levels of circulating adiponectin, which can be reduced by testosterone treatment (Nishizawa et al. 2002). Studies on differences in adiponectin expression and secretion between subcutaneous and visceral adipose tissue have produced somewhat conflicting results. Most studies have reported higher adiponectin expression or secretion in subcutaneous WAT than in visceral WAT in both rodents and humans (Lihn et al. 2004; Fisher et al. 2002), although this has not been observed in all studies (Atzmon et al. 2002; Motoshima et al. 2002; Perrini et al. 2008). While in general, adiponectin levels are low in obese individuals, the association between subcutaneous WAT, visceral WAT, and adiponectin levels is less clear. Some studies have reported a positive correlation between subcutaneous WAT and serum adiponectin (van der Poorten et al. 2008; Hanley et al. 2007), while other have reported a negative correlation (Fujikawa et al. 2008; Farvid et al. 2005). What is clear, however, in all of these studies is the negative correlation between visceral WAT and serum adiponectin. A recent study in young Danish men reported that abdominal subcutaneous fat, rather than intra-abdominal/visceral fat, is negatively associated with adiponectin levels, whereas fat in the thighs and lower extremities is positively associated with serum adiponectin levels (Frederiksen et al. 2009). As with leptin, stimulation of the β-adrenergic receptor decreases the expression and release of adiponectin by adipose tissue and may explain these depot-specific differences (Fu et al. 2007; Fasshauer et al. 2001; Delporte et al. 2002).

5.3.2.3 Other Adipocyte Secreted Factors

RBP4 belongs to the lipocalin family and is the principal transport protein for retinol (vitamin A) in the circulation (Yang et al. 2005). Production of RBP4 by adipose tissue was originally identified in mice with deletion of Glut4 in adipose tissue (AG4KO) (Yang et al. 2005). These mice are insulin resistant and glucose intolerant and have increased expression of RBP4 in adipose tissue and elevated circulating RBP4. High circulating levels of RBP4 have been found in insulin-resistant mice models and humans with obesity and type 2 diabetes, and in mice can be normalized by the insulin sensitizing agent rosiglitazone (Yang et al. 2005; Graham et al. 2006). In addition, injection of recombinant RBP4 in normal mice is sufficient to cause insulin resistance (Yang et al. 2005). In humans, serum RBP4 concentration has been reported to be negatively associated with insulin sensitivity and onset of type 2 diabetes (Graham et al. 2006; Stefan et al. 2007; Gavi et al. 2007; Kloting et al. 2007; Cho et al. 2006), although some studies observed no correlations (Promintzer et al. 2007; Broch et al. 2007). Circulating levels of RBP4 show a strong association with fat distribution. Thus, in healthy subjects, serum RBP4 is positively correlated with percentage of fat in the trunk, but not with percentage of total body fat (Gavi et al. 2007). RBP4 is also a strong measure of visceral fat accumulation in women (Lee et al. 2007).

At the level of mRNA expression, RBP4 is higher in visceral WAT than subcutaneous WAT. Furthermore, RBP4 expression in visceral adipose shows a stronger correlation with circulating RBP4 levels than expression in subcutaneous WAT (Kloting et al. 2007). Like adiponectin, plasma levels of RBP4 exhibit a sexual dimorphism; however, in this case, higher levels of circulating RBP4 are found in men (Cho et al. 2006). Although the mechanisms by which RBP4 might induce insulin resistance are not well understood, systemic and paracrine regulations have been described. Thus, RBP4 can act on the liver and the skeletal muscle to increase expression of phosphoenolpyruvate carboxykinase and decrease insulin signaling, respectively (Yang et al. 2005).

Resistin is a member of a family of resistin-like molecules, also known as the FIZZ family (Holcomb et al. 2000; Steppan et al. 2001b). When first discovered, this adipokine was proposed to be the link between obesity, insulin resistance, and diabetes, and hence the name resistin (Steppan et al. 2001a). Although initial reports indicated adipocytes as a main source of resistin, resistin mRNA is present in hypothalamus (Morash et al. 2002; Wilkinson et al. 2005; Tovar et al. 2005), pituitary (Morash et al. 2002, 2004), and pancreatic β-cells (Minn et al. 2003) in mice. Resistin expression in adipose tissue is increased in diet-induced and genetic mice models of obesity and is down-regulated by the insulin sensitizing agent rosiglitazone (Steppan et al. 2001a). In addition, treatment of normal mice with recombinant resistin impairs glucose tolerance and insulin action (Steppan et al. 2001a). Somewhat contrary to the view of an insulin resistance factor, resistin mRNA expression increases during differentiation of murine preadipocytes into adipocytes (Kim et al. 2001). In mice, resistin expression in adipose tissue decreases in response to fasting and greatly increases after refeeding (Kim et al. 2001). In addition, resistin expression is strongly upregulated in adipose tissue of streptozotocin-diabetic mice after insulin injection (Kim et al. 2001).

In humans, the role of resistin in insulin sensitivity is less clear. Human adipose tissue expresses only low levels of resistin, and adipocytes seem to contribute very little to its production (Nagaev and Smith 2001; Pagano et al. 2005; McTernan et al. 2002, 2003; Yang et al. 2003; Fain et al. 2003; Savage et al. 2001). In fact, in human WAT, the major source of resistin expression and secretion is the stromal-vascular fraction containing preadipocytes, vascular endothelial, smooth muscle cells, and inflammatory cells (Fain et al. 2003; Savage et al. 2001). In this fraction, macrophages have been identified as the primary source of resistin production (Savage et al. 2001; Curat et al. 2006). In addition, studies of the association between serum levels of resistin and obesity or type 2 diabetes in humans have yielded divided opinions. While many studies have reported a positive correlation between circulating resistin levels and obesity (Lee et al. 2005; Vendrell et al. 2004; Gawa-Yamauchi et al. 2003) or insulin resistance and type 2 diabetes (Fujinami et al. 2004; Silha et al. 2003; Smith et al. 2003; McTernan et al. 2003), others have observed no correlation (Heilbronn et al. 2004; Lee et al. 2003; Savage et al. 2001). Interestingly, there is growing evidence that resistin could be involved in other diseases, including atherosclerosis, non-alcoholic fatty liver, cancer, inflammatory bowel disease, chronic kidney disease, and asthma (Filkova et al. 2009).

TNFα was discovered in 1975 as a cytotoxic factor in the serum of mice infected with bacillus Calmette-Guerin and was given its name because it was able to induce necrosis of tumors (Carswell et al. 1975). TNFα is produced as a 26 kDa transmembrane protein and after cleavage by a metalloproteinase is released in the circulation as a 17 kDa soluble molecule (Black et al. 1997; Moss et al. 1997). TNFα was the first factor secreted by adipose proposed to represent a link between obesity and insulin resistance (Hotamisligil and Spiegelman 1994). TNFα expression is increased in adipose tissue of obese mice models and in human obese individuals (Hotamisligil and Spiegelman 1994; Hotamisligil et al. 1995; Kern et al. 1995; Yamakawa et al. 1995; Hofmann et al. 1994). Although adipocytes can make TNFα, it appears that infiltrating proinflammatory (M1) macrophages are responsible for almost all of the TNFα expression in adipose tissue (Weisberg et al. 2003). TNFα mediates its effects through the activation of two distinct receptors TNFR1 and TNFR2 which homodimerize in the presence of TNFα (Tartaglia and Goeddel 1992; Smith et al. 1990). While TNFR1 is ubiquitously expressed, TNFR2 is found only in cells of the immune system. Both receptors are found in a soluble form in the circulation and can block TNFα effects in vitro and in vivo (Van Zee et al. 1992).

A large number of studies have reported the multiple effects of TNFα on metabolism homeostasis. Thus, TNFα has been shown to impair insulin sensitivity in vitro and in vivo (Hotamisligil 1999). TNFα has also been shown to affect fatty acid metabolism by reducing LPL expression and activity (Hauner et al. 1995; Cornelius et al. 1988; Semb et al. 1987), decreasing expression of fatty acid transporter (Memon et al. 1998a), ACC and FAS (Doerrler et al. 1994; Pape and Kim 1988) acyl-CoA synthetase (Memon et al. 1998b), and increasing lipolytic activity (Green et al. 1994; Feingold et al. 1992; Hauner et al. 1995). TNFα is able to block adipocyte differentiation by preventing the induction of C/EBPα and PPARγ expression (Kurebayashi et al. 2001; Xing et al. 1997; Zhang et al. 1996), and to induce the dedifferentiation

of mature adipocytes (Petruschke and Hauner 1993; Torti et al. 1989; Xing et al. 1997). Finally, TNFα can induce apoptosis of preadipocytes and adipocytes (Qian et al. 2001; Prins et al. 1997). The regulation of metabolism homeostasis by TNFα is not limited to its action on adipose tissue. Indeed, TNFα can impair insulin sensitivity in muscle (Li and Reid 2001) and liver (Tilg and Moschen 2008).

IL-6 is a cytokine with pleiotropic biological effects in multiple organs (Kamimura et al. 2003). A large number of tissues and cell types, including WAT, secrete IL-6. Adipose tissue has been estimated to account for 10–35% of circulating IL-6 in healthy humans (Mohamed-Ali et al. 1997) and slightly more in obese individuals (Hoene and Weigert 2008; Bastard et al. 2002). Omental WAT releases 2–3 times more IL-6 than subcutaneous WAT (Fried et al. 1998). Although several studies have reported positive correlations between IL-6 levels and the presence of insulin resistance or type 2 diabetes (Pradhan et al. 2001; Fernandez-Real et al. 2001; Pickup et al. 1997), other studies have demonstrated that plasma IL-6 levels and increased fat mass are not independent risk factors for the development of insulin resistance (Corpeleijn et al. 2005; Carey et al. 2004; Kopp et al. 2003). Furthermore, whether IL-6 induces or has a beneficial effect on insulin sensitivity is still actively debated (Pedersen and Febbraio 2007; Mooney 2007; Spangenburg et al. 2007).

5.4 Depot-Specific Differences of WAT

5.4.1 Anatomical Distribution of Adipose Tissues

With evolution, the adipose organ has become more anatomically dispersed (Gesta et al. 2007). In vertebrates, the two major divisions of WAT are in subcutaneous and intra-abdominal locations. These were described as being distinct as early as 1871 by Flemming (1871). Today, this simple dichotomization of WAT is still referred to in a large number of metabolic studies, due to the different impacts of these depots on metabolism. However, this is an over-simplification and often leads to discordant observations due to an important heterogeneity within these two divisions. Additional difficulty in attempting to categorize WAT resides in the fact that fat distribution varies considerably between species and also between individuals from the same species. In this review, we will discuss only WAT distribution in mice and the corresponding depots in humans (Fig. 5.2).

5.4.1.1 Subcutaneous Adipose Tissue

In mice, subcutaneous WAT is referred to as the tissue that is located beneath the skin and outside the peritoneal cavity. It consists of two main depots, one which is anterior and the other posterior (Cinti 2005). The anterior depot lies in the interscapular region between and under the scapulae and projects into the axillary and

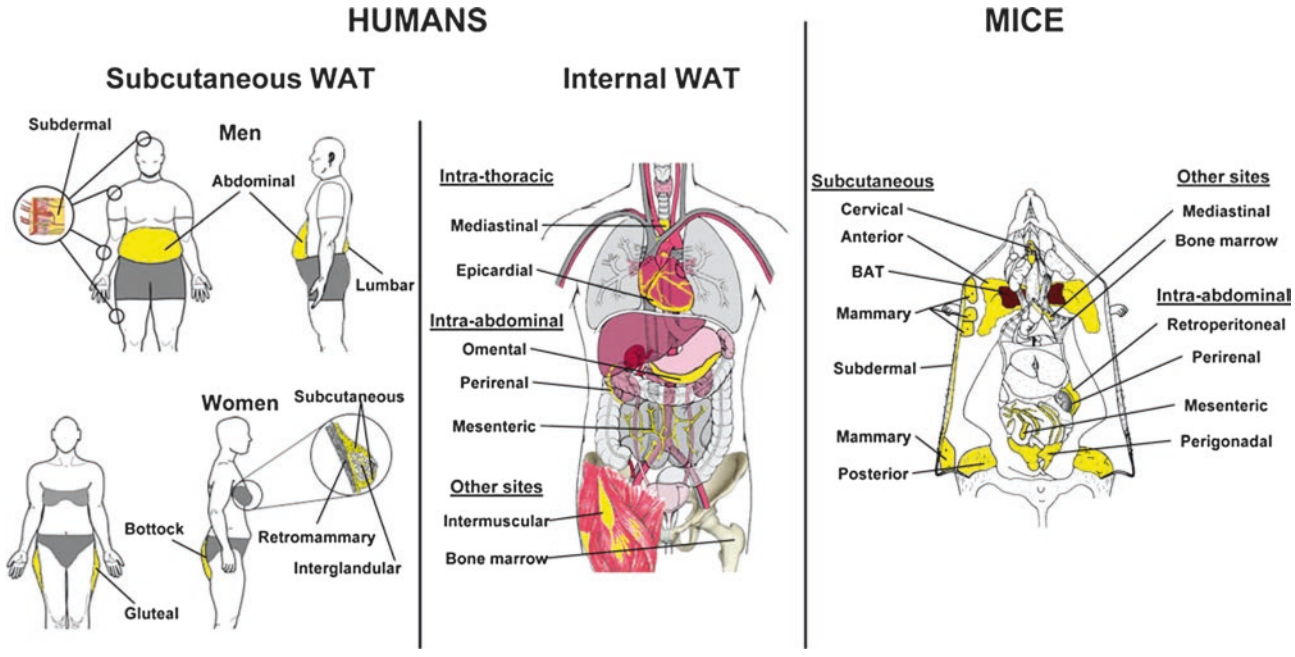

Fig. 5.2 White adipose tissue distribution in humans and mice. White adipose tissue is distributed throughout the body in both humans and mice. The two major compartments of WAT are located subcutaneously and intra-abdominally, although WAT can be found in other regions, such as the intra-thoracic region. While these two species share most of their WAT depots, some depots are species specific, such as the epicardial WAT in humans and the perigonadal WAT in mice

proximal regions of the forelimbs and the cervical area (Cinti 2005). BAT is also located within this interscapular region, the majority being embedded in the WAT. BAT also projects anteriorly into a deep cervical depot and laterally into subscapular and axillo-thoracic depots. Small amounts of BAT are also visible in the mediastinal and perirenal regions (Cinti 2005). Interestingly, although these two tissues share an intimate location, they are diametrically opposite in their function and their developmental origin (Yamamoto et al. 2010). In human fetuses and newborns, BAT can be found in this interscapular location in addition to axillary, perirenal, and periadrenal regions. In humans, these BAT depots decrease shortly after birth (Cannon and Nedergaard 2004), and in adults, only cervical, supraclavicular, axillary, and paravertebral BATs remain (Nedergaard et al. 2007; Cypess et al. 2009). In humans, enlargement of the subcutaneous WAT in these neck and upper back regions has been described as being part of Cushing's syndrome. In this disease, hypercorticism leads to an increase in fat mass forming a so-called "buffalo-hump," indicating the higher glucocorticoid responsiveness of this depot (Nieman et al. 1985). An increase in fat accumulation in these regions has also been observed in the acquired form of lipodystrophy that is associated with treatment for human immunodeficiency virus (Miller et al. 1998).

The posterior WAT of mice (also called inguinal or flank) consists of a long strip of tissue located around the hind legs. This tissue can be dissociated in three portions, starting from the dorsum at the lumbar level (dorso-lumbar portion). It then extends into the inguino-crural region (inguinal portion) up to the pubic level and into the gluteal region (gluteal portion). At the pubic level, this depot joins the contralateral depot (Cinti 2005). In humans, the distribution of subcutaneous fat is similar with large WAT depositions in the posterior lumbar, epidural, buttock, gluteal, and thigh regions. In lean subjects, these regions are dissociated from one other, whereas as obesity develops, especially lower body obesity, these regions appear to join. In addition, humans have a subcutaneous abdominal adipose depot which is absent in mice. This abdominal subcutaneous depot has a great expansion capacity and exhibits important differences in several biochemical pathways compared to other subcutaneous depots (Lafontan and Berlan 2003; Arner 1995). In addition to these two main subcutaneous depots in mice, some adipose tissue can be found at the root of the limb and at the level of the join in the middle of the limbs (Cinti 2005). This latter depot is called the popliteal adipose depot and is well-known by radiologists who try to suppress its lingering signal observed during magnetic resonance imaging (Moriya et al. 2010).

Besides these well-delimited subcutaneous depots, a subdermal layer of fat is present in both mice and humans throughout the body. Unfortunately, this layer of fat has been poorly studied in mice, as it cannot be easily dissected. However, this tissue seems to present properties which differ from the other subcutaneous depots. Indeed, as mentioned above, a recent genetic engineering study showed that deletion of the gene encoding for GAPT4 (also called AGPAT6) in mice leads to complete absence of subdermal adipose tissue, whereas the posterior (inguinal) subcutaneous fat pad was modestly reduced (Vergnes et al. 2006). In humans, increased fat accumulation in this subdermal adipose tissue causes skin dimpling

and nodularity, better known as cellulite. Although this fat accumulation has been of limited interest to metabolic researchers, its cosmetic interest is significant.

Recently, Sbarbati et al. defined three different types of subcutaneous WAT in humans, based on their structural and ultrastructural features (Sbarbati et al. 2010). Type 1 WAT or deposit WAT (dWAT) is a non-lobulated organized WAT with low collagen content and large adipocytes which tend to adhere to one other in parallel membrane plates. dWAT is considered as a metabolic depot due to its high lipid content. It mainly corresponds to the abdominal subcutaneous depot. Type 2 WAT or structural WAT (sWAT) is more polymorphous and variable from site to site. It is defined as a stromal depot with a non-lobular structure and smaller adipocytes. sWAT is located in limited adipose areas, usually rich in muscular tissue, including around trochanters, suprapubic, axillae, inner faces of the knees, thighs, hips, arms, pectoral, and mammary areas. Type 3 WAT or fibrous WAT (fWAT) has an important fibrous component and can be found in areas where a severe mechanic stress occurs. Adipocytes of fWAT are the smallest and surrounded by a thick collagen layer. fWAT is divided into two subtypes: lobular and non-lobular. Lobular fWAT can be found in the calcaneal region where mechanical constraint is important. It is organized into micro- and macro-chambers delimited by connective septae. Non-lobular fWAT is a hard adipose tissue with a major degree of fibrosis and low lipid content.

5.4.1.2 Intra-Abdominal Adipose Tissue

Internal adipose tissue is located in the thoracic and abdominal cavities. Mice and humans share the large majority of intra-abdominal adipose depots, but also have distinct depots. In the abdominal cavity, large amounts of adipose tissue accumulate around the digestive system in two main depots, namely mesenteric and omental adipose tissues. Mesenteric adipose tissue (often called visceral adipose tissue) is present in both species and is located in the connective tissue of the intestine, along with blood and lymph vessels. In mice, this connective tissue also contains the pancreas, which is diffuse and irregular, often leading to cross contamination during dissection (Caesar and Drevon 2008). Omental adipose tissue is hardly detectable in mice; however, in humans, this depot can be substantial. This adipose tissue develops in the greater omentum, a serous membrane hanging from the greater curvature of the stomach. This depot can enlarge in obese humans to cover the entire intestine and form a pannus, or apron, of fat.

Two other adipose tissues are present in the intra-abdominal cavity and present in both humans and mice: the retroperitoneal and the perirenal adipose tissue. In mice, the retroperitoneal adipose tissue lies in the paravertebral position between the spine and the posterior abdominal wall. Perirenal adipose tissue is found around the kidney and can be separated from the retroperitoneal adipose tissue by a peritoneal fold (Cinti 2005). In humans, in addition to the perirenal adipose tissue, also called the adipose capsule of the kidney, there is an additional depot located superficially to the renal fascia termed the pararenal adipose tissue (or paranephric body).

The last adipose depot present in the intra-abdominal cavity is found surrounding reproductive organs and is called perigonadal adipose tissue. This is only present in mice. In females, this tissue surrounds the uterus, bladder, and ovaries and is called periovarian adipose tissue. In males, this tissue surrounds the epididymis, projecting anteriorly in the intra-abdominal cavity along the peritoneum and is termed epididymal adipose tissue. Interestingly, although this tissue is absent in humans, it has been the most studied WAT depot because of its easy access and dissectability in mice. However, this does raise some questions about the relevance of certain physiological and pathophysiological studies in mice to humans (Harris and Leibel 2008).

Two adipose tissues have been described in the thoracic cavity: epicardial and mediastinal adipose tissues. Epicardial WAT develops at different sites around the heart: on the free wall of the right ventricle, on the left ventricular apex, around the atria, from the epicardial surface into the myocardium, following the adventitia of the coronary artery branches and around the two appendages (Iacobellis et al. 2005). Epicardial WAT is usually found only in large mammals, such as humans, and is almost absent in mice or rats (Marchington et al. 1989), which explains why epicardial adipose tissue has been so poorly studied. However, in humans, the size of epicardial adipose tissue has been related to left ventricular mass and other features of the metabolic syndrome (Iacobellis et al. 2005). Indeed, increases in epicardial adipose tissue are strongly associated with abdominal obesity and visceral adiposity as opposed to overall adiposity (Iacobellis et al. 2003a, b; Silaghi et al. 2008). The mediastinal adipose tissue is located in the superior and posterior mediastinum in both mice and humans. Although the presence of this tissue in humans during android obesity was observed almost 250 years ago by Joannes Baptista Morgagni, this tissue has also been poorly studied in mice (Morgagni 1765). Interestingly, in rats, this tissue appears to be a mixture of WAT and BAT (Osculati et al. 1989; Giordano et al. 2004).

5.4.1.3 Mammary Adipose Tissue

In mice, there are five pairs of mammary glands, three of which are located in the thoracic region and two in the inguinal region. All are surrounded by subdermal adipose tissue. In the lipodystrophic mouse model A-ZIP/F-1 transgenic mice, rudimentary mammary anlagen were able to form, but were unable to grow and branch normally (Couldrey et al. 2002). However during gestation, even in the absence of adipocytes, a tremendous amount of epithelial cell division and alveolar cell formation occurred, illustrating that adipose tissue was not required for mammary gland differentiation (Couldrey et al. 2002). Adipose tissue represents an important component of the human breast. Using ultrasound imaging, Ramsay et al. have calculated that in non-lactating women, the ratio of glandular tissue to adipose tissue is 1:1, and this rises to 2:1 during lactation (Ramsay et al. 2005). Although the distribution of adipose tissue shows a wide variation between women, they identified several adipose tissue sub-depots within the breast: one located directly under the skin (subcutaneous fat), another within the glandular tissue (intraglandular fat) and

a third behind the glandular tissue in front of the pectoral muscle (retromammary fat) (Ramsay et al. 2005). Mammary adipose tissue represents an important source for the synthesis of many diverse molecules involved in the development and the function of mammary glands (Hovey et al. 1999). In addition, several studies have implicated mammary adipose tissue in the metastatic progression of breast tumors (Elliott et al. 1992; Chamras et al. 1998; Manabe et al. 2003). Interestingly, over 350 unique proteins have been identified in the interstitial fluid of mammary adipose tissue from high-risk breast cancer patients (Celis et al. 2005).

5.4.1.4 Intermuscular Adipose Tissue

Lipid deposition is present in muscle and can be separated into two compartments: intermuscular and intramuscular. Intramuscular fat is the result of ectopic lipid accumulation within myocytes and therefore by definition cannot be considered as adipose tissue. However, intermuscular fat is the visible muscle fat marbling resulting in infiltration of adipose tissue between the muscle fibers that can be observed in mice, human and other mammals. In mice, this depot has been poorly studied, but it has been reported to increase in mice deficient in CC chemokine receptor 2 following ischemic injury (Contreras-Shannon et al. 2007) and decrease in mice overexpressing the mitochondrial uncoupling protein-3 (Changani et al. 2003). In addition, a BAT depot, with regulatable expression of the uncoupling protein-1, has been recently observed within the muscles of a strain of mouse that is resistant to diet-induced obesity and metabolic disorders, providing a genetically based mechanism for this protection (Almind et al. 2007). In humans, intermuscular adipose tissue has been well documented to increase with age and obesity, with higher levels found in women than in men (Ryan and Nicklas 1999; Kelley et al. 1999). Its function is not clear, but a high amount of intermuscular adipose tissue appeared to be associated with muscle weakness in the elderly (Katsiaras et al. 2005; Goodpaster et al. 2001).

5.4.1.5 Bone Marrow Adipose Tissue

Bone marrow is the site of production of red blood cells, platelets, and most white blood cells. While bone marrow usually has a red color due to its high content in hematopoietic cells, the color changes from red to yellow when adipose tissue develops in the bone marrow. Adipose tissue-rich marrow (also called yellow marrow) increases with age and in patients with osteoporosis, but unlike the other adipose depots, it does not increase with obesity (Justesen et al. 2001; Kugel et al. 2001). The function of adipose tissue in the bone marrow is still unclear and subject to controversy. It has been suggested to serve simply as a passive space filler, to be an active participant in lipid metabolism, energy storage, or even contribute to cell differentiation within the bone marrow (Gimble et al. 1996). Interestingly, thiazolidinediones have been reported to increase adipocyte and decrease osteoblast

formation in the bone marrow of mice (Rzonca et al. 2004) and diabetic women, but not men (Schwartz et al. 2006). A recent study reported that bone marrow adipocytes are negative regulators of the hematopoietic microenvironment, suggesting that antagonizing bone marrow adipogenesis may enhance hematopoietic recovery after bone-marrow transplantation (Naveiras et al. 2009).

5.4.2 Fat Distribution and Associated Risks

As noted above, adipose tissue is distributed throughout the body in humans, but this distribution can vary considerably from one individual to another. In lean individuals, when body fat accumulation increases leading to overweight and/or obesity, fat deposition can be exacerbated in specific regions of the body, leading to altered fat distribution. These changes have an important impact on metabolism and lead to the development of metabolic disorders such as type 2 diabetes and metabolic syndrome. This has resulted in several classifications of different types of obesity.

At the end of the 1940s, a French physician from Marseille, Jean Vague, noted that "fat excess is dangerous because of its metabolic complications and a woman normally has twice a man's fat mass, i.e., the mass of an obese man. Though she is often as obese as a man or is fatter, she dies later and less often from metabolic complications of obesity." He then proposed in *La presse medicale* the existence of sexual dimorphism as a determining factor for two different patterns of fat distribution in obese patients (Vague 1947). He classified these two patterns of obesity as android (or upper-body) vs. gynoid (or lower-body) obesity using the brachio-femoral adipo-muscular ratio, which was based on ratios of skinfolds and circumferences of the arms and thighs. In 1956, he reported that a high brachio-femoral adipo-muscular ratio in obese individuals (android obesity) was associated with an increased risk of type 2 diabetes, atherosclerosis, and gout, whereas gynoid obesity was not (Vague 1956). Three decades later, a new classification was made based on the calculated ratio between the waist circumference (WC) (measured midway between the lowest rib and the iliac crest) and the hip circumference (measured at the level of the great trochanters with the legs together) (Kissebah et al. 1982; Bjorntorp 1987). In a 12-year longitudinal study, Larson et al. reported that abdominal obesity, determined by a high waist–hip ratio (WHR), was associated with an increased risk of myocardial infarction, stroke, and premature death, whereas no association was found when indices of generalized obesity, such as body mass index (BMI), were used (Larsson et al. 1984). Interestingly, in this study, individuals with a low BMI but high WHR exhibited the highest risk of developing myocardial infarction and premature death, indicating the deleterious consequences of "pure" abdominal fat accumulation (Larsson et al. 1984).

Since that time, an impressive number of studies have recognized that abdominal obesity, assessed by WHR or simply WC, is associated with adverse health risks,

including insulin resistance, type 2 diabetes mellitus, dyslipidemia, hypertension, atherosclerosis, hepatic steatosis, cholesterol gallstones, several cancers (esophagus, pancreas, colorectum, breast, endometrium, cervix, and kidney), and overall mortality (Carey et al. 1997; Wang et al. 2005; Zhang et al. 2008; Baik et al. 2000; Pischon et al. 2008; Seidell 2010). The most common cutoffs used for WC are 102 cm for men and 88 cm for women, while those for WHR are 0.95 for men and 0.80 for women. However, concerns have been raised about using the same upper limits of these indicators in all ethnic groups. Strong evidence has been presented that lower WC cutoffs should be used for Asians (85 and 80 cm for men and women, respectively) for assessment of diabetes and hypertension risk, whereas the normal limits for WHR may be similar. In addition, the use of specific cutoffs for African–American, Hispanic, and Middle Eastern populations has been recommended (Lear et al. 2010).

Fat distribution is determined by multiple factors in addition to ethnicity. Gonadal steroids have been shown to affect adipose tissue mass and distribution in humans. For example, a decrease in intra-abdominal adipose tissue and increase in subcutaneous adipose tissue mass are observed in men that have been treated with testosterone. Interestingly, this adipose tissue redistribution has been shown to be associated with increased insulin sensitivity (Mayes and Watson 2004). In addition, while premenopausal women often have increased amounts of subcutaneous WAT (Lear et al. 2010; Loomba-Albrecht and Styne 2009; Wells 2007), postmenopausal women are prone to increases in intra-abdominal fat (Turgeon et al. 2006), and this is attenuated by hormone replacement therapy (Mayes and Watson 2004). In ovariectomized mice, adipose tissue mass and adipocyte size increase in both subcutaneous and perigonadal depots, and this has been associated with impaired glucose uptake and insulin sensitivity (Macotela et al. 2009). In male mice, castration has no effect on fat mass in either depots (Macotela et al. 2009).

In addition, genetics play an important role in both obesity and distribution of WAT. Twin and population studies have revealed that both BMI and WHR are heritable traits, with genetics accounting for 30–70% of the variability (Nelson et al. 2000). Such genetic control of body fat distribution is most evident in Hottentot/Khoisan women, who have a marked accumulation of fat in the buttocks (steatopygia) (Krut and Singer 1963). Striking differences in WAT distribution can also be observed in individuals with heritable forms of partial lipodystrophy (Agarwal and Garg 2006). For example, in congenital generalized lipodystrophy (Berardinelli-Seip Syndrome), adipose tissue is almost completely absent from subcutaneous depots, intra-abdominal depots, intra-thoracic region, and bone marrow. However, these individuals still have a relatively normal amount of adipose tissue in the buccal region, palms, soles, and other areas. By contrast, individuals with familial partial lipodystrophy of the Dunnigan type have a marked loss of subcutaneous adipose tissue in the extremities and trunk, but no loss of visceral, neck, or facial adipose tissue. These partial lipodystrophies, which are the result of mutations in different genes, indicate the developmental heterogeneity of the different adipose depots.

5.4.3 Causes for the Deleterious Impact of Abdominal Obesity

Several theories have been proposed to explain the link between intra-abdominal/visceral adipose tissue and the increased risk for metabolic complications, such as insulin resistance, glucose intolerance, and dyslipidemia. Historically, the "Portal circulation Theory" has been the most actively discussed. In this theory, it has been noted that intra-abdominal/visceral adipose tissue drains into the portal vein, allowing preferential access of FFA to the liver (Bjorntorp 1990). This high level of FFA could stimulate hepatic gluconeogenesis and reduce hepatic insulin sensitivity by decreasing the number of insulin receptors and altering intracellular insulin signaling through activation of protein kinase C and other pathways. This theory was also supported by the fact that intra-abdominal adipose depots possess higher lipolytic rates than subcutaneous adipose tissue, and therefore release more FFA directly into the portal vein to feed the liver. Indeed, it is now well-established that in humans, intra-abdominal adipose tissue depots show a significantly greater lipolytic activity when stimulated by catecholamines than subcutaneous adipose depots. This difference is due primarily to the presence of a higher level of lipolytic β-adrenergic receptors and a much lower level of anti-lipolytic α_2-adrenergic receptors on the surface of adipocytes from intra-abdominal adipose depots compared to those from subcutaneous depots.

This theory of FFA being released in the portal vein as the major mechanism to explain the association between intra-abdominal fat accumulation and metabolic disorders has been subject to challenge. One major argument against the theory is that any adipose depot with a high continuous rate of FFA release should ultimately disappear, and presumably the metabolic disorders associated with it would also disappear. However, in reality the converse is true. Thus, as central obesity develops, fat accumulation in intra-abdominal adipose depot tends to increase rather than disappear and the metabolic disorders worsen rather than improve. One possibility to explain the increase in intra-abdominal WAT as obesity develops would be the presence of a high FFA turnover such that at certain times of the day, e.g., postprandially, there would be high triglyceride accumulation, whereas during periods of fasting or stress, this would be followed by episodes of high lipolysis. Interestingly, in healthy individuals, intra-abdominal WAT has a 30% higher FFA uptake rate per gram of tissue than abdominal subcutaneous WAT (Hannukainen et al. 2010). However, when tissue FFA uptake per gram of fat is multiplied by the total tissue mass, total FFA uptake is almost 1.5 times higher in abdominal subcutaneous WAT than in visceral WAT, indicating that subcutaneous rather than visceral fat storage plays a more direct role in systemic FFA availability (Hannukainen et al. 2010). In addition, measurement of FFA in the portal vein has been found to be very close to those in arterial plasma (Hagenfeldt et al. 1972; Bjorkman et al. 1990; Blackard et al. 1993). Finally, it appears that in central obesity, the higher level of FFAs delivered to the liver originate from upper-body, non-splanchnic adipose depots (probably the subcutaneous abdominal depot), but not a visceral depot (Guo et al. 1999).

In addition to FFAs, adipokines and cytokines, such as interleukin-1, IL-6, TNFα, resistin, and others, which have been associated with reduced insulin sensitivity, are also potential mediators for the portal mechanism of insulin resistance

(Lafontan and Girard 2008; Girard and Lafontan 2008). These cytokines, whose secretion from adipose tissue is increased in obese individuals, are produced at higher levels from intra-abdominal than subcutaneous adipose depots. From this observation, a cell biological theory has emerged based on the concept that fat cells in different depots possess different intrinsic properties, and possibly have a different developmental origin, causing them to be more or less associated with metabolic alterations. This hypothesis is supported by the fact that at a molecular level, significant differences in expression of hundreds of genes have been reported between distinct adipose tissue depots in both rodents and humans, and these depot-specific variations in gene expression appear to be intrinsic (Gesta et al. 2007). Therefore, although there is no doubt that the anatomical location of intra-abdominal adipose depots draining into the portal circulation plays a critical role, the intrinsic properties of these depots are also one of the causes for the association between central obesity and metabolic disorders.

5.5 Adipogenic Lineage of Different WAT Depots

Intrinsic property differences between adipocytes from various WAT depots have recently lead to theories about the existence of different adipogenic lineages that are responsible for the development of the various WAT depots. Indeed, substantial evidence supporting the theory that different white adipose depots may be derived from distinct precursors exists (Vohl et al. 2004; Cantile et al. 2003; Gesta et al. 2006; Tchkonia et al. 2007). In rodents, the different WAT depots appear after birth. The first depots to develop are the intra-abdominal perigonadal and the anterior and posterior subcutaneous, while the intra-abdominal mesenteric, retroperitoneal, and perirenal depots usually develop later. In humans, although the development of subcutaneous and intra-abdominal WAT starts during early to mid-gestation, at birth, newborn babies have greater amounts of subcutaneous than intra-abdominal WAT. In healthy newborns, only 10% of the total WAT mass is intra-abdominal, whereas 90% is subcutaneous, with 70% being non-abdominal subcutaneous WAT (Modi et al. 2009). In addition, several intrinsic properties have been observed in cells taken from different white adipose depots. Thus, cloned human preadipocytes from subcutaneous adipose tissue exhibit a greater ability to differentiate and accumulate lipids in culture than those from mesenteric or omental adipose depots. These attributes are associated with differences in expression of C/EBPα, PPARγ, and many other adipocyte-related genes (Tchkonia et al. 2002). Furthermore, these inter-depot differences have been shown to be conserved after multiple generations of cell replication in culture (Tchkonia et al. 2006). Similarly, intrinsic variations in gene expression have been observed in adipocyte and preadipocyte fractions taken from different intra-abdominal and subcutaneous adipose tissue depots in mice (Gesta et al. 2006). In addition, Wu et al. have shown that administration of monoclonal antibodies raised against adipocyte plasma membranes in chick embryos significantly reduces abdominal adipose tissue weight without affecting femoral or

pectoral fat depots (Wu et al. 2000), suggesting that the adipocytes in these depots have different membrane protein antigens. Together, these observations indicate that the adipogenic lineage for the development of WAT differs from one depot to another (Fig. 5.3).

Recent gene expression profiling approaches have provided insights into the molecular mechanisms involved in the early development and patterning of the different adipose depots. Thus, using this approach, several fundamental developmental genes have been found to be differentially expressed between intra-abdomi-

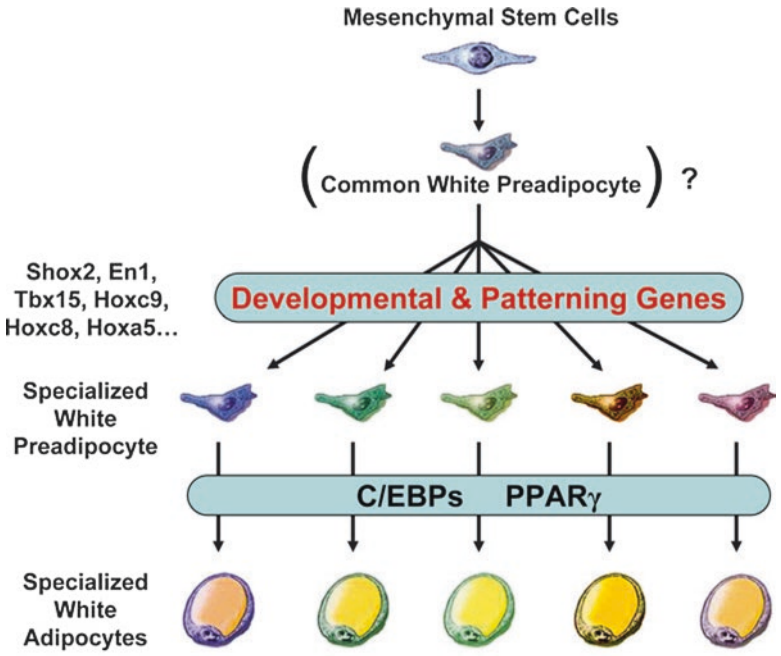

Fig. 5.3 Hypothetical scheme of the adipogenic lineage of the different WAT depots. Under the influence of developmental and patterning genes including *Shox2*, *En1*, *Tbx15*, *HoxC9*, *HoxC8*, and *HoxA5*, mesenchymal stem cells or a pool of common white preadipocyte precursors give rise to different specialized white preadipocytes which will form the various WAT depots. The adipocytes in these depots have specialized functions which are at least in part, therefore, cell autonomous. Thus, WAT depots develop as separate mini-organs with different functions and a specific developmental signature

nal and subcutaneous adipose depots in both humans and mice (Vohl et al. 2004; Cantile et al. 2003; Gesta et al. 2006; Tchkonia et al. 2007). Among those genes, intra-abdominal WAT of rodents expressed higher levels of several members of the homeobox gene family HOX, including *HoxA5*, *HoxA4*, *HoxC8*, as well as other developmental genes including, *Glypican 4* (*Gpc4*) and *nuclear receptor subfamily 2 group F member 1* (*Nr2f1* also known as Coup-TF1). Conversely, subcutaneous WAT has been shown to express higher levels of other members of the HOX family, including *HoxA10*, *HoxC9*, and the developmental genes *Twist1* (twist homolog 1), *Tbx15* (T-box15), *Shox2* (Short stature homeobox 2), *En1* (Engrailed 1), and *Sfpr2* (Secreted frizzled-related protein 2). Interestingly, a recent study in mice demonstrated that this profile of expression is not simply dichotomized between intra-abdominal and subcutaneous WAT depots, as these developmental genes have specific patterns of expression when one compares multiple depots throughout the body (Yamamoto et al. 2010).

The precise role played by these developmental genes in adipose tissue is still unclear; however, in humans, *HoxA5*, *Gpc4*, and *Tbx15* expression has been shown to be highly correlated with both obesity (measured by BMI) and fat distribution (measured by WHR) (Gesta et al. 2006). The most striking correlations were observed with *Tbx15*, for which in visceral adipose tissue, a robust exponential negative relationship is observed, with *Tbx15* expression exhibiting a marked decrease as BMI progressed from normal to overweight or obese levels. In addition, a strong exponential negative relationship between *Tbx15* expression and WHR in this tissue has been found, with markedly lower levels of expression observed when WHR is above 1.05 in males and above 0.95 in females. In contrast, *Tbx15* expression in subcutaneous adipose tissue shows a modest, but significant positive correlation with both BMI and WHR in subcutaneous adipose tissue of both males and females (Gesta et al. 2006). Recently, a study in human subjects also reported differential expression of *Tbx15* between subcutaneous (gluteal) and visceral (omental) fat depots. In this study, the authors performed a meta-analysis of genome-wide association studies and observed a single nucleotide polymorphism in the *Tbx15* allele to be strongly associated with WHR in men and women (Heid et al. 2010). Interestingly, another recent study has reported that overexpression of *Tbx15* in murine preadipocytes impairs adipocyte differentiation and mitochondrial mass and respiration, suggesting that differential expression of *Tbx15* between WAT depots plays an important role in controlling both adipocyte development and function that may contribute to the risk of diabetes and metabolic disease (Gesta et al. 2011).

5.6 Is There a Good Fat: An Alternative View

The anatomical location and biological intrinsic properties of intra-abdominal omental and mesenteric WAT both appear to be responsible for their deleterious effects on health. Consistent with this notion, removal of WAT from these depots should therefore be sufficient to improve metabolic dysfunction associated with central obesity. The impact of surgical removal of omental WAT (omentectomy) on

several metabolic parameters has been tested in humans, however, with mixed results. Thorne et al. observed that omentectomy leads to an improvement in glucose tolerance and insulin sensitivity in individuals undergoing adjustable gastric banding. However, in addition to these metabolic improvements, these subjects lost more weight than the group of individuals with adjustable gastric banding alone, complicating conclusions regarding the specific effects of omentectomy (Thorne et al. 2002). Two other studies have reported that omentectomy in addition to a Roux-en-Y gastric bypass procedure in humans exerted no beneficial effects on various metabolic parameters (plasma glucose, plasma insulin, plasma adiponectin, plasma C-reactive protein, lipid profile, blood pressure and glucose tolerance) (Csendes et al. 2009; Herrera et al. 2010). However, these studies are also limited, since weight loss induced by Roux-en-Y gastric bypass surgery could have masked any potential therapeutic effects of the omentectomy (Klein 2010).

Several experiments employing WAT transplantation in mice have provided further insights. Indeed, a recent study demonstrated that transplantation of intra-abdominal WAT into the mesentery (conferring a portal venous drainage) leads to the development of glucose intolerance and hepatic insulin resistance, whereas transplantation of intra-abdominal WAT into the parietal peritoneum (conferring a caval/systemic venous drainage) has no effect (Rytka et al. 2011). These deleterious effects of portally drained intra-abdominal transplantation appeared to be mediated by the production of IL-6, as these effects are abolished when transplants are derived from IL-6 knockout mice. Several studies involving the transplantation of subcutaneous WAT into the visceral cavity have provided some novel perspectives. Indeed, in contrast to intra-abdominal WAT, transplantation of subcutaneous WAT leads to a decrease in total adipose tissue mass, improved glucose tolerance, and improved whole body and hepatic insulin sensitivity (Tran et al. 2008; Hocking et al. 2008). These results strongly suggest that the nature of the WAT rather than the anatomical location per se appears to have a major influence on whole body metabolic homeostasis. Although the mechanisms mediating these beneficial effects remain unknown it seems likely that one or more factors are secreted specifically from subcutaneous adipose tissue which can act on nearby tissues, such as the liver, to improve insulin sensitivity. Whether these interesting findings can be extrapolated to humans remains to be determined.

5.7 Conclusions

WAT is a complex heterogeneous organ with multiple compartments (e.g., intra-abdominal WAT, subcutaneous WAT) and with multiple functions (e.g., metabolic and endocrine). Recent advances in the understanding of these heterogeneities have led to the conclusion that the different WAT depots should be considered as separate mini-organs which most likely arise from different developmental lineages and have different metabolic functions. These intrinsic differences have clearly shown that intra-abdominal and subcutaneous WAT have diametrical consequences on the risk of developing metabolic complications during obesity. The discovery of molecular

mediators of these effects, together with a better characterization of the different developmental lineages of the various WAT depots, will be the next challenge in the development of new therapeutics to fight obesity and its adverse complications.

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Chapter 6
The Physiological Significance of Brown Adipose Tissue and the Beiging of White Adipose Tissue in People

Maria Chondronikola and Labros S. Sidossis

Abstract The recent discovery of functional brown adipose tissue (BAT) in adults has stimulated intense interest for its therapeutic potential against obesity and its related metabolic conditions. Although rodent studies support the importance of brown and beige adipocytes in metabolic health, a smaller number of clinical investigations have examined their physiological significance in people. This chapter provides a critical overview of the published research regarding the metabolic role of BAT and the beiging of white adipose tissue (WAT) in people. In summary, the current available evidence supports the physiological relevance of human BAT in energy, glucose and lipid metabolism. The ability of human WAT to undergo beiging has only recently been demonstrated in patients with severe burn trauma, while its metabolic role remains currently unknown. Future research is needed to gain deeper understanding of the regulating mechanisms and metabolic role of brown and beige adipocytes in health and disease.

Keywords Brown adipose tissue • Beiging • White adipose tissue • Glucose • Lipid • Energy expenditure • Insulin sensitivity • Thermogenesis • Burn trauma

M. Chondronikola
Center for Human Nutrition, Washington University School of Medicine, St. Louis, MO, USA

Harokopio University of Athens, Kallithea, Greece

L.S. Sidossis (✉)
Departments of Medicine and Exercise Science and Sports Studies, Rutgers University, New Brunswick, NJ, USA

Harokopio University of Athens, Kallithea, Greece

University of Texas Medical Branch, Galveston, TX, USA
e-mail: lss133@rci.rutgers.edu

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6.1 Introduction

The prevalence of obesity and its associated metabolic abnormalities (diabetes, insulin resistance, hyperlipidemia) has risen dramatically over the past few decades in the United States (2014) and worldwide. Obesity is characterized by excessive adipose tissue accumulation and ectopic fat deposition resulting in metabolic abnormalities. The current traditional approaches—targeting dietary intake and/or physical activity to induce a negative energy balance—have yielded modest results. Therefore, there is need for novel approaches to combat obesity and its related metabolic pathological conditions.

Thermogenesis comprises a significant component (~15–20%) of the daily energy expenditure (Rolfe and Brown 1997; van Marken Lichtenbelt and Schrauwen 2011), which has been only minimally manipulated as a therapeutic target against obesity and its related metabolic complications. Brown adipose tissue (BAT) appears to be the primary tissue responsible for non-shivering thermogenesis, at least in animals (Cannon and Nedergaard 2004). The unique characteristic of brown adipocytes is the large number of mitochondria enriched with uncoupling protein 1 (UCP1), that uncouples oxidative phosphorylation from chemical energy production, resulting in heat production (thermogenesis).

A few years ago, the (re)-discovery of BAT in adult subjects (Cypess et al. 2009; van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009) triggered intense scientific interest in the potential role of BAT in metabolic regulation in people. More recently, it was confirmed for the first time in people that subcutaneous white adipose tissue (WAT) can adopt a phenotype resembling BAT, including increased UCP1 expression, the presence of smaller multilocular adipocytes and increased respiratory capacity (Sidossis et al. 2015). This phenomenon has been defined as the "*browning*" or "*beiging*" of WAT (Kajimura et al. 2015).

The physiological role of BAT or the beiging of WAT in people is largely unknown and, thus, controversial. The purpose of this chapter is to review the current scientific literature regarding: (1) the physiological relevance of human BAT, (2) the metabolic role of the beiging of WAT in people, and (3) the intrinsic and environmental factors promoting activation (increased metabolic activity), recruitment and/or maintenance of brown and beige adipocytes in humans.

6.2 Human Brown Adipose Tissue Physiology

Brown adipocytes are morphologically and physiologically distinct from white adipocytes. They have a smaller cell size, richer enervation and vascularization, and multilocular appearance compared to white adipocytes (Fruhbeck et al. 2009). A unique feature of the brown adipocyte is the abundance of numerous, large mitochondria that contain copious amounts of the trans-membrane UCP1 (Fig. 6.1). UCP1 allows protons to re-enter the mitochondrial matrix independent of ATP

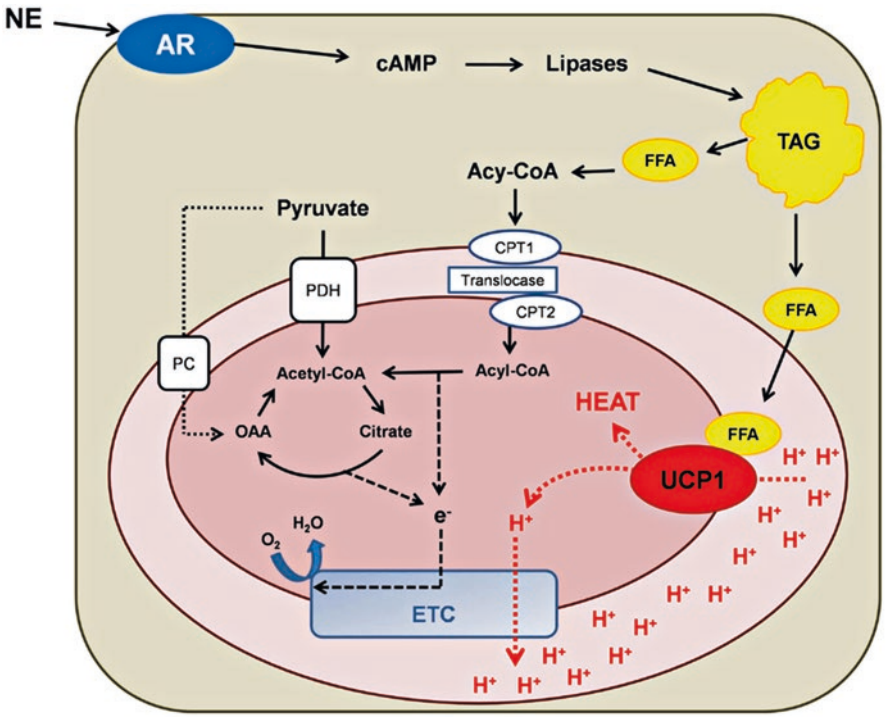

Fig. 6.1 Schematic overview of mitochondrial energy transduction within a brown adipocyte mitochondrion. Norepinephrine (NE) activation of adrenergic receptors (AR) causes an increase in cytosolic cyclic adenosine monophosphate (cAMP) levels. The subsequent activation of lipases results in the lipolysis of triacylglycerol (TAG), which increases intracellular free fatty acid (FFA) concentrations. FFAs fulfill two principal metabolic fates: (i) FFAs bind to and activate uncoupling protein 1 (UCP1), thus switching on the UCP1-mediated proton (H+) conductance; and (ii) activated FFAs (acyl-CoA) can be transported into the mitochondrion via the carnitine palmitoyl transferase (CPT) system and oxidize to acetyl-CoA, thereby potentiating anaplerosis and providing reducing equivalents for the electron transport chain (ETC). Glucose in the form of pyruvate also participates in mitochondrial anaplerosis and the production of reducing equivalent by being decarboxylated to acetyl-CoA by pyruvate dehydrogenase (PDH) or being carboxylated by pyruvate carboxylase (PC), forming oxaloacetate (OAA). Image from Porter et al. (2015). Reprinted with permission

synthase, thus uncoupling mitochondrial respiration from ATP production (Cannon and Nedergaard 2004). Upon stimulation (via cold or other means), BAT consumes glucose and lipids to produce heat (thermogenesis). Brown adipocytes initially mobilize intracellular energy substrates, but the intracellular lipid stores in BAT are insufficient to sustain thermogenesis for a prolonged time. Therefore, upon prolonged stimulation, the activated brown adipocytes rely on circulating plasma fuel substrates [glucose, triglycerides (TG) and free fatty acids (FFA)].

6.3 Developmental Origins of BAT

Since the sampling of BAT in people is laborious (Chondronikola et al. 2015), the majority of the evidence regarding the developmental origins of BAT comes from animal studies. According to tracing studies, brown adipocytes (also known as "*classical*" or "*constitutional*" brown adipocytes) first derive from the same precursors as skeletal muscle cells (dermomyotomal precursors) expressing *engrailed-1* (*En1*) (Atit et al. 2006), and the myogenic factors *myogenic factor 5* (*Myf5*) (Seale et al. 2008) and *paired box protein* (*Pax7)* (Lepper and Fan 2010). Consistent with the results of the cell lineage studies, transcriptome analysis studies also support the idea that the gene-expression pattern of brown adipocytes resembles that of skeletal muscle cells more than that of white adipocytes (Timmons et al. 2007; Walden et al. 2009).

However, results from animal studies support the idea that, under the influence of different adrenergic and other stimuli, UCP1-positive adipocytes can emerge within the epididymal adipose tissue (also known as "*beige*" or "*brite*" adipocytes from brown in white or "inducible") (Wu et al. 2012). Similar to white adipocytes, beige adipocytes derive from the mesodermal stem cell line (Sidossis and Kajimura 2015). Although the developmental origin of beige adipocytes is currently a matter of scientific debate, brown and beige adipocytes appear to be functionally similar (Shabalina et al. 2013).

6.4 Definition of Human BAT

Currently the cellular identity of human BAT remains controversial. Wu et al. first reported that the gene-expression profile of human BAT resembles most murine beige adipose tissue (Wu et al. 2012). By contrast, Lindell et al., after analyzing intra-scapular adipose tissue samples collected postmortem from infants, concluded that the gene expression pattern of those samples was similar to classical brown adipocytes (Lidell et al. 2013). Further studies to clarify the cellular lineage of BAT yielded mixed results (Jespersen et al. 2013; Sharp et al. 2012). In an attempt to explain this inconsistency, Cypress et al. found that the genetic signatures of the adipose tissue depended on the depth of the tissue sampled (Cypess et al. 2013). However, the inconsistent results between the different studies appear to be attributable to the heterogeneity of the tissue, i.e. brown, beige and white adipocytes coexist in the adipose tissue samples. To overcome this limitation, Shinoda et al. isolated UCP1-positive adipocytes from supraclavicular adipose tissue samples collected from adult subjects (Shinoda et al. 2015). Interestingly, the gene-expression patterns of those cells resembled recruitable beige adipocytes more than the classical brown adipocytes, indicating that adult human BAT consists of recruitable UCP1-positive adipocytes.

Considering the results of all the previously mentioned studies, human BAT deposits most probably are comprised of at least three co-existing types of cells (i.e., white, beige and brown adipocytes). The brown and beige adipocytes appear to be functionally similar (Shabalina et al. 2013), although no currently available imaging method can discern the two cell types *in vivo*. Thus, to avoid any confusion, for the rest of this chapter the term BAT in people will be used to refer to any adipose tissue deposits containing significant amounts of UCP1-positive cells (Cypess et al. 2014).

6.5 The Rediscovery of Human BAT

Since the beginning of the twentieth century, anatomists have noted that the adipose tissue is not just a homogenous lipid-containing connective tissue (Shattock 1909), but it is highly heterogeneous. Human BAT has been described as "gland-like lobulated" adipose tissue (Shattock 1909), located in the back between the shoulder blades (Bonnot 1908). Later, other investigators localized this different type of fat in the intrascapular, axillae, paravertebral, and peri-renal areas both in infants (Ito and Kuroshima 1967) and adults (Cramer 1920; Heaton 1972). Although these early studies supported the idea that BAT is present in adults (Bouillaud et al. 1983; Heaton 1972; Huttunen et al. 1981; Huttunen and Kortelainen 1990) up to the ninth decade of life (Lean 1989), the notion that only animals and newborns possess physiologically significant amounts of BAT prevailed (Cunningham et al. 1978; Lean 1989) and the scientific interest in human BAT did not gain traction.

It was not until 2002 when radiologists, conducting positron emission tomography/computed tomography (PET/CT) scans using radiolabeled 2-Deoxy-2-[^{18}F] fluoroglucose (^{18}F-FDG) for cancer diagnostic purposes, reported an unusual, symmetrical, increased radiolabeled glucose uptake in the adipose tissue near the base of the neck, thorax and shoulder area that did not correspond to cancerous lesions (Hany et al. 2002). Like malignant tumors, the metabolically active BAT takes up significant amounts of glucose from the circulation, and thus, it can be easily visualized in PET/CT images. BAT became known in the nuclear medicine literature as "USA" fat (upper supraclavicular area fat) (Cohade et al. 2003b). The prevalence of this increased ^{18}F-FDG uptake pattern was associated with lower outdoor temperature (Christensen et al. 2006; Cohade et al. 2003a; Kim et al. 2008), leanness (Sturkenboom et al. 2004) and female gender (Cohade et al. 2003a; Truong et al. 2004). Propranolol administration abolished the increased ^{18}F-FDG uptake in the supraclavicular adipose tissue (Jacobsson et al. 2005; Tatsumi et al. 2004). Although radiologists were convinced that the observed increased metabolic activity corresponded to hypermetabolic BAT deposits (Hany et al. 2002), the rest of the scientific world continued to ignore its presence.

As recently as 2007, Nedergaard et al. summarized the previously mentioned findings supporting the long-neglected notion that adults possess significant

amounts of BAT (Nedergaard et al. 2007). In 2009, three independent studies provided solid evidence that adults possess significant amounts of functional BAT (Cypess et al. 2009; van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009).

6.6 Anatomical Localization of Human Brown Adipose Tissue

According to its anatomical localization, human BAT can be divided in to two categories: subcutaneous BAT and visceral BAT (Sacks and Symonds 2013). *Subcutaneous* BAT is localized in the supraclavicular area and around the neck muscles (Cypess et al. 2013), clavicles and axillary areas, inguinal fossa and the anterior abdominal wall (Heaton 1972). *Visceral* BAT is located around vessels (neck vessels, intercostal vessels, mammary vessels and the paraortic area) (Wei et al. 2015), around hollow muscular structures (trachea-esophagus, heart, lung hilium, omental fat, mesocolon and diaphragm) (Bar-Shalom et al. 2004; Chechi et al. 2013) and around solid organs (pancreas, kidneys, paravertebral area, adrenals and spleen) (Astrup et al. 1985; Cunningham et al. 1985; Heaton 1972). From an evolutionary perspective, the localization of the BAT in specific anatomic areas, adjacent to large vessels and vital organs, would maximize its thermogenic efficiency (i.e., protecting the vital organs of the body from hypothermia).

6.7 Physiological Significance of Human BAT in Energy Expenditure

The role of human BAT in energy balance remains highly controversial. The first evidence supporting the role of BAT in energy expenditure came from acute cold exposure studies involving no or minimal shivering. Results from these studies indicated that mild cold exposure increased resting energy expenditure (REE) but the reported effect size was quite variable. In the majority of clinical trials conducted, the investigators reported that non-shivering cold induces BAT activation, leading to a 13–27% increase in REE (Chondronikola et al. 2014; van Marken Lichtenbelt et al. 2009; Yoneshiro et al. 2011). Ouellet et al. reported an 80% increase in REE, a result approximately threefold higher than those from other investigations and possibly attributable to muscle shivering (Ouellet et al. 2012). Muzik et al. used oxygen 15 (^{15}O)-PET/CT in an attempt to more directly quantify the contribution of BAT to energy expenditure (Muzik et al. 2012, 2013). In contrast to previous studies, BAT only minimally contributed to the reported increase in energy expenditure (15–25 kcal per day). Although these last two studies shed doubt on the role of BAT in energy expenditure in people, the very short cold stimulation protocol (30 min) may not have been adequate to maximally activate

BAT. Nevertheless, one important point to consider is that chronic weight gain is a result of a small and chronic positive energy imbalance (e.g., an energy surplus of as little as 25 kcal per day can result in a long-term weight gain of 10 kg in a decade).

Experimental studies, so far, have focused on the role of BAT in energy expenditure and not on the other part of the equation, i.e., energy intake. Currently, only one study has attempted to investigate the role of BAT activation in appetite regulation using appetite- and hunger-related visual analog scales. According to the results of this study, BAT activation did not contribute to an increased appetite (Lee et al. 2014). However, this question remains to be further investigated, since the sample in the previous study was very small and the measures used to determine appetite have high variability.

Indices of adiposity can be used as surrogate markers of long-term energy balance. Retrospective studies support the notion that having detectable levels of BAT is inversely associated with body mass index (BMI) (Cypess et al. 2009; Lee et al. 2010; Ouellet et al. 2011) and non-alcoholic fatty liver disease (Yilmaz et al. 2011). Similarly, prospective studies have correlated BAT volume and activity with BMI (Saito et al. 2009; van Marken Lichtenbelt et al. 2009), total fat mass (Saito et al. 2009; van Marken Lichtenbelt et al. 2009) and abdominal subcutaneous and visceral adiposity (Saito et al. 2009). In line with the previous data, BAT recruitment using daily exposure to cold exposure at 17 °C, 2 h a day for 6 weeks has been associated with a decrease in body fat, supporting the temporal relation of BAT activity with adiposity (Yoneshiro et al. 2013).

Overall, the current evidence supports the role of BAT in obesity and energy metabolism in people. However, whether BAT activation leads to weight loss remains to be determined by long-term studies in clinically relevant populations.

6.8 Physiological Significance of Human BAT in Glucose Metabolism

The involvement of human BAT in glucose metabolism is rather apparent, as the first evidence for the presence of functional BAT in adults came from the increased ^{18}F-FDG uptake (radiolabeled glucose) noted in the images of PET/CT scans performed for diagnostic purposes (Hany et al. 2002). Moreover, evidence from retrospective medical record review studies indicates an inverse relationship between BAT activity and diabetes and glycemia (Cypess et al. 2009; Jacene et al. 2011; Matsushita et al. 2013; Ouellet et al. 2011). Furthermore, results from animal studies indicate that BAT is responsible for ~75% of total glucose clearance (higher than muscle), while cold-induced BAT activation has been shown to improve glucose tolerance in obese mice (Bartelt et al. 2011). Similarly, transplantation of BAT improves whole-body glucose metabolism in mice (Stanford et al. 2013). However, the evidence in rodents and results from retrospective

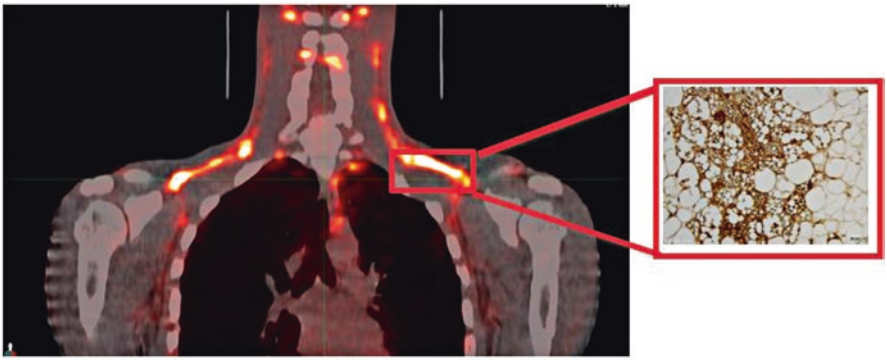

Fig. 6.2 Coronal 2-deoxy-2-[18F]fluoro-D-glucose (18F-FDG) positron emission tomography-computed tomography (PET-CT) image from a volunteer during cold exposure. The intense orange color in the supraclavicular area corresponds to brown adipose tissue. The tissue image represents paraffin-blocked supraclavicular adipose tissue stained for uncoupling protein 1. Image from Chondronikola et al. (2014). Reprinted with permission

investigations do not themselves establish the role of BAT in whole-body glucose homeostasis in people.

Therefore, prospective studies were conducted to investigate the role of BAT in glucose metabolism. According to those studies, acute cold stimulation increased glucose uptake in BAT, while the glucose uptake rate was higher than that noted in muscle or WAT (Orava et al. 2011, 2013; Ouellet et al. 2012). Specifically, Orava et al. reported that cold exposure resulted in a 12-fold increase in glucose disposal in BAT, but not in other tissues (Orava et al. 2011). This result suggests that, upon activation, BAT clears glucose from the circulation. Figure 6.2 presents the PET/CT images of a representative individual with increased ^{18}F-FDG uptake in the supraclavicular adipose tissue deposit during cold exposure corresponding to BAT. The results from the aforementioned studies raise the question of the role of BAT in whole-body metabolism in people. Ouellet et al. demonstrated that BAT minimally contributed to whole-body plasma glucose utilization (Ouellet et al. 2012), which might be due to the relatively short duration of cold exposure and the presence of mild shivering (muscle contraction) during cold exposure activating glucose uptake from the skeletal muscle (Blondin et al. 2015). To address the question of the physiological role of BAT in whole-body glucose metabolism, we studied men with significant amounts of BAT and those with no or minimal BAT (Chondronikola et al. 2014). According to our results, tissue-specific glucose uptake increased significantly only in BAT, while only individuals with high amounts of BAT demonstrated a significant increase in whole-body glucose uptake.

Overall, the current evidence supports the physiological role of BAT in whole-body glucose metabolism in people. Since emerging evidence in rodents support the cross-talk of BAT with other metabolically-active tissues (Berbee et al. 2015; Stanford et al. 2013), it will be important for future studies to clarify the indirect effects of BAT in glucose metabolism.

6.9 Physiological Significance of Human BAT in Lipid Metabolism

Results from rodent studies recently confirmed the role of BAT in lipid metabolism (Bartelt et al. 2011; Berbee et al. 2015). FFAs constitute the primary substrate for BAT, while they activate UCP1 to produce heat (Ma and Foster 1986). Upon adrenergic stimulation, BAT initially uses the intracellular lipid stores to produce heat (Cannon and Nedergaard 2004). Nonetheless, prolonged BAT activation leads to increased uptake of FFA derived from lipolysis. BAT activation has been recently reported to increase the systemic clearance of TG-rich lipoproteins in rodents, lending credence to the notion that BAT may protect against hyperlipidemia (Bartelt et al. 2011; Berbee et al. 2015). Activated BAT is thought to be responsible for approximately 50% of triglyceride clearance (Bartelt et al. 2011; Nedergaard et al. 2011). Moreover, rodent BAT has been shown to release factors that increase vascular permeability, allowing TG-rich lipoproteins to enter the interstitial space (Bartelt et al. 2011).

The role of BAT lipid metabolism remains largely unexplored in people. The majority of studies (Orava et al. 2011; van Marken Lichtenbelt et al. 2009)—but not all (Yoneshiro et al. 2011)—report a decreased respiratory quotient during cold exposure, indicating a shift of the substrate metabolism towards fat oxidation and a potential role for BAT metabolism in people. Additionally, Ouellet et al. (2012) conducted a more targeted study in six young healthy adults to investigate the role of human BAT in lipid metabolism using PET/CT and a fatty acid tracer, ^{18}F-fluoro-thiaheptadecanoic acid (^{18}FTHA). According to their results, cold exposure increased FFA uptake in BAT, but it accounted for less than 1% of the total FFA turnover (Ouellet et al. 2012). The conclusion of this study was that thermogenic BAT relies predominantly on the limited intracellular substrates (i.e., triglycerides stored inside BAT) to fuel mitochondrial thermogenesis (Ouellet et al. 2012). However, this result might be attributable to the relatively short duration of the study. Moreover, Blondin et al. recently reported that BAT activity was correlated with cold-induced lipolysis in young, lean adults (Blondin et al. 2015). To comprehensively investigate the role of BAT in whole body lipid kinetics, we studied individuals with various amount of BAT during prolonged (5–6 h) mild cold exposure and thermoneutral conditions. Our findings support the notion that BAT volume is associated with increased whole-body lipolysis, TG-FFA cycling and FFA oxidation, while functional and molecular analyses of BAT samples further support the role of BAT in lipid mobilization and clearance (Chondronikola et al. 2016a). Moreover, we observed that prolonged (8 h) non-shivering cold exposure led to decreased plasma very low-density lipoprotein and plasma concentrations, indicating that BAT activation may have a delayed effect on triglycerides and lipoprotein metabolism (Chondronikola et al. 2016a). Future studies are needed to investigate the role of BAT in lipoprotein metabolism in people.

6.10 Physiological Significance of Human BAT in Inulin Sensitivity

Results from studies in people and rodents support the role of BAT in insulin sensitivity. Insulin resistance constitutes one of the major factors contributing to the pathogenesis of diabetes and its associated metabolic disorders (Defronzo 2009). Insulin regulates numerous metabolic processes in various tissues. Therefore, multiple organs contribute to the development of insulin resistance (Conte et al. 2012). Insulin resistance refers to the responsiveness of different tissues in the action of insulin (Kahn 1978). In rodents, BAT transplantation increases the insulin-stimulated glucose uptake in BAT and other tissues (WAT and heart muscle) (Stanford et al. 2013). In people, hyperinsulinemia acutely increases glucose uptake in BAT similar to muscle, indicating that BAT is an insulin sensitive tissue (Orava et al. 2011). In healthy young adults, cold acclimated BAT has been associated with an increased insulin sensitivity response to a mixed meal (Lee et al. 2014). Moreover, in patients with type 2 diabetes, prolonged acclimation increased BAT activity, improved insulin-stimulated glucose uptake and decreased fasting FFA levels (an indirect measure of adipose tissue insulin sensitivity) (Hanssen et al. 2015a). Our recent results further support the role of BAT in multi-organ insulin sensitivity. Specifically, we recently demonstrated that BAT activation is linked to improved whole-body insulin sensitivity in people (Chondronikola et al. 2014). Moreover, we recently reported that BAT activation is also associated with an increase in adipose tissue insulin sensitivity during cold (Chondronikola et al. 2016a). Liver constitutes a master regulator of whole-body metabolism, and some evidence supports a potential cross-talk between BAT and the liver (Berbee et al. 2015; Yilmaz et al. 2011). Further research is needed to investigate the relationship of BAT and liver metabolism in people.

6.11 Physiological Significance of Human BAT in Thermoregulation

Thermoregulation is a vital homeostatic mechanism that helps maintain body core temperature within a narrow range, despite large fluctuations in ambient temperature (Mekjavic and Eiken 2006). Deviation from this normal range may indicate the presence of a pathological condition, and can be lethal in extreme cases. In rodents, BAT has been confirmed as the primary thermoregulatory tissue during non-shivering cold exposure in mammals (Cannon and Nedergaard 2004), while the numerous mitochondria containing high amounts of UCP1 (also known as *thermogenin*) are considered to be the primary mechanism responsible for the heat production. Hence, other metabolic processes appear to be also involved in the thermogenic role of BAT [e.g., futile cycling of creatine and creatine phosphate (Kazak et al. 2015)].

The role of human BAT in thermoregulation has not been studied systematically in people. Historically, BAT has been reported to play a thermoregulatory role in infants (Dawkins and Scopes 1965; Ito and Kuroshima 1967; Silverman et al. 1964). Acute cold exposure activates BAT (Chondronikola et al. 2014; Ouellet et al. 2012; van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009), while cold acclimation for several days can further increase BAT activity (Blondin et al. 2014; Hanssen et al. 2015a, c; Lee et al. 2014; van der Lans et al. 2013; Yoneshiro et al. 2013) and increase thermal comfort and trunk skin temperature (Hanssen et al. 2015a, c; van der Lans et al. 2013). However, the "dose-response" link between those thermoregulatory responses and BAT activity has been missing. When we studied subjects with a wide range of detectable BAT, we found that the presence of high amounts of detectable BAT was associated with a higher tolerance to cold (core body temperature during cold exposure and a lower tolerated cold temperature without shivering) (Chondronikola et al. 2016b), supporting a physiologically significant role for BAT in thermoregulation in people. Moreover, we (Chondronikola et al. 2016b) and others (Boon et al. 2014; Jang et al. 2014; Symonds et al. 2012; van der Lans et al. 2016) have demonstrated that the skin temperature over the supraclavicular depot during cold exposure is correlated with BAT activity, providing evidence for the thermogenic role of BAT and a potential surrogate marker of BAT activity.

6.12 Evidence of the Browning of Subcutaneous WAT in People

The ability of white adipocytes to adopt a thermogenic phenotype similar to the brown adipocytes ("beiging" of WAT) in response to prolonged exposure to cold has been reported more than three decades ago (Young et al. 1984). Later studies demonstrated that other adrenergic stimuli (beta 3 adrenergic agonists) also trigger the beiging of WAT (Cousin et al. 1992). The thermogenic capacity of acclimated WAT is estimated to be at maximum, approximately 50% that of BAT (Shabalina et al. 2013). Considering that WAT constitutes a significant proportion of the total body weight in adults (about 10–35% of body weight for a normalweight individual depending on gender), it becomes apparent that the beiging of WAT may have important physiological and clinical implications for obesity and its associated metabolic abnormalities.

Interspecies differences in adipose tissue bioenergetics do not always allow the direct translation of rodent studies to people. Therefore, confirmation of the previously mentioned findings in humans is pending. Studies in which abdominal subcutaneous WAT samples were obtained before and after acclimation (duration 10 days to 4 weeks) failed to report evidence of WAT beiging (evaluated as the expression of thermogenic genes and respiratory capacity of the tissue) (Hanssen et al. 2015a; Lee et al. 2014; van der Lans et al. 2013). These results suggest that a more intense or prolonged stimulation than that used in the previously mentioned

studies may be necessary to induce the beiging of WAT. Alternatively, WAT depots other than the subcutaneous abdominal WAT may undergo beiging.

Results from several small case-control studies including patients with wasting and hypermetabolic pathological conditions such as pheochromocytoma (English et al. 1973; Frontini et al. 2013) and cancer cachexia (Petruzzelli et al. 2014) have reported evidence of the beiging of WAT depots. Although these results suggested that the beiging of subcutaneous WAT is possible in people, the cross-sectional study design of these studies did not allow establishment of a temporal relationship. Moreover, the histological and/or genetic analysis of the collected tissues neither allowed for the functional assessment of these tissues nor provided evidence for their physiological significance.

Burn trauma, which involves prolonged and high adrenergic stress, has been shown to induce increased BAT metabolic activity in rodents (Carter et al. 2011). Therefore, we hypothesized that burn trauma-related adrenergic stress may induce beiging of WAT in people. To this end, we prospectively studied patients with severe burn injury shortly after the injury and approximately 2 weeks after admission to the hospital for acute treatment (Sidossis et al. 2015). Approximately 2 weeks after the burn trauma, remodeling of the subcutaneous adipose tissue occurred, including the presence of smaller multilocular adipocytes with high UCP1 staining (Fig. 6.3). The histological changes were accompanied by functional and molecular changes, including increased subcutaneous WAT UCP1 mRNA and protein expression,

Fig. 6.3 (continued) (**a***i*) sWAT from a healthy child obtained at the time of elective surgery. No UCP1 staining was evident. The adipocytes contain one large lipid droplet and there is very little tissue between the adipocytes. (**a***ii*) Representative appearance of sWAT from a burned patient (at 45 days postburn; 14-year-old male). The largest observed adipocytes are smaller than those in the control tissue. Multiple fat droplets are observable in some adipocytes, and some cells between the large adipocytes contain multiple small fat droplets and are positive for UCP1. (**a***iii*) Negative control from a burn patient. (**b**) Prospective evidence of UCP1 induction in human WAT. (**b***i*–**b***vi*) Morphological and immunohistochemical evidence of browning over time in a representative patient. One day after burn, the adipocytes appear similar to the control samples. Small blood vessels appear to stain positively for UCP-1. At 9–21 days after burn, a peripheral rim of UCP1-positive cytoplasm containing many tiny fat droplets is present at the periphery of a large fat droplet. A few small cells staining positive for UCP1 are observed between the adipocytes. At 27–31 days after burn, large adipocytes are separated by an interstitium that contains multiple elongated cells positive for UCP1, some containing small lipid droplets. In addition, scattered large adipocytes contain multiple fat droplets rather than one large fat droplet per cell (multilocular and paucilocular cells). (**c**) Ultrastructural evaluation of white adipocytes from control and burn patients. Composite electron micrograph of a typical adipocyte (diameter 95.8 mm) from a healthy child undergoing elective surgery (**c***i*). This image of an entire lipid droplet was produced from a collage of six separate electron micrograph images. The nucleus is out of the plane of the section. As shown at higher magnification below, only a very thin rim of cytoplasm with few mitochondria (M) separates the lipid droplet (L) from extracellular collagen fibers (C). Representative adipocyte (diameter 46.6 mm) from a child 21 days after burn. (**c***ii*) Surrounding a single large lipid droplet is a rim of cytoplasm (C) that contains many small (0.5–1 mm) lipid droplets (L). The adipocyte nucleus (N) and many small and medium size electron-dense mitochondria (M) are also present in the cytoplasmic rim (C). Image from Sidossis et al. (2015). Reprinted with permission

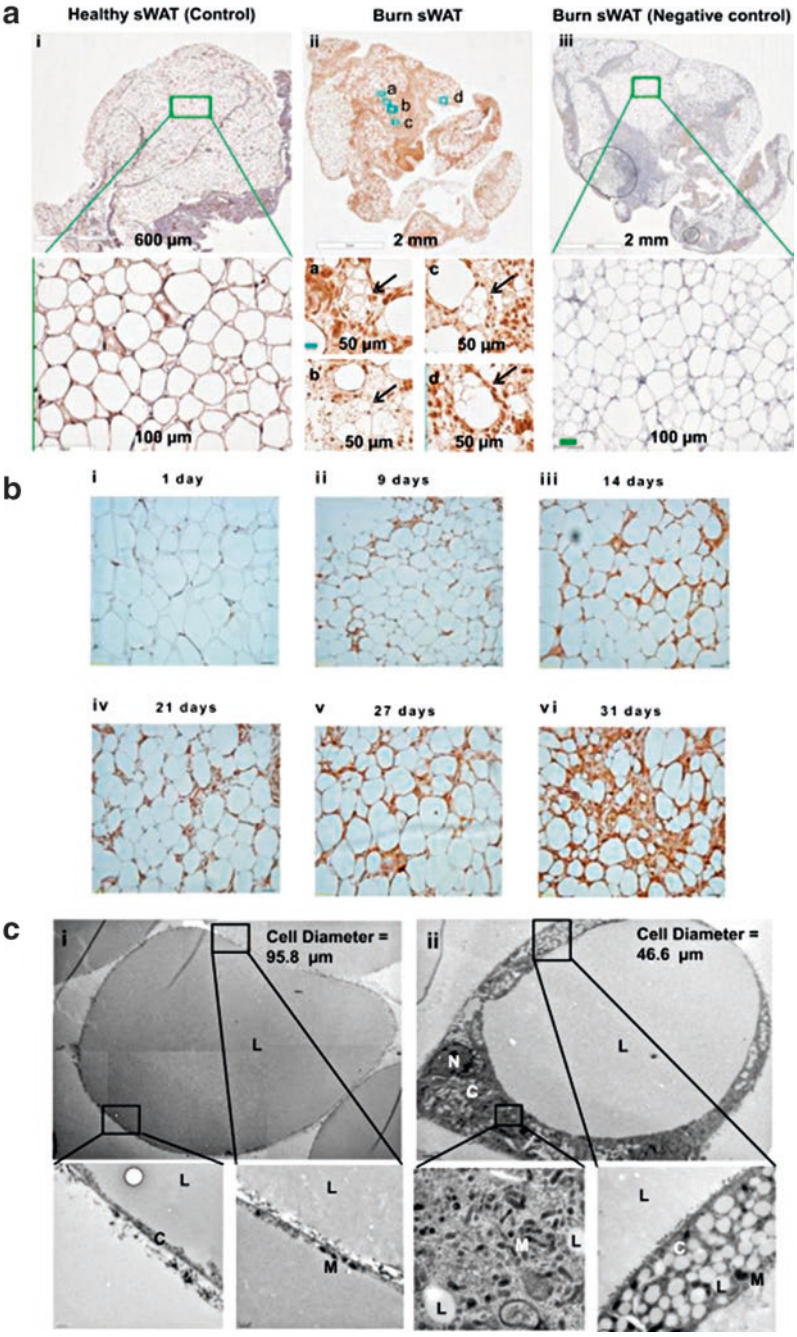

Fig. 6.3 Burn injury alters adipocyte morphology. (**a**) Morphological and immunohistochemical evidence of browning. Micrographs of paraffin sections of WAT from burned and healthy children (**a**). All images show immunohistochemical staining for uncoupling protein 1 (UCP1) protein.

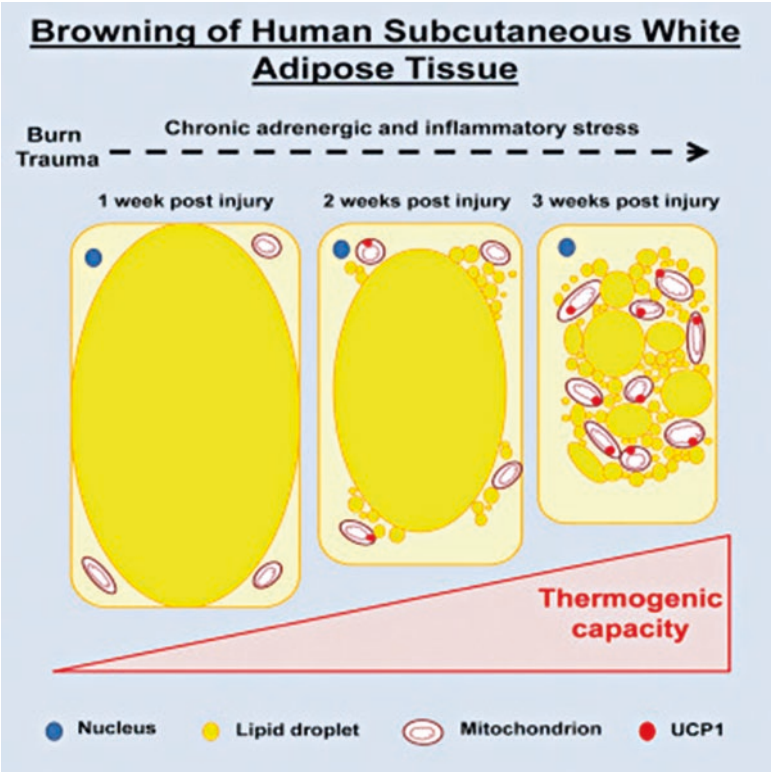

Fig. 6.4 Schematic overview of the browning of white adipose tissue in patients with severe burn trauma. Image from Sidossis et al. (2015). Reprinted with permission

respiratory capacity and whole-body energy expenditure (Fig. 6.4) (Sidossis et al. 2015). To the best of our knowledge, these results support for the first time that beige adipocytes can emerge within the subcutaneous WAT in people. Another study published a few months later independently confirmed these results (Patsouris et al. 2015).

6.13 Physiological Significance of the Beiging of Human WAT

The beiging of WAT has attracted intense scientific interest as a potential therapeutic target against obesity and metabolic syndrome. Results from different rodent models support the physiological significance of the beiging of WAT (Cohen et al. 2014; Ohno et al. 2012; Seale et al. 2011).

The physiological significance of the beiging of WAT in people remains unknown and highly controversial. The association of the beiging of WAT with hypermetabolic

conditions involving significant involuntary weight loss [i.e., burn trauma (Sidossis et al. 2015) and cancer cachexia (Petruzzelli et al. 2014)], suggests a potential role for the beiging of WAT in energy metabolism in people. Using results from our previous studies in healthy individuals and patients with burn trauma along with the estimated contribution of the major metabolically active tissues in the hypermetabolic response noted in burn individuals, we estimated the contribution of the beiging of WAT in whole-body REE. Patients with larger burns experience a significant increase in REE (Wilmore et al. 1974). Several tissues are known to contribute to this increase, while it is estimated that ATP requiring processes account for up to 60% of the increased metabolic rate (Yu et al. 1999). This indicates that thermogenesis accounts for a significant proportion of the increased energy expenditure, presumably to compensate from the accelerated heat loss from the loss of the skin barrier. According to our estimations [described in a greater detail elsewhere (Porter et al. 2015)], the beiging of WAT could explain approximately 10% of the burn-induced hypermetabolism. Moreover, our preliminary results support the idea that the change in the uncoupled respiratory capacity and the coupling control ratio (the index of thermogenic capacity per mitochondrion) of WAT is correlated with the rate of the appearance of FFA (lipolysis). These results indicate that the beiging of WAT may have implications in the regulation of lipid metabolism at the whole-body level (Chondronikola et al. unpublished observations).

Although this early evidence supports the potential role of the beiging of WAT in energy metabolism and potentially in lipid turnover, we need to emphasize that the severe adrenergic stress in burn patients (which may mediate the beiging of WAT) has a deleterious impact in many other tissues. Therefore, the development of efficacious interventions to safely stimulate the beiging of WAT is needed for its therapeutic potential to be realized.

6.14 Factors Affecting Brown Adipose Tissue Activation and Recruitment and Beiging of WAT

The (re)-discovery of human BAT has triggered scientific interest in the ability of different intrinsic and environmental factors to either activate BAT or result in the beiging of WAT. Numerous factors have been shown to activate BAT or induce the browning of WAT. Here, we will focus on the factors that have been shown to be relevant in humans.

Cold Temperature: Environmental exposure to temperatures below thermoneutrality is currently the most widely studied approach to activating BAT thermogenesis, both in people and rodents. Cold exposure has been shown to induce BAT hypertrophy and *UCP1* expression in BAT, and to promote the beiging of WAT in rodents (Barbatelli et al. 2010; Cannon and Nedergaard 2004). However, the results of rodent studies cannot always be translated in people, especially considering the usual animal housing conditions (~22 °C) that are below the thermoneutral zone for rodents (Cannon and Nedergaard 2010).

Studies using ^{18}F-FDG-PET/CT imaging have reported that chronic cold acclimation increases BAT volume and/or BAT glucose uptake and oxidation in the supraclavicular area (Blondin et al. 2014; van der Lans et al. 2013; Yoneshiro et al. 2013). These results have been reported in young and lean (van der Lans et al. 2013), obese (Hanssen et al. 2015b) and older individuals with type 2 diabetes (Hanssen et al. 2015a). This increase was reversible upon the removal of the stimulus (Lee et al. 2014). Since the BAT of these patients was not sampled before and after acclimation, it remains unknown whether the reported increase in BAT activity is attributable to a higher capacity of those cells for glucose uptake, the transformation of existing white adipocytes into brown adipocytes or the recruitment of brown progenitor cells.

Whether cold exposure leads to the beiging of WAT remains debatable in people. In a recent study, increased *UCP1* expression was reported during winter and after an acute cold stimulus in subcutaneous abdominal WAT samples collected from healthy young adults (Kern et al. 2014), but no additional molecular or functional data were reported to support the beiging of WAT. When abdominal subcutaneous WAT samples were obtained and analyzed before and after acclimation (10 days to 4 weeks), no evidence was reported of the beiging of WAT in young healthy and older diabetic individuals (evaluated as the expression of thermogenic genes and the respiratory capacity of the tissue) (Hanssen et al. 2015a; Lee et al. 2014; van der Lans et al. 2013). These results suggest that a more intense or prolonged stimulation than that used in these protocols may be necessary to induce the beiging of WAT. Alternatively, WAT depots other than the subcutaneous abdominal WAT may undergo beiging.

Exercise: Rodent studies support the hypothesis that exercise induces the beiging of WAT (Bostrom et al. 2012; Xu et al. 2011). Several factors have been proposed to mediate the exercise-induced beiging of WAT, including the secretion of several myokines (irisin, meteryn-like hormone, interleukin 6 [IL6]) or lactate (Bostrom et al. 2012; Carriere et al. 2014; Knudsen et al. 2014; Rao et al. 2014). Although the reported evidence on the role of exercise in the browning of WAT in rodents are convincing, the results for exercise interventions in humans are not. Specifically, when endurance athletes were compared to sedentary individuals (Vosselman et al. 2015), athletes appeared to have lower BAT activity as measured by ^{18}F-FDG-PET/CT imaging, while no differences have been noted in the expression of thermogenic gene markers. Moreover, results of a retrospective data analysis from a training intervention also failed to report evidence of beiging of WAT (Norheim et al. 2014). Although the current literature does not support the idea that exercise activates BAT or induces the beiging of subcutaneous WAT in people, future studies are needed to prospectively investigate the role of exercise in the browning of WAT.

Food Compounds: Naturally occurring compounds and food ingredients have been proposed to stimulate BAT thermogenesis. Capsinoids are naturally occurring substances in red peppers and several other spices which are responsible for the hot sensation of those food items. Acute administration (Yoneshiro et al. 2012) and 6-week supplementation (Yoneshiro et al. 2013) with 9 mg of non-pungent capsinoids per day increased cold-induced thermogenesis (a surrogate marker of BAT

activity) in healthy lean participants. Similarly, acute supplementation with grains of paradise (also known as *Aframomum melegueta*, a species in the ginger family) has been shown to increase cold-induced thermogenesis in adults with detectable levels of BAT (Sugita et al. 2013), implying that grains of paradise may activate human BAT. Capsinoids and grains of paradise have been proposed to activate BAT thermogenesis via the transient receptor potential cation channel subfamily V member 1 (TRVP1). Both compounds contain a vanilloid moiety that stimulates TRVP1, which interacts with the sympathetic nervous system leading to increased BAT thermogenesis (Saito and Yoneshiro 2013). Preliminary studies in people suggest that supplementation with naturally occurring compounds such catechins (Nirengi et al. 2015), menthol or other similar compounds may also increase BAT activity in healthy lean adults. Further studies should determine the long-term efficacy and the effectiveness of those interventions in populations of clinical interest.

Adrenergic Stimulation: Different pharmaceutical compounds, acting on the sympathetic nervous system, have been shown to activate/recruit or block BAT metabolic activity in healthy normal-weight adults. Administration of the non-selective beta-blocker propranolol has been shown to decrease ^{18}F-FDG-PET/CT BAT activity (Parysow et al. 2007; Soderlund et al. 2007). On the other hand, oral administration of 2.5 mg/kg ephedrine (sympathomimetic amine) induces BAT activity (Carey et al. 2013), while causing a pronounced increase heart rate and blood pressure, which precludes the routine use of this agent as a therapeutic intervention to activate BAT. However, oral administration of 200 mg mirabegron (a beta 3 adrenergic agonist) significantly increased BAT activity in healthy adults while causing only a small increase in heart rate and blood pressure (Cypess et al. 2015), making administration of mirabagron a potentially viable therapeutic modality to activate BAT thermogenesis. However, evidence of the long-term effectiveness/efficacy of this agent in clinically relevant populations (such as individuals with obesity or diabetes) remains to be determined.

Currently, there is no evidence to suggest that the administration of sympathomimetics can promote the beiging of WAT in people. Although the beiging of WAT may be attributable to the trauma-related catecholamine surge (Wilmore et al. 1974), it remains unknown if the catecholamines are solely responsible for the observed phenotype, as discussed in the *Burn Injury* section below.

Burn Injury: We (Sidossis et al. 2015) and others (Patsouris et al. 2015) have recently reported evidence of browning of WAT patients with large burns. Although the trauma-related increase in the circulating levels of catecholamines might be the major pathway responsible for the beiging of WAT, burn patients also demonstrate increases in circulating concentrations of several circulating factors and immune cell infiltration of adipose tissue factors that may also contribute to the beiging of WAT. Elucidation of the underlying mechanism is of major importance and it may potentially lead to the discovery of a novel pathway for the browning of WAT in people. Finally, understanding the mechanism of WAT browning in burn patients may also have therapeutic implications for patients with severe burn who experience hypermetabolism.

Bariatric Surgery: Bariatric surgery is thought to be a highly effective approach for the treatment of obesity and diabetes (Bradley et al. 2012b). The bariatric surgery procedures, especially those involving upper intestinal bypass, result in changes in the circulating levels of bile acids (Kohli et al. 2013), glucagon-like peptide 1 (Bradley et al. 2012a), natriuretic peptides (Neinast et al. 2015) or other factors, which have been also shown to increase BAT activity and/or induce the beiging of WAT (Beiroa et al. 2014; Bordicchia et al. 2012; Broeders et al. 2015; Watanabe et al. 2006). Recently, Neinast et al. reported increased *UCP1* expression in WAT after Roux-en-Y gastric bypass in rodents, while no changes were noted in sham operated mice that lost equal weight with caloric restriction (Neinast et al. 2015). Currently, there is no evidence to support the beiging of WAT in patients after bariatric surgery. However, weight loss after bariatric surgery has been shown to lead to increased BAT activity in obese patients who do not have type 2 diabetes (Rachid et al. 2015; Vijgen et al. 2012). Further research is needed to confirm the potential role of bariatric surgery in the beiging of WAT and the potential contribution of increased BAT activation to the metabolic improvements that can occur.

Natriuretic peptides: The natriuretic peptides are a family of hormones [atrial NP (ANP), B-type NP (BNP) and C-type NP] thought to be secreted by the heart and the endothelium (Lafontan et al. 2008). They exert their physiological actions by binding to NP receptor A (NPRA) and C (NPRC). The guanylyl cyclase activity of NPRA generates the second messenger cGMP (Chinkers et al. 1989; Waldman et al. 1984), while NPRC is responsible for the clearance of NPs from the circulation (Maack et al. 1987). Bordicchia et al. have demonstrated the role of natriuretic peptides in the regulation of the beiging of WAT in rodents (Bordicchia et al. 2012). Although the role of NPs and their reciprocal receptors in the beiging of WAT has not been reported, recent evidence supports a link between the levels of *NPRA* and *NPRC* gene expression and UCP1 expression in WAT (Kovacova et al. 2016).

Bile Acids: Bile acids are important signaling molecules that play a regulatory role in lipid metabolism. Bile acids bind to the TGR5 receptor and the nuclear farnesoid X receptor (FXR). The interaction of bile acids with TGR5, which is also a G protein-coupled receptor, leads to increased cAMP levels and increased type 2 deiodinase (DIO2) activity (Watanabe et al. 2006). DIO2 promotes the conversion of thyroxine to active thyroid hormone, triiodothyronine, resulting in increased BAT thermogenesis. On the other hand, FXR agonism has been shown to induce the beiging of WAT in rodents, indicating that bile acid administration may provide an alternative pathway by which to induce the beiging of WAT in people (Fang et al. 2015). Results from a recent randomized controlled trial support the notion that a 2-day administration of chenodeoxycholic acid (15 mg/kg) leads to a significant increase (~60%) in BAT activity measured by ^{18}F-FDG-PET/CT (which is several-fold lower than the observed increase in cold-induced BAT activity) compared to the placebo group (Broeders et al. 2015). Chenodeoxycholic acid administration was also accompanied by a ~5% increase in energy expenditure, indicating that bile acid administration might be an alternative method by which to induced thermogenesis in UCP1-positive adipocytes. Further studies are need to clarify whether bile

acid supplementation can induce the beiging of subcutaneous WAT and the metabolic significance of the bile acid-induced increase in BAT activity in terms of whole body metabolic regulation.

Other factors: A number of animal studies support the notion that different endocrine/paracrine factors and other metabolites can regulate BAT metabolic activity and/or affect the recruitment of thermogenic UCP1-positive adipocytes. These factors include leptin (Buyse et al. 2001), adiponectin (Puerta et al. 2002), fibroblast growth factor 21 (Hondares et al. 2011), thyroid hormones (Silva and Larsen 1983), bone morphogenic proteins 4, 7 and 8 (Tseng et al. 2008; Whittle et al. 2011), prostaglandins (Vegiopoulos et al. 2010), IL6 (Stanford et al. 2013) and parathyroid hormone-related protein (Kir et al. 2014). The physiological significance of the previously mentioned factors in BAT thermogenesis and the beiging of WAT in people remain to be determined.

6.15 Conclusion

BAT and the beiging of WAT have attracted intense interest as therapeutic targets against obesity and its related metabolic abnormalities. The physiological significance of brown and beige adipocytes in metabolic regulation in people remains unknown, and thus, highly controversial. Currently available evidence supports the physiological relevance of human BAT in energy, glucose and lipid metabolism. The ability of human WAT to undergo beiging has only recently been demonstrated in patients with severe burn trauma, while its metabolic role remains currently unknown. Future research studies are needed to: (1) establish minimally invasive methods for the quantification and identification of BAT and the beiging of WAT *in vivo*, (2) determine the direct and indirect role of BAT and the beiging of WAT in metabolic thermogenesis and beyond, (3) understand the factors and signaling pathways modulating the different metabolic processes in brown and beige adipocytes, and (4) develop efficacious lifestyle and pharmacological interventions to safely stimulate BAT activity and the beiging of WAT.

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Chapter 7
Macrophages and Inflammation

Elise Dalmas, Joan Tordjman, Michèle Guerre-Millo, and Karine Clément

Abstract Adipose tissue has been under focus in the last decades, and pivotal concepts have emerged from the studies of its complex biology. White adipose tissue is composed of mature adipocytes, precursors (preadipocytes), endothelial cells, macrophages, and other immune cells. The phenotype, amount, and biology of each adipose tissue component are profoundly altered in human obesity. Low-grade inflammation both at the local and systemic levels characterizes obesity and appears to have a key role in mediating the consequence of increased adipose tissue mass on metabolic and vascular comorbidities. Among the different cell types contributing to inflammation, this chapter focuses on the mechanisms and consequences of macrophage accumulation in obese adipose tissue. While differences probably exist between rodent models and human cases, macrophage cells have a very complex phenotype able to change with weight modification. It is not fully established whether macrophages exert a rather beneficial or deleterious role in the adipose tissue. In any case, the presence of these cells modifies the biology of adipose specialized cells such as preadipocytes and adipocytes. This chapter reviews the current knowledge regarding the contribution of monocytes/macrophages in development and maintenance of obesity and related complications both in mouse and human situations.

Keywords Obesity • Inflammation • Macrophages • Weight loss

E. Dalmas (✉) • J. Tordjman • M. Guerre-Millo • K. Clément
INSERM, U872, Paris 75006, France

Centre de Recherche des Cordeliers, Université Pierre et Marie Curie-Paris 6, UMR S 872, 15 rue de l'école de Medecine, Paris 75006, France

Nutrition and Endocrinology Department, Assistance Publique-Hôpitaux de Paris, Pitié-Salpêtrière Hospital, Paris 75013, France

CRNH-Ile de France, Paris 75013, France
e-mail: elise.dalmas@crc.jussieu.fr

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7.1 Introduction

Inflammation is a physiological response aiming at defending the organism against injurious stimuli and initiating the healing process in order to restore tissue homeostasis. A typical acute inflammatory response involves triggering molecules, also known as inducers, which are recognized by cellular sensors, leading to increased production of a large panel of mediators acting on target tissues. Inflammatory response ends with a highly regulated process known as resolution of inflammation allowing transition to the homeostatic state. When this resolution phase cannot occur for any reason, a chronic inflammatory state ensues (Medzhitov 2008). For a decade now, obesity is seen as an inflammatory disease characterized by low-grade chronic inflammatory state. Obesity associates with increased circulating concentrations of inflammatory cytokines and acute-phase proteins and decreased concentrations of molecules, such as adiponectin, with anti-inflammatory properties. Also, local up-regulation in genes encoding inflammatory proteins has been described in enlarged adipose tissue associated with a marked accumulation of macrophages in the adipose tissue (Yudkin et al. 1999; Weisberg et al. 2003; Curat et al. 2004).

As mentioned by Hotamisligil et al., chronic inflammation can lead to vicious cycles as it intrinsically connects inflammation to the pathological process it accompanies (Hotamisligil 2006). To understand the deleterious consequences of chronic inflammation in obesity, we need to get deeper insights into the contributing cellular and molecular mechanisms. Particular attention is given to obesity-associated immune response that may influence local and systemic biology. Although many types of inflammatory cells, such as neutrophils (Nijhuis et al. 2009), mast cells (Liu et al. 2009), and lymphocytes (Nishimura et al. 2009) might be involved in white adipose tissue inflammation, this review specifically focuses on the contribution of monocytes and macrophages.

7.2 Adipose Tissue Inflammation: A Myriad of Actors But the "Egg or Chicken" Question Remains Unanswered

Among others, a still unanswered question is what triggers inflammation and immune cells accumulation in the adipose tissue. Several actors and signaling pathways have been proposed to explain the pathogenesis of inflammatory cell accumulation. Adipocytes themselves have been put into the scene since they are able to produce various mediators, including cytokines, chemokines, and adipocyte-specific molecules known as adipokines. One hallmark of obesity is adipocyte hypertrophy (i.e., increased adipocyte volume). These hypertrophied cells are prone to secrete large quantities of inflammatory cytokines (Skurk et al. 2007). Markers associated with increase adipocyte size have been recognized as, for

example, serum amyloid A (SAA). It has been suggested that this acute phase protein could participate into local dialogue between adipocyte and inflammatory cells. In vitro, SAA contributes to local inflammation, adipocyte lipolysis, and to the regulation of adipocytes cholesterol efflux (Yang et al. 2006; Poitou et al. 2009). Adipocyte hypertrophy can also lead to necrosis-like adipocyte death. Cell contents are released in the extracellular space where they trigger inflammatory responses from neighboring cells, especially macrophages typically surrounding moribund adipocytes (Cinti et al. 2005). Thus, adipocyte hypertrophy and its related perturbed biology could be directly involved in the development of chronic low-grade inflammatory state by secreting proinflammatory molecules and/or liberating intracellular components after death.

Nutrition derived factors can contribute to the stimulation of local inflammation. Among them, fatty acids are able to bind and activate toll-like receptor-4 (TLR4) in adipocytes and macrophages. The capacity of fatty acids to induce inflammatory signaling in adipose tissue is blunted with the deletion of TLR4 in mouse models (Shi et al. 2006; Davis et al. 2008). Fatty acids released from hypertrophied adipocytes could also serve as a naturally occurring ligand for TLR4 to promote inflammation. Endotoxemia, i.e., increased circulating concentration of lipopolysaccharide (LPS) originating from intestinal microbiota, could represent another triggering factor of proinflammatory cytokines when it binds to TLR4 at the surface of innate immune cells (Cani et al. 2007). Finally, hypoxia is able to induce proinflammatory gene expression in adipocytes and macrophages and may represent an additional mechanism for chronic inflammation in obesity (Ye 2009). This list is certainly not complete, as shown by the recent identification of reticulum endoplasmic stress as a critical mechanism underlying obesity-induced inflammatory responses (Hummasti and Hotamisligil 2010).

While the overall mechanisms inducing inflammatory cell accumulation remain to be fully deciphered, there is probably no unified theory. Obesity-related inflammation is likely to be explained by complex overlapping and complementary inflammatory signaling pathways (Table 7.1). Presumably, obesity could be termed as "sterile" inflammation, since no pathogen or pathogen-derived molecules have been yet clearly identified. However, potential antigenic reactions, for, e.g., against circulating LPS or fatty acids, cannot be excluded so far (Chen and Nunez 2010). Whatever the initiating mechanisms, inflammation definitely leads to a vicious cycle where macrophages and adipocytes organize a paracrine loop. Paracrine dialogs play in turn the role of inducers and sensors aggravating and auto-maintaining inflammatory changes in adipose tissue (Suganami et al. 2005).

It is now recognized that inflammatory cells are present in expanded adipose tissue. Both cells of the innate and the adaptive immune system have been described in obese animal models and human patients. Monocytes and macrophages are part of the innate immune system and represent a large proportion of the stroma-vascular fraction, i.e., the non adipocyte fraction in adipose tissue. In 2003, accumulation of adipose tissue macrophages in both human and diet-induced obese (DIO) mice was described and found to be directly proportional to measures of adiposity (Weisberg et al. 2003; Xu et al. 2003).

Table 7.1 Causes and pathophysiological outcomes of chronic inflammation in obesity

Inducers	Sensors	Mediators	Target tissues	Induced pathologies
Exogenous	Innate immunity	Cytokines	Local	Type-2 diabetes
Excess dietary factors	Circulating monocytes	TNF-α, IL-1, IL-6, IL23…	Adipose tissue	NAFLD – NASH
LPS from intestinal flora	Macrophages	Chemokines	Endothelium	Cardiovascular diseases
Endogenous	Other cells…	MCP-1, RANTES, IL-8, CXCL14…	Systemic	
Adipocyte hypertrophy	Adaptative immunity	Metabolites	Muscle	
Adipocyte death	Circulating lymphocytes	ROS, NO, fatty acids…	Liver	
Local hypoxia	Other cells	Others	Endothelium	
ER stress	Endothelial cells	SAA, Cathepsin S…		
ECM remodeling	Preadipocytes			
Immune cells activation				
Endothelium activation				

Adipose tissue changes during the onset of obesity are a great source of proinflammatory molecules that are likely to trigger sensors cells located both in the circulation and in adipose tissue. These sensor cells secrete in turn inflammatory mediators that can act locally and systemically leading to diverse pathologies (adapted from Medzhitov 2008)

7.3 Accumulation of Macrophages in Adipose Tissue in Obesity

Macrophages provide the immediate defense against foreign pathogens and coordinate leukocyte infiltration. They contribute to the balance between antigen availability and clearance through phagocytosis and subsequent degradation of microbes, senescent, or apoptotic cells. Their role is essential in triggering, instructing, and terminating the adaptive immune response. Macrophages collaborate with T and B cells, through both cell–cell interactions via their major histocompatibility complex II and fluid-phase-mediated mechanisms mostly based on the release of cytokines and chemokines. Macrophages derived from the differentiation of circulating monocytes after extravasation through the endothelium of a blood vessel within tissue where they undergo local activation. At sites of infection or wound healing for example, intense recruitment of monocytes and precursors from bone-marrow pools results in the accumulation of tissue macrophages (Gordon and Taylor 2005).

7.3.1 *Monocytes Trafficking and Phenotypes*

Circulating monocytes are released from the bone marrow as non-differentiated cells and circulate in the blood for 1–3 days. Monocytes are known to display heterogeneous phenotypes characterized by different markers as shown in Fig. 7.1. The specific surface marker for human monocyte population is membranous CD14 (mCD14). Thanks to flow cytometry analysis, subgroups of monocytes have been defined based on the level of expression of mCD14. The additional separation of monocytes is defined by the surface marker CD16 antigen, also known as the FC receptor γIII. Based on these markers, two subsets of circulating monocytes have been identified. The main monocyte population in humans is $CD14^{hi}CD16^{-}$ subset (corresponding to antigens $7/4^{hi}$/Ly-$6C^{hi}$ in mouse). These cells are considered as the inflammatory monocytes recruited to inflamed areas. A second subset has been proposed to be a resident cell population in tissues recruited independently of inflammatory stimuli (e.g., alveolar or splenic macrophages, Kupffer cells, etc.). These cells are $CD14^{+}CD16^{+}$ (corresponding to antigens $7/4^{low}$/Ly-$6C^{low}$ in mouse) and show a macrophage-like phenotype with enhanced antigen-presenting capacities and higher endothelial affinity as reviewed in Pandzic Jaksic et al. (2010). These $CD14^{+}CD16^{+}$ cells appear to be potent producers of proinflammatory cytokines. Their increase was noted in inflammatory disorders such as sepsis, HIV infection, or atherosclerosis (Ziegler-Heitbrock 2007). Of note, a third population has been recently identified as CD14dim CD16+, so-called "patrolling" monocytes that could be implicated in local surveillance of damaged or infected tissues. It is currently unknown whether or not they infiltrate the adipose tissue (Cros et al. 2010). Several studies described a significant rise in overall $CD14^{+}$ circulating monocytes but also in the $CD14^{+}CD16^{+}$ subset in human obesity (Cottam et al. 2002; Rogacev et al. 2010). In 2004, Ghanim et al. described monocytes from obese patients as being in a proinflammatory state with increased transcription of proinflammatory genes regulated by nuclear factor-kappa B, including tumor necrosis factor-alpha (TNF-α),

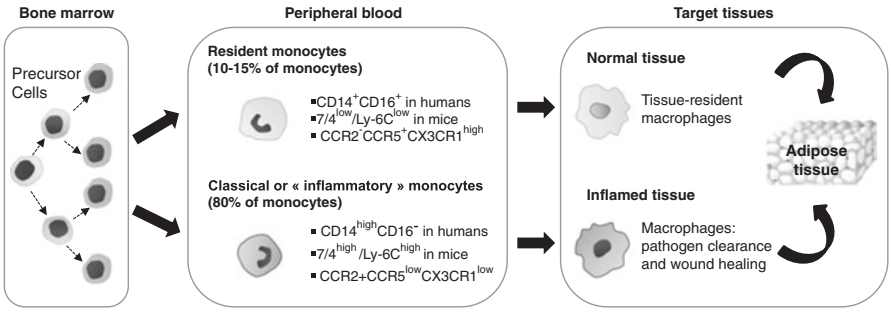

Fig. 7.1 Trafficking of monocytes from bone marrow to peripheral blood and target tissue. Under steady-state conditions, resident monocytes enter the tissues to replenish the pool of tissue-resident macrophages. Inflammatory monocytes immigrate into inflamed tissue and differentiate into so-called newly recruited macrophages

and interleukin-6 (IL-6) (Ghanim et al. 2004). Thus, preferential trafficking of $CD14^{+}CD16^{+}$ monocytes subset in addition to the usual inflammatory $CD14^{hi}CD16^{-}$ monocyte accumulation may contribute to significant recruitment of macrophages in obese adipose tissue. Monocytes recruitment is typically directed by chemokines that attract cells through activation of their cognate receptor. The different monocyte subsets appear to display different chemokine-receptor expression profiles that directly mediate their distinctive recruitment properties. For example, in human, the classical $CD14^{hi}CD16^{-}$ monocytes express high amounts of CCR2, low levels of CCR5 (the receptor of CCL3), and medium amounts of CX3CR1 (the receptor of fractalkine). On the contrary, $CD16^{+}$ subset is CCR2 negative but displays high levels of CX3CR1 and CCR5 receptors (Ziegler-Heitbrock 2007).

7.3.2 Mediators of Monocytes Recruitment

The mechanism of monocyte diapedesis in the adipose tissue has not been clearly defined, but it presumably involved the secretion of chemotactic molecules or chemokines, known to be overexpressed in mice and human adipose tissue depots. These chemokines are thought to be derived from cells of the stromal vascular fraction, although their secretion from adipocytes has also been reported (Dahlman et al. 2005).

Mice models gave the opportunity to study different chemoattractant molecules that mediate monocytes mobilization from the bone marrow and recruitment into the adipose tissue. Westcott et al. have identified the galactose-type C type lectin 1 (Mgl1) as being critical for the survival and migration of $7/4^{hi}$/Ly-$6C^{hi}$ monocytes, the population classically recruited to sites of inflammation (Westcott et al. 2009). Animals deficient in Mgl1 are protected from macrophage accumulation in fat due to a reduction in circulating levels of these $7/4^{hi}$/Ly-$6C^{hi}$ proinflammatory monocyte subsets. CCR2, the receptor for Monocytes Chemotactic Protein-1 (MCP-1/CCL2), has also been implicated in the mobilization of cells from the bone marrow to the peripheral circulation. Tsou et al. showed that $CCR2^{-/-}$ mice have a marked decrease in blood $7/4^{hi}$/Ly-$6C^{hi}$ monocytes, although the bone marrow contained normal or increased numbers of monocyte progenitors, suggesting a defect in mobilization rather than monocyte differentiation impairment (Tsou et al. 2007). Accordingly, transgenic obese mice deficient for CCR2 on bone marrow cells displayed reduced number of macrophages in adipose tissue (Ito et al. 2008). A number of studies further showed that the CCR2/MCP-1 system plays a crucial role in macrophages accumulation in the obese adipose tissue. MCP-1 gene and protein are up-regulated in white adipose tissue of DIO mice with the highest level found in mesenteric depots (Yu et al. 2006). In vitro migration assay showed that mesenteric adipose tissue-conditioned medium-induced macrophage migration and proinflammatory activation, which were inhibited upon MCP-1 neutralization. Transgenic mice overexpressing MCP-1 in adipose tissue displayed higher macrophage accumulation in adipose tissue (Kamei et al. 2006; Kanda et al. 2006), while disruption of MCP-1

gene by a homozygous knock-out model or the expression of a dominant-negative mutant reduced macrophage accumulation (Kanda et al. 2006). Similarly, genetic deficiency or pharmacological inhibition of CCR2 reduced the macrophage content and inflammatory profile of adipose tissue (Weisberg et al. 2006). Yet, contradictory results do exist, and the physiopathological relevance of the MCP-1/CCR2 duo is still discussed (Chen et al. 2005; Inouye et al. 2007; Kirk et al. 2008).

Another CC motif chemokine, CCL5, also known as RANTES (Regulated on Activation Normal T cells Expressed and Secreted) has recently been studied for its emerging role in regulating the recruitment of inflammatory cells in adipose tissue. RANTES is expressed in mouse adipose depots and increased in obesity, along with elevated level of its receptor CCR5 (Wu et al. 2007). Studies conducted in humans also showed statistical association between CCL5 expression and macrophage accumulation in adipose tissue. In vitro, cellular studies using human primary cells have demonstrated the contribution of CCL5 in mediating monocyte/macrophage adhesion and transmigration though endothelial barrier (Keophiphath et al. 2010). CCL3, also commonly referred to macrophage inflammatory protein-1α (MIP-1α), and its potential receptors CCR1 and CCR5 show a significant increase in gene and protein expressions in genetically and DIO obese mice (Xu et al. 2003).

Surprisingly, however, MIP-1α-deficient (MIP-1α$^{-/-}$) mice were not protected from macrophage accumulation in adipose tissue (Surmi et al. 2010). MIP-1α deficiency was associated with a relative decrease in RANTES and MIP-1β expression. The absence of inflammatory improvement in this model suggests that the function of these chemokines can be compensated by other factors that promote macrophage accumulation (Surmi et al. 2010). The chemokine (CXC motif) ligand 14 (CXCL14) and its receptor CXCR2 are also known to be involved in macrophage attraction. They were found to be up-regulated in white adipose tissue of obese mice. Besides, CXCL14-deficient mice have impaired macrophages accumulation in adipose tissue (Nara et al. 2007). There is no doubt that involvement of new chemoattractant molecules will be enlightened in future studies. However, these chemokines appear to have redundant functions such that the sole alteration of one chemokine or one receptor may have only minor effects on macrophages accumulation. Further studies should give insights into the mechanisms by which these chemokines work together to promote macrophage infiltration in the adipose tissue. Human studies have confirmed the increase in gene and protein expression of a variety of chemokines and associated receptors in obese adipose tissue. Indeed, MCP-1, MCP-2 (CCL7), MCP-3, MIP-1α, RANTES along with CCR1, CCR2, CCR3, and CCR3 were up-regulated in obese compared to lean subjects (Huber et al. 2008). As shown for MCP-1 and RANTES, these factors are preferentially secreted by non-fat cells in the adipose tissue (reviewed in Fain 2010). Strikingly, these molecules positively correlated with monocyte/macrophage markers such as CD14 and CD68 expressed in adipose tissue (Bruun et al. 2005). Human studies showed that most of these chemokines and associated receptors were overexpressed in omental adipose tissue compared to subcutaneous adipose tissue of obese patients, in line with macrophage content that was found to be higher in omental adipose depots (Cancello et al. 2006) (Tordjman et al. 2009).

7.3.3 *From Monocytes to Tissue Macrophages*

Tissue infiltration of blood monocytes is complex and involves steps including the activation and transmigration of monocytes through the endothelium as illustrated in Fig. 7.2. The vascular endothelium serves as a barrier to monocyte trafficking and as a sentinel to instruct their adhesion and transmigration. Classically, the extravasation of monocytes consists of five steps starting with the accumulation of circulating monocytes on the luminal surface of the endothelium. Monocytes undergo transient rolling interactions mediated by selectin cell adhesion molecules such as E-selectin or CD62L (step 1). This facilitates the sensing of and the responses to chemokines presented on the surface on the endothelium (step 2). This phenomenon triggers high-affinity interaction of monocyte integrin receptors (e.g., lymphocyte function-associated antigen 1, macrophage 1 antigen, and very late antigen 4) with their

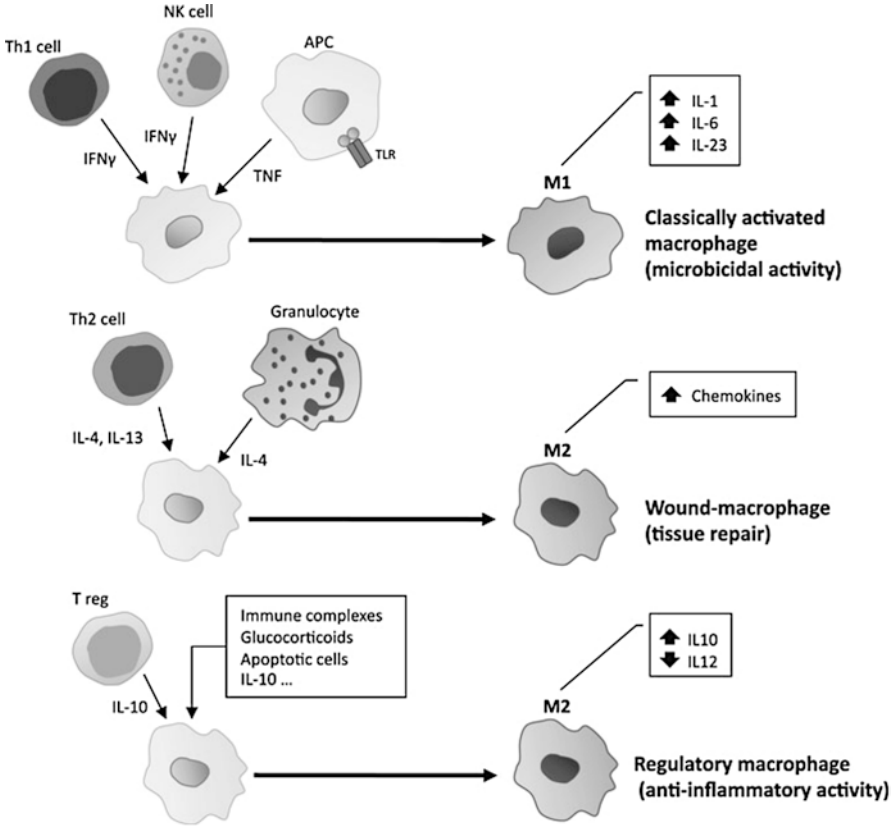

Fig. 7.2 Distinct phenotypes of tissue macrophages, depending of the microenvironment produced by surrounding cell cytokine release. Each macrophage subgroup is associated with typical secretory responses and functional characteristics (adapted from Mosser and Edwards 2008). *APC* antigen presenting cell; *T reg* regulatory T cell

endothelial ligands (e.g., intercellular adhesion molecule – ICAM -1 and 2 – and vascular cell adhesion protein – VCAM-1) resulting in monocyte immobilization (step 3). Subsequently, monocytes undergo actin-dependent spreading, polarization and integrin-dependent lateral migration on the luminal surface of the endothelium (step 4). This activity allows monocytes to seek for permissive sites enabling the penetration of the endothelial barrier. Then, the monocyte formally breaches and transmigrates across the endothelium (step 5), a process referred as diapedesis. Until recently, only one basic pathway for diapedesis was widely recognized, the paracellular route, in which leukocytes and endothelium cooperate to locally disassemble the interendothelial junctions to open a paracellular gap for cell transmigration. Recent studies have shown that a second pathway termed as transcellular route exists and consists in monocytes passing directly through individual endothelial cells via the formation of a transcellular pore (Kamei and Carman 2010).

Obesity is characterized by the increased production of many adhesion molecules (i.e., P-selectin, E-selection, ICAM, VCAM). This supports the fact that increased fat mass is associated with a systemic endothelial activation that increased overall monocytes diapedesis. However, the precise mechanisms of extravasation across adipose tissue endothelial cells are not known. Curat et al. noted that mature human adipocytes released soluble factors that directly increase the diapedesis of human blood monocytes across a layer of adipose tissue-derived capillary endothelial cells in a transwell migration assay. This effect was actually reproduced with human recombinant leptin alone but at supraphysiological doses. Adipocyte-conditioned media could also directly induce the up-regulation of platelet/endothelial cell adhesion molecule-1 and ICAM-1 from endothelial cells (Curat et al. 2004). In a subsequent study, endothelial cells were found to be in a more marked proinflammatory state in visceral than in subcutaneous human adipose tissue (Villaret et al. 2010), suggesting a role in regional differences in macrophage accumulation (Cancello et al. 2006; Tordjman et al. 2009).

7.4 Macrophages in Adipose Tissue

7.4.1 Macrophage Plasticity

Macrophages are well known to be versatile cells that can adopt specialized functions at particular tissue locations. They adapt themselves and actively respond to the local microenvironment (Gordon and Taylor 2005). Their amazing plasticity is reflected by the different phenotypes they can display (Fig. 7.3). In line with the current understanding of monocyte heterogeneity and in an effort to mimic the T cells Th1/Th2 nomenclature, a M1/M2 macrophage activation classification was created where M1/M2 are the extreme of a continuum of functional states (Gordon 2003). Stimulation of macrophage with Th1 cytokines such as interferon-gamma alone or in concert with cytokines (e.g., TNF-α and Granulocyte Macrophage-Colony Stimulating Factor) and bacterial stimuli (e.g., LPS) promotes maturation of

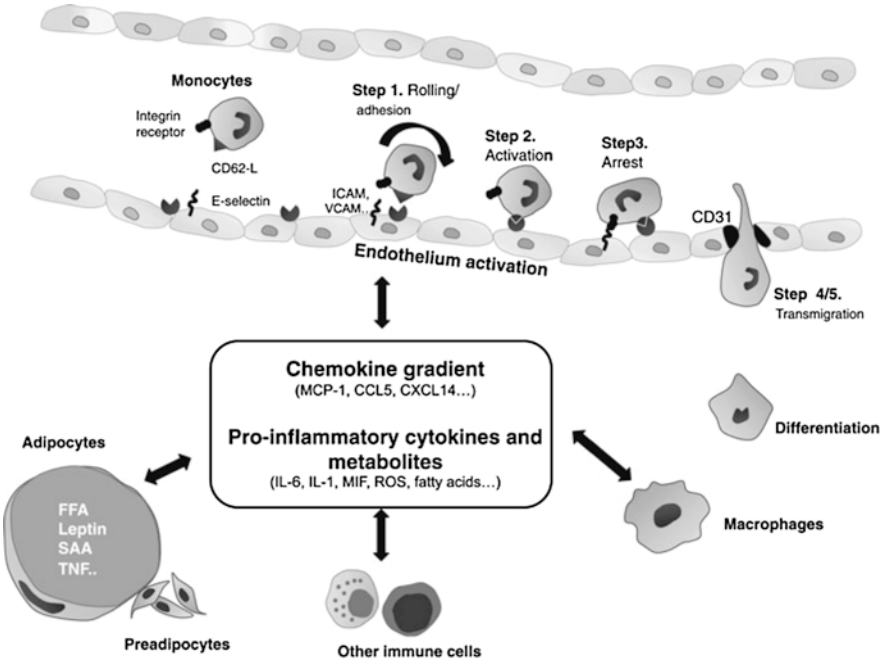

Fig. 7.3 Monocyte recruitment in the adipose tissue. Upon obesity, adipose tissue macrophages together with hypertrophic adipocytes, preadipocytes, and probably other immune cells produce a panel of chemokines, proinflammatory cytokines, and metabolites that participate into monocyte recruitment. Endothelium activation causes endothelial cells to produce various cellular adhesion molecules. Though a rolling/adhesion process, monocytes slow down and eventually bind tightly to the endothelium until they transmigrate into the adipose tissue. *Double arrows* suppose that cells are both producers and targets of the associated chemokines and cytokines

"classically" activated M1 macrophages. These cells are characterized by high secretion of IL-12 and IL-23, high production of toxic intermediates (e.g., reactive oxygen species, nitric oxides [NOs]), and high capacity to present antigens. In contrast, various signals (e.g., IL-4, Il-13, glucocorticoids, adiponectin…) induce distinct M2 functions able to tune inflammatory responses and to promote angiogenesis, tissue remodeling, and repair (Gordon and Taylor 2005; Ohashi et al. 2010). However, the M2 term was used in a loose and confusing way. Martinez et al. (2008) thus proposed three forms in the M2 nomenclature: M2a, induced by IL4 or IL13 and involved in killing or encapsulation of parasites; M2b, induced by exposure of immune complexes and involved in immunoregulation; and M2c, induced by IL-10 and glucocorticoids and preferentially implicated in matrix deposition and tissue remodeling. In the mean time, a new foundation for macrophages classification was recommended based on their functions: host defense (close to a M1 phenotype with microbicidal activity), wound healing (promoted by IL-4 from Th2 cells), and immune regulation (preferentially induced by IL-10 from regulatory T cells) (Mosser and Edwards 2008).

7.4.2 *Complex Phenotype of Adipose Tissue Macrophages: Mouse Studies*

The presence of macrophages in obese adipose tissue have been described in 2003 (Xu et al. 2003; Weisberg et al. 2003). The authors described transcript expression profiles of adipose tissue and found that inflammation and macrophage-specific genes were dramatically up-regulated in mouse models of diet-induced and genetic obesity. Body mass and adipocyte sizes appeared to be strong predictors of the percentage of the F4/80 and CD68 expressing macrophages in the adipose tissue. Macrophage contents were estimated to range from less than 10% of total cell nuclei count in lean mice to over 50% in extremely obese leptin-deficient mice (Weisberg et al. 2003). In the obese adipose tissue, aggregates of F4/80 positive cells were described surrounding a single adipocyte. These clusters contained many oil-red O-staining vesicles, indicating intracytoplasmic lipid accumulation, consistent with phagocytic activities of macrophages (Xu et al. 2003).

Following these pioneer studies, the next challenge was to determine the phenotype that macrophages acquire during the setting of obesity, using membranous and intracellular markers considered specific hallmarks of M1- or M2-polarized macrophages (Table 7.2). In 2007, Lumeng et al. demonstrated that obesity induces a phenotypic switch in macrophages from an anti-inflammatory M2-polarized state to

Table 7.2 Markers of macrophage polarization: effects on surface antigens and metabolic changes in mice and human adipose tissue

Reactive species	Pan macrophage marker	M1-polarized macrophage	M2-polarized macrophage
Mice	•F4/80, a glycoprotein with homology of the G-protein linked transmembrane 7 hormone receptor family	•CD11c, a dendritic cells marker, also called integrin alpha X, member of beta 2 family of integrin receptors •iNOS, inducible Nitric Oxide Synthase, enzymes that catalyze the production of nitric oxide (NO) from L-arginine	•MGL1, macrophage galactose-type C-type lectins are unique lectins for the carbohydrate specificity toward galactose and N-acetylgalactosamine as monosaccharides •Ym-1, secretory lectin chitinase 3-like ¾ •ArgI, arginase isoform I, a cytosolic enzyme that transforms L-arginine into L-ornithine, a precursor of polyamines and proline •Lyve 1, lymph vessels endothelial hyaluronan receptor-1
	•Microsialin (CD68 mouse equivalent) •mCD14, a co-receptor for the detection of bacterial lipopolysaccharide (LPS)	•ArgII, arginase isoform II, a mitochondrial enzyme that transforms L-arginine into L-ornithine, a precursor of polyamines and proline	

(continued)

Table 7.2 (continued)

Reactive species	Pan macrophage marker	M1-polarized macrophage	M2-polarized macrophage
Human	•CD68, a member of the lysosomal-associated membrane protein family with a macrophage-specific mucin-like extracellular domain	•CD11c, a dendritic cells marker, also called integrin alpha X	•CD206, the mannose receptor, a C-type lectin carbohydrate binding protein involved in the uptake of mannose-containing particles (controversial: might also be expressed by M1-like macrophages as described in crown-like structures) •CD163, a hemoglobin/haptoglobin scavenger receptor •CD150, as known as signal lymphocyte activation molecule (SLAM), a costimulatory molecule on B lymphocytes and dendritic cells •CD51, integrin αV (in particular heterodimer αVβ5) •CD34, intercellular adhesion protein and cell surface glycoprotein (ligand: CD62L), highlights M2-like macrophage angiogenic properties
		•CD40, a costimulatory protein found on antigen presenting cells and is required for their activation and lymphocyte activation (ligand: CD154)	
	•mCD14, a co-receptor for the detection of bacterial LPS, a co-receptor for the detection of bacterial LPS	•CD86, a costimulatory protein found on antigen presenting cells and is required for lymphocyte activation (ligands: CD28 and CD152)	
		•HLA-DR, human leukocyte antigen DR, one of the major histocompatibility complex class II-antigens responsible for antigen presentation to $CD4^+$ T lymphocytes	

a proinflammatory M1 state (Lumeng et al. 2007a, b). They identified a population of proinflammatory cells expressing $F4/80^+$ and the integrin $CD11c^+$ recruited in the adipose tissue of DIO mice. These cells preferentially secreted IL-6 and inducible NO synthase. $CD11c^-$ macrophages of lean mice, called resident macrophages, expressed a majority of anti-inflammatory factors such as IL-10 and Arg1. The authors then explored whether M1 macrophages were newly recruited or resulted from repolarization of M2 resident cells. Using pulse PKH26 labeling, a dye staining efficiently macrophages but not monocytes, they purified and compared gene expression profiles between recruited and resident macrophages, demonstrating that recruited macrophages displayed inflammatory properties and increased accumulation of lipids. The proportion of resident macrophages expressing the M2a marker macrophage MGL1 remained stable with obesity, while newly recruited M1 proinflammatory macrophages expressing CD11c but not MGL1 rapidly accumulate in adipose tissue (Lumeng et al. 2008). Another study in DIO mice showed that obesity associated with an increase in both M1 ($CD11c^+CD206^-$) and M2 ($CD11c^-CD206^+$) macrophages, although the ratio M1/M2 macrophages was switched towards M1 macrophages (Fujisaka et al. 2009). These studies suggest that obesity is associated with accumulation of proinflammatory M1 macrophages in the adipose tissue, which occurs in parallel to the maintenance or slight increase

in the number of M2 anti-inflammatory resident macrophages that are believed to help maintaining tissue homoeostasis.

Surprisingly, in mice deficient in the M2 marker MGL1, the trafficking of M2 macrophages was normal, while the number of M1 macrophages drastically decreased in adipose tissue (Westcott et al. 2009). MGL1 is known to bind to Lewis X, a protein specifically expressed in obese mice adipose tissue, with highest concentrations in crown-like structures. It was therefore suggested that MGL1/Lewis X interactions provide a mean for the circulating $MGL1^{+}7/4^{high}$ monocytes precursors of M1 macrophages to traffic to crown-like structures (Westcott et al. 2009). This study raised the hypothesis that monocytes subsets have specific fates and are committed to differentiate into M1 or M2 macrophages, independently of the local microenvironment (Geissmann et al. 2010).

The "M1/M2 paradigm" fitting with the DIO mice model presented by Lumeng et al. (2007a, b) might be more complex than initially proposed. Shaul et al. demonstrated that a high fat diet (HFD) did not elicit classical M1 polarization macrophages, but rather a mixed M1/M2-like pattern of gene expression (Shaul et al. 2010). Three cell populations were identified: $MGL1^{+}CD11c^{-}$ (M2a cells), $MGL1^{-}CD11c^{+}$ (M1 cells), and a new $MGL1^{med}/CD11c^{+}$ population with an intermediate phenotype. When the HFD was prolonged, macrophages exhibited global changes in gene expression with an up-regulation of M2 markers and a down-regulation of M1 markers. Besides, the $MGL1^{med}/CD11c^{+}$ subgroup showed adipogenic and angiogenic properties (Shaul et al. 2010). Using a 20-week course of HFD feeding in mice, Strissel et al. demonstrated that frequency of adipocyte death along with adipocyte size increased until peaking at week 16 where it coincided with maximum expression of CD11c and proinflammatory genes (Strissel et al. 2007). By week 20, adipocyte number was restored with a state of hyperplasia, corresponding to reduced adipocyte death and down-regulation of CD11c. Thus, adipocyte death in adipose tissue is a progressive event that is temporally linked to macrophage recruitment and to their phenotype switch from M2 to M1 macrophages. Eventually a return to M2-like polarization seems to occur under extended HFD course, potentially corresponding to an adaptive response to restore adipose tissue homeostasis.

7.4.3 Adipose Tissue Macrophage Phenotypes: Human Studies

Several groups have addressed the question of adipose tissue macrophages phenotype in human adipose tissue. Flow cytometry analysis showed the existence of a $CD14^{+}CD206^{+}$ double positive population of macrophages correlated with subjects' BMI. Besides being CD206 positive, these macrophages expressed the hemoglobin scavengor receptor CD163 and integrin heterodimer αVβ5 and produced anti-inflammatory cytokines (IL-10 and IL1-RA), which are all hallmarks of M2-like macrophage phenotype. Nevertheless, these cells also produce large amounts of proinflammatory molecules such as TNF-α, IL-1b, IL-6, MCP-1, and MIP-1α suggesting a M1-like polarization. Thus, adipose tissue macrophages show a particular M2-like surface marker expression while they are able to produce amounts of

proinflammatory cytokines (Zeyda et al. 2007). These results were confirmed by Bourlier et al., who also observed that human $CD14^+CD206^+$ adipose tissue macrophages expressed both M1 (TNF-α, IL8, MCP-1, COX-2) and M2 (IL-10, TGF-B) markers (Bourlier et al. 2008). In an immunohistochemistry-designed study, obese adipose tissue was shown to contain more $CD40^+$ cells, another protein marker of M1 macrophages, than lean adipose tissue. There was also more $CD40^+$ stained cells in visceral depots compared to subcutaneous depots. Meanwhile, the number of CD206 and CD163 positive cells were unchanged with severe obesity (Aron-Wisnewsky et al. 2009). In another human study combining immunohistochemistry, immunofluorescence, and flow cytometry, adipose tissue macrophages were defined as resident $CD206^+CD11c^-$ macrophages in the parenchyma and as crown aggregated cells with high expression of CD11c and low expression of CD206 ($CD11c^+CD206^{low}$). Further characterization of these cells showed high expression of the antigen-presenting molecules CD1c and HLA-DR, of the T-cell costimulatory molecule CD86, and high levels of proinflammatory mediators (IL-8 and MIP-1α). Confirming these observations, a recent publication showed that macrophages in crown-like structures immunoreacted with CD86 and CD40 with low staining for CD206. Meanwhile, interstitial macrophages stained strongly for CD206 but slightly for CD86. They also specifically stained for the lymphocyte activation molecule (SLAM or CD150), a marker of M2c macrophage subclass known to be involved in wound healing. The count of adipose tissue macrophages was performed and nearly 60% of noncrown macrophages stained for both CD86 and CD206 in lean subjects while obese patients tend to have more CD206 positive macrophages, suggesting a shift from a mix M1/M2 to a more M2-oriented phenotype with the worsening of obesity (Spencer et al. 2010).

In conclusion, observations in humans suggest that the macrophages accumulating in the adipose tissue have a complex phenotype. Overlapping M1/M2 macrophage phenotypes may be the consequences of the incapacity to study separately the resident and inflammatory cell subsets in human adipose tissue. Also, it is possible that using the simplified macrophage M1/M2 nomenclature, if useful, cannot be adapted to the development of human adipose tissue known to be an ongoing dynamic process. Whether the mixed M1/M2 macrophage phenotypes described in obese human adipose tissue could be partly explained by a repolarization of resident M2 cells in M1-like macrophages is currently not known. Such a phenotypic switch has been proposed during the course of atherosclerotic lesions development in mice (Khallou-Laschet et al. 2010). In that way, newly recruitment of M1 macrophages as described in PKH26 mice experiment might not be entirely relevant in human obesity.

7.4.4 Do Macrophages Limit Obesity Development in Human?

As described above, potential inducers that trigger macrophage accumulation in adipose tissue are numerous but once macrophages are established, their phenotype and functional role remains unclear. A pending question is whether macrophage accumulation in obese adipose tissue could have some beneficial purposes. One of

the discussed hypotheses is that M1-like macrophages infiltrate adipose tissue to limit the expansion of adipocytes. This can be illustrated by the CCR2 knock-out mice model. These mice show decreased macrophage content and less systemic inflammation but increased fat pad weight (Lumeng et al. 2007a, b). In culture experiments, human preadipocytes exhibit impaired adipogenesis and increased extracellular matrix deposition when cultured with conditioned media from LPS-activated monocytes-derived macrophages or adipose tissue isolated macrophages (Keophiphath et al. 2009). Hence, inhibition of adipogenesis combined with a pro-fibrotic phenotype of preadipocytes strengthens the hypothesis that proinflammatory macrophages aim at limiting adipocyte hypertrophy. Nevertheless, another suggested scenario would be that macrophages serve to positively support adipose tissue growth and angiogenesis by secreting proangiogenic factors such as platelet-derived growth factor (Pang et al. 2008). Mirroring the situation in cancer, M2-polarized macrophages might be responsible for this effect (Sica et al. 2008). Finally, the main function of macrophages is to phagocyte necrotic debris from dead adipocytes and especially to metabolize fatty acids, preventing lipotoxicity. A recent study showed that increasing macrophage lipid storage capacity by overexpressing the enzyme diacylglycerol acyltransferase 1 in both macrophages and adipocytes protected the mice from macrophage accumulation and activation in adipose tissue (Koliwad et al. 2010). Thus, macrophages could very likely be the cells that ensure adipose tissue homeostasis and remodeling throughout obesity.

7.5 Adipose Tissue Inflammation and Obesity-Associated Complications

It is well-established that obesity is associated with a myriad of metabolic and cardiovascular complications. Currently, clinical studies and experimental evidences suggest a link between macrophage infiltration and insulin resistance, cardiovascular risk, and hepatic alterations (Fig. 7.4).

7.5.1 Insulin Resistance

The central involvement of the visceral adipose tissue in metabolic and cardiovascular diseases is well known (Hotamisligil 2006). Obese adipose tissue is a major source of inflammatory mediators that are linked to insulin resistance, such as TNF-α and proinflammatory cytokines (IL-6, IL-1β) that are released by both adipocytes and macrophages (Scherer 2006). TNF-α and IL-6 are known to promote lipolysis and to increase systemic free fatty acids, which then contribute to an increase in hepatic glucose production (Hotamisligil et al. 1995). Several cytokines and chemokines produced by inflamed adipose tissue activate intracellular pathways that promote the development of insulin resistance and Type 2 diabetes (Shoelson et al. 2006). In animal models, a role for adipose tissue macrophages in

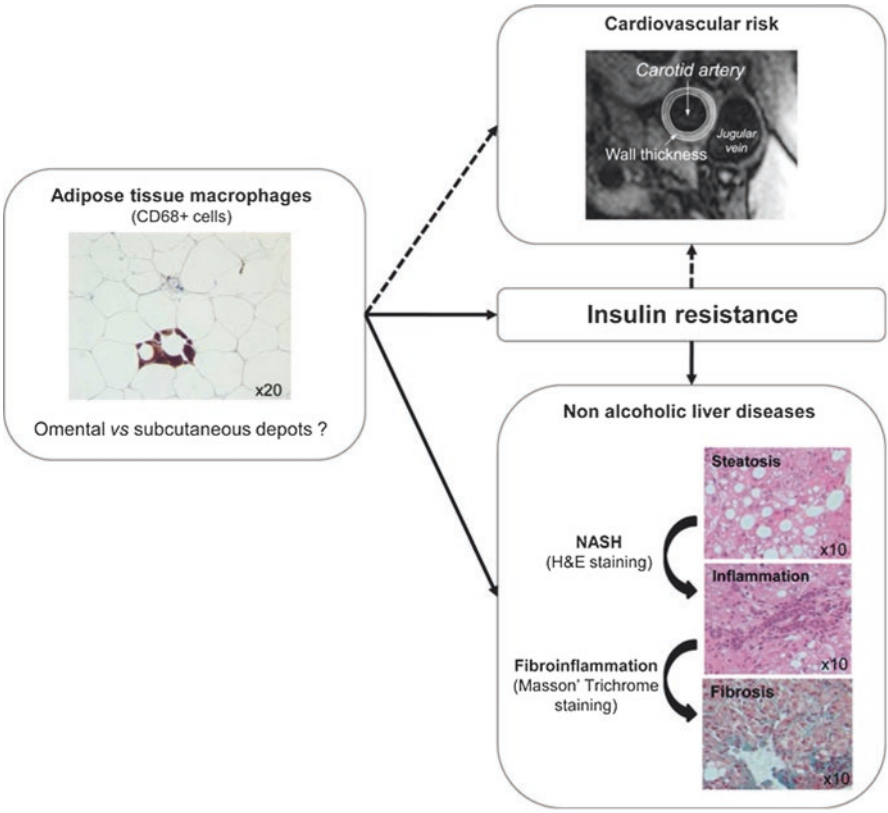

Fig. 7.4 Potential relationship between adipose tissue infiltrated macrophages and obesity comorbidities. Insulin resistance is a dependent factor implicated in the link between adipose tissue macrophages and non-alcoholic liver disease. The relative contribution of omental vs. subcutaneous inflammation might be distinct depending on the comorbidity. *Dotted arrows* indicate lack of clinical and experimental evidence for the relationship. *H&E* hemotoxylin and eosin staining. Cross section of carotid artery by magnetic resonance imaging taken from Skilton et al. (2011)

inducing systemic insulin resistance has been demonstrated through diet-induced, genetic, or pharmacological manipulations of macrophage numbers in adipose tissue (Xu et al. 2003; Weisberg et al. 2003, 2006; Kanda et al. 2006; Kamei et al. 2006). In these studies, accumulation of macrophages in adipose tissue was consistently associated with alteration of glucose homeostasis. However, in humans, the pathological consequences of macrophage infiltration in adipose tissue are more difficult to prove. Clinical studies have shown an inverse correlation between the expression of the macrophage marker CD68 in subcutaneous fat and whole body insulin sensitivity (Di Gregorio et al. 2005; Makkonen et al. 2007). It has been also shown that preferential macrophage infiltration into visceral adipose tissue was mainly observed in a subgroup of subjects with impaired glucose homeostasis (Harman-Boehm et al. 2007). Recently, obese subjects with more crown-like

structures of macrophages in subcutaneous adipose tissue were shown to be more insulin resistant than those without such cells aggregates (Apovian et al. 2008). However, observations in morbid obesity do not support such a relationship, since no correlation was found between adipose tissue macrophages and blood-derived parameters of insulin resistance (Cancello et al. 2005; Tordjman et al. 2009). Additionally, an overfeeding challenge rapidly installed an insulin-resistant state in healthy subjects, despite no significant change in macrophage accumulation in the adipose tissue (Tam et al. 2010).

7.5.2 Cardiovascular Diseases

Proinflammatory factors and/or adipokines produced by adipose tissue are thought to play a role to increase cardiovascular risks, although only a few supporting experimental or clinical evidences are currently available. This hypothesis has been tested in mice deficient for CD14, a co-receptor of toll-like receptor 2 and 4. When submitted to a HFD, these mice show reduced macrophages accumulation in the adipose tissue, associated with improvement of glucose homeostasis and reduction of blood pressure (Roncon-Albuquerque et al. 2008). Other studies support the implication of adipokines such as leptin, adiponectin, resistin, or visfatin (Ahima and Osei 2008). Indeed, adiponectin plays a crucial role in vascular homeostasis, in part by counteracting the negative effect of TNF-α in aortic endothelial cells (Matsuda et al. 2002; Kobashi et al. 2005; Andersson et al. 2008) or by inhibiting the formation of foam cells (Yokota et al. 2000). Since adiponectin production by adipose tissue is decreased in obesity, its protective effects are thought to be reduced in obese subjects. In healthy human, infusion of free fatty acids was used to mimic elevated blood lipidemia (Kishore et al. 2010). This challenge was shown to induce the expression of PAI-1, a well-established promoter of blood coagulation, in adipose tissue macrophages in association with TNF-α and IL-6. Finally, since MCP-1 is both involved in macrophage recruitment in adipose tissue and in the promotion of atherosclerosis, this chemokine has been proposed as a potential therapeutic target to reduce cardiovascular risk in human obesity (Ohman and Eitzman 2009).

7.5.3 Non-Alcoholic Fatty Liver Disease

Non-alcoholic fatty liver disease (NAFLD) is a frequent complication of human obesity (Utzschneider and Kahn 2006; Westerbacka et al. 2004). The relationship between adipose tissue secreted products and hepatic damage has been recently evaluated in humans. In a population of severely obese patients, neither leptin nor TNF-α circulating levels were significantly associated with the severity of hepatic lesions. However, patients with significant hepatic fibroinflammation had reduced adiponectin levels (Cancello et al. 2006). A similar association of low serum

adiponectin with worsening grades of hepatic necroinflammation has been reported in different populations (Marra et al. 2005; Hui et al. 2004; Musso et al. 2005).

The link between adipose tissue macrophages and NAFLD in human obesity is poorly understood. A study addressed this point by focusing on non-alcoholic liver pathology. In a large group of morbidly obese subjects, visceral adipose tissue macrophages accumulation was associated with the severity of hepatic fibroinflammatory lesions. No association was found with the number of macrophages in subcutaneous adipose tissue, thus suggesting a specific link between visceral macrophages and liver damage (Cancello et al. 2006). Insulin resistance contributes to the pathological mechanisms leading to hepatic steatosis, inflammation and fibrosis. Taking into account the glycemic status, Tordjman et al. further showed that accumulation of macrophages in omental adipose tissue is insufficient alone to promote liver steatosis, although it contributes to its aggravation in conjunction with insulin resistance. By contrast, the severity of fibroinflammation associated with higher numbers of macrophages in omental adipose tissue, irrespective of the degree of insulin resistance. This suggests that obesity-driven macrophage accumulation specifically in this adipose depot is an independent determinant of liver fibrosis and inflammatory damages (Tordjman et al. 2009). These observations support recent findings in humans showing that the amount of visceral fat can associate with liver inflammation and fibrosis independent of insulin resistance (Van der Poorten et al. 2007). The actual factors (proinflammatory cytokines, free fatty acids, adipokines) conveying the inflammatory signals from omental adipose tissue to the liver must be identified. Increased IL-6 concentrations measured in the portal vein of obese subjects suggests a role for this proinflammatory cytokine in promoting liver damage in obesity (Fontana et al. 2007).

7.6 Adipose Tissue Remodeling and Inflammation

Components of the extra cellular matrix (ECM) are particularly crucial for maintaining structural integrity of adipocytes. To accommodate the changes induced by increased adipocyte size in obesity, remodeling of the ECM occurs by degradation of the existing ECM and production of new ECM components. Implication of proteases such as metalloproteases (MMPs) or disintegrin and metalloproteinases with thrombospondin motifs in these processes are not fully deciphered. Other proteins might be involved, including SPARC, a collagen-binding matricellular protein initially found to be increased in the adipose tissue of obese mice (Tartare-Deckert et al. 2001) and in humans (Kos et al. 2009). The consequences of ECM modification in normal and pathological growth of adipose tissue have been mostly investigated in mice. Gene invalidation of the pericellular collagenase MT1-matrix metalloproteinase (MT1-MMP) leads to the formation of a rigid network of collagen fibrils, which compromises adipocyte differentiation and lipid accumulation (Chun et al. 2006). In genetically obese mice, various types of collagen are overexpressed in the adipose tissue. The predominantly expressed collagens are types I, IV,

and VI, the latter being the most abundantly expressed (Halberg et al. 2009). In this context, the authors generated collagen VI-null obese mice, showing that this manipulation resulted in increased adipose tissue mass, due to uninhibited expansion of individual adipocytes. Interestingly, a similar phenotype of increased adipose cell size was reported in SPARC-null mice (Bradshaw et al. 2003). Thus, accumulation of ECM in adipose tissue might contribute to a failure to expand adipose tissue mass to accommodate excess caloric intake. Subsequently, this causes fibrosis and increases inflammatory stress in adipose tissue (Halberg et al. 2009). However, another study in DIO mice suggests that inflammation and collagen deposition occur concomitantly in the adipose tissue (Strissel et al. 2007), leaving unresolved the kinetic of events involved in the structural and inflammatory alterations of adipose tissue in obesity.

In humans, adipose tissue remodeling and fibrosis are poorly documented. In 2008, Henegar et al. showed for the first time that major changes in the expression of a subset of genes encoding ECM components occur in adipose tissue of obese subjects and in response to weight loss (Henegar et al. 2008). As a follow-up of these observations, picrosirius labeling of adipose tissue slides revealed that amount of fibrosis in subcutaneous adipose tissue was increased in obesity, along with increased inflammatory state. More recently, Pasarica et al. reported that type VI collagen gene expression was elevated in moderately obese subjects, and that obese subjects with high collagen VI display increased adipose tissue inflammation and increased visceral adipose tissue mass (Pasarica et al. 2009). In morbid obese individuals, the presence of different patterns of fibrous depots and detailed collagen fibers organization in the adipose tissue was reported (Divoux et al. 2010). Macrophages of both M1 and M2 phenotype and mast cells were the main immune cells found in fibrotic areas, where T lymphocytes were less frequent. Fibrosis is typically considered a fibroproliferative disorder with the uncontrolled production of ECM components by fibroblasts activated by an inflammatory microenvironment. Recent studies suggest that adiponectin exerts antifibrotic effects partly by reducing profibrotic TGF-β signaling in experimental models of liver or cardiac fibrosis (Kamada et al. 2003; Fujita et al. 2008). Thus, reduced adiponectin production could contribute to promote fibrosis deposition in adipose tissue. The pathophysiological relevance of fibrosis in the adipose tissue, which might differ between fat depots, is yet to be explored in detail.

7.7 Adipose Tissue Inflammation and Weight Loss

7.7.1 Moderate Weight Loss

Moderate weight loss induced by caloric restriction improves insulin sensitivity and other complications associated with obesity (Wing et al. 1987; Tuomilehto et al. 2001). Diet-induced obesity is associated with a reduction of systemic inflammation and specific metabolic adaptations, suggesting an interaction between nutrition, the

immune system and metabolism (Heilbronn et al. 2006; You and Nicklas 2006). Interactions between adipose tissue macrophages and weight loss after caloric restriction have been investigated using large-scale transcriptomic analyses. In 2004, Clement et al. observed that weight loss induced by a very-low-calorie diet decreases the expression of inflammatory markers in white adipose tissue of obese subjects and leads to the concomitant increased expression of molecules with anti-inflammatory properties (Clement et al. 2004). In another clinical study, transcriptomic analysis of subcutaneous adipose tissue following a specific dietary intervention program revealed that inflammatory pathways and macrophages markers were unchanged or up-regulated during energy restriction and down-regulated during weight stabilization (Capel et al. 2009). In a third study, adipose tissue macrophages number was unchanged during short-term caloric restriction but substantially decreased after a 6-month period of weight maintenance, without detectable change in macrophage phenotype (Kovacikova et al. 2011). These observations in humans indicate that improvement of adipose tissue inflammation following caloric restriction is a complex phenomenon, partly independent of body weight reduction. Kosteli et al. addressed this question in a model of DIO mice submitted to caloric restriction. They showed that macrophage recruitment to adipose tissue initially increased following caloric restriction and then declines (Kosteli et al. 2010). The early increase in macrophages accumulation was not associated with a concomitant rise in inflammatory gene expression. A series of experimental studies therefore indicated that these macrophages phagocytose excess lipids without causing inflammation, thereby contributing to restore local and systemic lipid homeostasis during the initial phases of caloric restriction.

7.7.2 Drastic Weight Loss Induced by Bariatric Surgery

Bariatric surgery is the most effective treatment to combat morbid obesity and deleterious metabolic complications (Sjostrom et al. 2004; Dixon et al. 2008). It is well-established that weight loss induced by bariatric surgery improves inflammatory status in obesity (Cottam et al. 2004; Esposito et al. 2003). In 2005, Cancello et al. reported that weight loss is associated with major modifications of infiltrating macrophages in subcutaneous adipose tissue. They show that fat mass reduction was associated with decreased numbers of adipose tissue macrophages and reduction of crown-like structures. After weight loss, remaining macrophages stained positive for the anti-inflammatory cytokine IL10. The expression of chemoattractant genes (MCP-1, colony-stimulating factor-3, and plasminogen activator urokinase receptor) was reduced after weight loss (Cancello et al. 2005). These pioneering observations suggested a switch from a proinflammatory M1 phenotype towards an M2 macrophage polarization in response to weight loss. This point was reevaluated in an immunochemistry-based human study, where the authors showed that the M1/M2 balance, estimated by the ratio of $CD40^+/CD206^+$ macrophages in subcutaneous adipose tissue, decreased after weight reduction

(Aron-Wisnewsky et al. 2009). Gastric by-pass induced weight loss improves an individual's metabolic and inflammatory profile (Buchwald et al. 2004). While factors derived from M1 proinflammatory macrophage induce insulin resistance and inflammation in preadipocytes and/or adipocytes (Suganami et al. 2005; Lacasa et al. 2007), it is tempting to speculate that amelioration of the M1/M2 balance towards a less proinflammatory state after weight loss contributes to the amelioration of metabolic condition.

7.8 Conclusion

Adipose tissue inflammation and macrophage infiltration are well-established features of obesity with different stages of severity and only partial reversion with weight loss (Fig. 7.5). The whole spectrum of instigators and physiopathological consequences of this inflammation is yet to be defined. Actually, it remains to be fully established whether macrophages exert a rather beneficial or deleterious role in the adipose tissue. In lean conditions, M2-like macrophages may contribute to maintain adipose tissue homeostasis. Obesity, which is associated with different stresses such as nutrient excess or adipocyte hypertrophy, could be considered an illustration of "para-inflammation" according to the definition given by Medzhitov (2008). Para-inflammation refers to an adaptive response induced by tissue stress or malfunction that is intermediate between basal and inflammatory states. In this context, intense recruitment of macrophages (both M1 and M2) into adipose tissue might be part of adaptive mechanisms aimed at restoring tissue functionality and homeostasis. Thus, whether or not adipose tissue inflammation could be a suitable therapeutic target in obesity remains an open question.

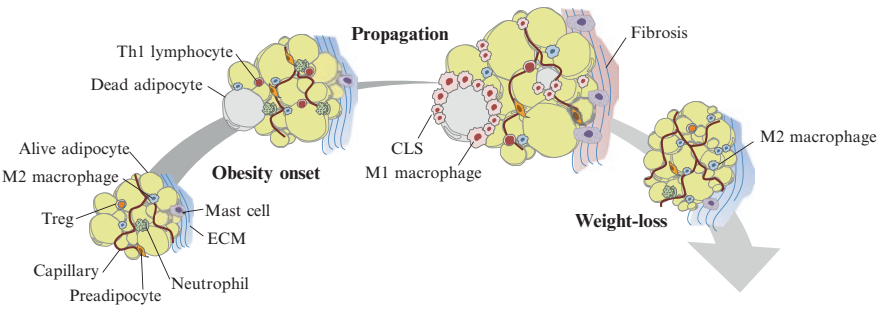

Fig. 7.5 Cellular and structural alterations in adipose tissue during obesity and weight loss. Healthy adipose tissue contains resident M2-polarized macrophages and other immune cells that help to maintain tissue homeostasis. During weight gain, hypertrophic adipocytes become inflammatory and/or necrotic and contribute to recruit M1 macrophages of crown-like structure around moribund adipocytes. Weight loss intervention could lead to remodeling of adipose tissue via relief of inflammation, ECM reorganization and macrophage repolarization from an M1 toward an M2 phenotype (adapted from Surmi et al. 2010; Lee et al. 2010)

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Chapter 8
Sex Differences in Body Fat Distribution

Mohamed Fouad Mansour, Chon-Wai Jeremy Chan, Sofia Laforest, Alain Veilleux, and André Tchernof

Abstract Although obesity is an important determinant of metabolic disease, specific accumulation of visceral fat is strongly and independently associated with important metabolic alterations such as insulin resistance, hypertension and dyslipidemia. Excess accumulation of visceral fat is a strong predictor of cardiometabolic risk in both sexes, but a marked dimorphism and large interindividual variations are observed in body fat distribution. Women are more likely to store lipids in lower-body fat compartments through adipocyte hyperplasia, while visceral adipose tissue depots of men are more prone to manage incoming lipids through adipocyte hypertrophy. Adipocyte hypertrophy appears as a critical determinant of sex-related and depot-related differences in lipid metabolism and may contribute to the chronic, low-grade inflammation observed in abdominally obese individuals. Regarding the hormonal etiology of abdominal obesity, active androgens are known to inhibit adipogenesis and lipogenesis in adipose tissue. Estrogens have important central effects

M. Fouad Mansour
Endocrinology and Nephrology, CHU de Québec Medical Center, Québec, QC, Canada

Department of Biochemistry, Faculty of veterinary Medicine, Zagazig University, Zagazig, Egypt

Québec Heart and Lung Institute Research Center, Université Laval, Québec, QC, Canada

C.-W.J. Chan
University of British Columbia, Vancouver, BC, Canada

S. Laforest
Endocrinology and Nephrology, CHU de Québec Medical Center, Québec, QC, Canada

Québec Heart and Lung Institute Research Center, Université Laval, Québec, QC, Canada

School of Nutrition and Institute of Nutrition and Functional Foods, Université Laval, Québec, QC, Canada

A. Veilleux
School of Nutrition and Institute of Nutrition and Functional Foods, Université Laval, Québec, QC, Canada

A. Tchernof, Ph.D. (✉)
Endocrinology and Nephrology, CHU de Québec Medical Center, Québec, QC, Canada

Québec Heart and Lung Institute Research Center, Université Laval, Québec, QC, Canada
e-mail: andre.tchernof@crchul.ulaval.ca

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on energy balance, but may also directly modulate central fat accumulation through direct effects on adipose tissue metabolism. Moreover, a relatively high adipose tissue glucocorticoid reactivation by 11β-hydroxysteroid dehydrogenase type 1 appears to promote specific accumulation of visceral fat and to alter adipocyte function in humans. Interventions targeting visceral fat accumulation such as moderate weight loss are known to exert beneficial effects on cardiometabolic disease risk.

Keywords Abdominal obesity • Visceral fat • Omental • Subcutaneous • Adipocyte size • Men • Women

8.1 Introduction

High obesity rates are expected to result in elevated prevalence of many chronic diseases and adverse events including premature death, musculoskeletal problems and metabolic complications (Katzmarzyk 2002; Ng et al. 2014), the latter which includes type 2 diabetes as well as cancer (reviewed in (Must and McKeown 2000)). However, excessive total body fat content does not always lead to cardiometabolic disease. Many studies have now established that body fat distribution more accurately predicts cardiometabolic risk factors associated with obesity (reviewed in (Tchernof and Despres 2013)). More specifically the presence of a central pattern of fat distribution (Fig. 8.1) with large fat stores within intra-abdominal anatomical structures such as the mesentery and greater omentum, also termed visceral obesity,

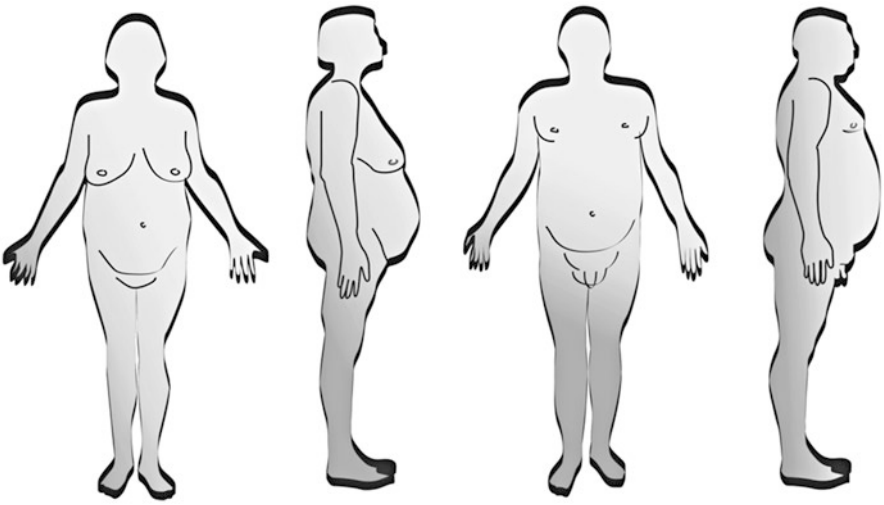

Fig. 8.1 Android obesity in male and female. Central pattern of fat distribution in a female (*Left Panels*) and a male (*Right Panels*) (Adapted from Vague, 1956)

has now clearly emerged as one of the most prevalent manifestations of the metabolic syndrome and represents an essential feature of the current obesity epidemic (Despres and Lemieux 2006). This chapter will review studies which have documented sex differences in body fat distribution, and how depot-specific characteristics of abdominal adipose tissues in men and women relate to cardiometabolic disease risk. The impact of sex hormones on body fat distribution is also addressed.

8.2 Sex Differences in Body Composition and Fat Distribution

Over the course of childhood, weight gain is slightly higher in boys than girls. Lean mass appears to be relatively similar in both sexes, although boys weigh slightly more before puberty. Total body fat mass is also comparable between boys and girls before the age of seven. After adrenarche, girls accumulate fat mass more rapidly and eventually reach slightly higher values than boys (Taylor et al. 2010; Veldhuis et al. 2005; Wells 2007). Hence, differences in body composition can be observed, but are relatively small in magnitude before puberty.

Between ages 10 and 20 years, boys accumulate approximately twice the amount of lean mass compared to girls (33 kg vs. 16 kg respectively) (Van Loan 1996). Conversely, total fat mass increases proportionately more in girls (Van Loan 1996). Despite the minor variation prior to puberty, studies had demonstrated that pre-puberty females had a smaller waist compared to pre-puberty males (Taylor et al. 2010). As a result, adult women have significantly higher fat mass values and relatively lower lean mass compared to men (Siervogel et al. 2003; Wells 2007). Average percent body fat mass values range 10–15% for men and 20–30% in women in healthy subjects, although values can obviously reach higher levels in different populations (Karastergiou et al. 2012; Van Loan 1996; Wells 2007). With aging, women tend to have a slightly higher propensity to gain fat mass than men (Wells 2007). This may be attributable to hormonal changes of the menopause, although the impact of such changes remains controversial and difficult to demonstrate consistently (Crawford et al. 2000; Keller et al. 2010; Lovejoy et al. 2008). Available studies rather show that the impact of menopause may manifest more specifically on abdominal fat accumulation (Guthrie et al. 2003, 2004; Keller et al. 2010; Lovejoy et al. 2008).

The sex dimorphism in body fat distribution becomes apparent at puberty (Karastergiou et al. 2012). The amount of fat that accumulates at the abdominal level can be estimated using imaging techniques such as computed tomography and magnetic resonance imaging (Kvist et al. 1987; Ross et al. 1992; Sjöström et al. 1986). These studies have shown that despite having higher percent body fat masses than men, women generally have significantly lower visceral adipose tissue accumulations. Cross-sectional data from the Quebec Family Study (Hajamor et al. 2003) and the Heritage Family study (Desmeules et al. 2003) enabled us to examine this sex dimorphism in the adult Caucasian population. For example, in a Quebec Family Study subsample of 203 men and 219 women that were on average 40 years

old, the sex dimorphism in body composition was readily apparent with 32% fat in women vs. 23% in men. Conversely, body fat-free mass weighed 61 kg in men vs. 46 kg in women. Despite such highly significant differences, men had a 37% higher visceral adipose tissue area compared to women. Conversely, abdominal subcutaneous adipose tissue area was 50% higher in women (Hajamor et al. 2003). Very similar differences can be observed in other Caucasian populations (Desmeules et al. 2003; Lear et al. 2007a). Other ethnicities also generally show this pattern of sex differences, although there are marked ethnicity-related disparities in total adiposity and the propensity to store visceral fat. In African American individuals (Després et al. 2000), a lower proportion of visceral fat is observed for any given total body fat mass value, suggesting a reduced susceptibility to visceral obesity in this population. The opposite is true for other ethnic groups such as the South East Asians and Canadian Aboriginals (Lear et al. 2007a, b; Sniderman et al. 2007). In our analysis of available literature on computed tomography studies in a variety of ethnic subgroups (Tchernof and Despres 2013), we reported that for any given adiposity level, the ratio of visceral-to-subcutaneous adipose tissue areas (reflecting the relative amount of visceral fat) was higher in Asian and Caucasian compared to African American populations. The tendency of some populations to preferentially accumulate visceral adipose tissue may have an impact on their susceptibility for type 2 diabetes and cardiovascular diseases.

A striking element is the very large inter-individual variability in visceral adipose tissue area in both men and women. Despite a generally lower visceral adipose tissue accumulation in women, relatively important and physiologically significant visceral fat accumulations can still be observed in this sex, even in the normal BMI range. As previosuly mentioned, convincing evidence is now available supporting the notion that not only in men but also in women, abdominal, visceral obesity is closely associated with a cluster of metabolic abnormalities including dyslipidemia, insulin resistance as well as a chronic, low-grade inflammatory state (Després 1993, 1994; Despres and Lemieux 2006; Lemieux and Despres 1994). Hypertriglyceridemia and low HDL cholesterol are two main abnormalities of the blood lipid profile associated with visceral adiposity. Such a dyslipidemic state, associated with visceral obesity is a main risk factor in the pathogenesis of cardiovascular diseases (reviewed in (Tchernof and Despres 2013). The following sections will describe sex differences in adipose tissue cellularity and function.

8.3 Sex Differences in Adipose Tissue Cellularity and Markers of Adipose Tissue Function

8.3.1 Adipose Tissue Morphology

The size of each fat compartment results from the integration of adipocyte number and cell size. Important inter-individual variation is noted in these parameters. However, for study purposes, two distinct adipose tissue phenotypes have often

been recognized in the human population: (1) individuals with fewer but larger fat cells are characterized by adipocyte hypertrophy; and (2) individuals with an increased number of small fat cells are characterized by adipocyte hyperplasia (Arner et al. 2010; Hoffstedt et al. 2010). In women and men, both adipose tissue phenotypes are observed at various adiposity levels across the range of BMI values (Arner et al. 2010; Hoffstedt et al. 2010). Susceptibility to adipocyte hypertrophy may generate large adipocytes even in non-obese individuals (Arner et al. 2010; Tchoukalova et al. 2008). In contrast, some severely obese women and men are characterized by smaller mean adipocyte size than expected (Arner et al. 2010; Tchoukalova et al. 2008).

In general, adipocytes from all anatomical locations and in both sexes increase in size along with adiposity level, but reach a plateau in massively obese subjects (Arner et al. 2010; Boivin et al. 2007; Hoffstedt et al. 2010; Mundi et al. 2010; Tchernof et al. 2006; Tchoukalova et al. 2008; Weyer et al. 2000) (Fig. 8.2). This

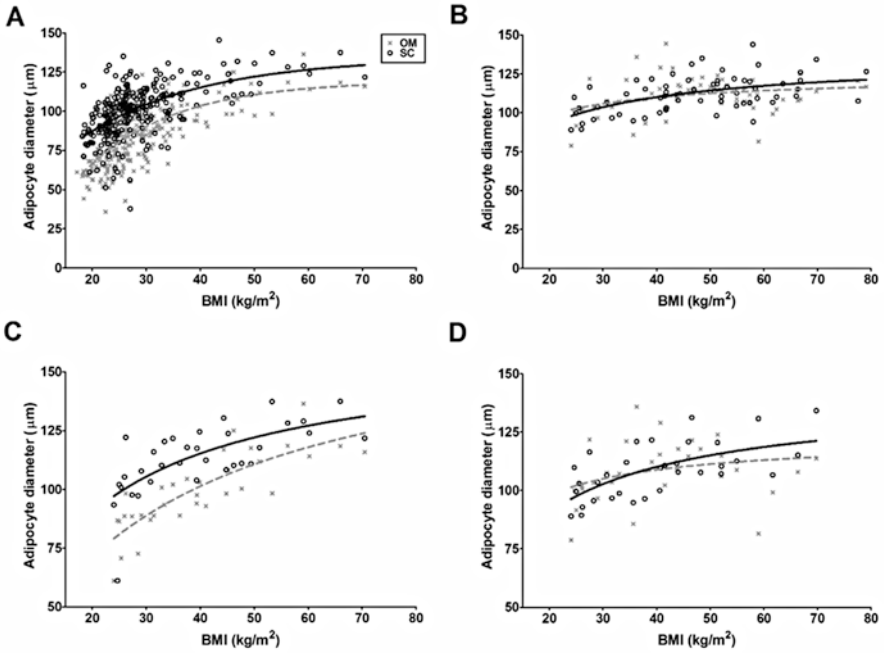

Fig. 8.2 Adipocyte size in subcutaneous and omental adipose tissue of women and men. Mean adipocyte diameter of subcutaneous and omental adipose tissue according to BMI in women (n=207) and men (n=54) undergoing abdominal elective surgery or bariatric surgery. Women were 46.9 ± 5.6 years old (range: 30–68.3 years) with a mean BMI of 28.2 ± 7.8 kg/m^2 (range: 17.2–70.5 kg/m^2). Men were 45.7 ± 9.8 years old (range: 22.6–66.4 years) with a mean BMI of 46.6 ± 13.5 kg/m^2 (range: 24–79.9 kg/m^2).A. OM and SC AD in women (n=301). B. OM and SC AD in men (n=71). C. Women and D. Men covering the whole range of the BMI values were matched for age and BMI (n=33). Matched participants still showed a sex-specific pattern of depot differences (or lack thereof)

plateau indirectly suggests that the presence of large adipocytes triggers the generation of new adipocytes to store excess dietary fat in severely obese individuals. Adipocyte size starts to reach a plateau at a lower BMI value for men (~25 kg/m^2) compared to women (~35 kg/m^2) (Fig. 8.2) and (Laforest et al. 2015). Omental fat cells of massively obese women reach a higher maximal cell diameter value (~130 μm) when compared with massively obese men (~120 μm) (Tchernof and Despres 2013). Furthermore, omental fat cell size appears to be lower than subcutaneous fat cell size by ~20% in women, whereas this depot difference was not observed in men (Laforest et al. 2015) and (Fig. 8.2). In fact, omental and abdominal subcutaneous adipocytes only tend to reach similar sizes at very high BMI values (>60 kg/m^2) (Tchernof and Despres 2013). The differences in adipocyte size in specific depots observed in women further supports the notion that adipocyte sizes and their pattern of expansion are influenced by sex (Laforest et al. 2015).

Accordingly, adipocyte number is positively associated with adiposity indices and adipose tissue cell populations appear to regenerate constantly during adulthood (Spalding et al. 2008). Early-onset obesity, as opposed to short term weight gain, has been suggested as an important predictor of hyperplasia in obese adults. This notion is supported by the fact that individuals characterized by hyperplasic or hypertrophic adipocytes may be uniquely distinguished by the age of obesity onset (Salans et al. 1973). However, this is not supported in all studies (Noppa et al. 1980). In adulthood, low generation rates of new adipocytes are associated with adipose tissue hypertrophy, whereas high generation rates are associated with adipose tissue hyperplasia in middle-age women and men (Arner et al. 2010). Failure to increase the generation of new adipocytes during long-term weight gain in adulthood could favor the development of hypertrophic adipose cells. We may also assume that short term changes in energy homeostasis are more strongly reflected in adipose tissue cell size than adipocyte number.

The absolute number of adipocytes and cell sizes are distinctly related to obesity in adipose tissues of women and men. More adipocytes are found in the lower-body adipose tissue compartments (i.e. gluteal and femoral) of obese women than of lean women (Tchoukalova et al. 2008). Lower-body adipocytes of obese men have been shown to be larger, but there is no report of adipocyte hyperplasia in obese men compared to lean men (Tchoukalova et al. 2008). These results indirectly suggest that during weight gain, lower-body adipose tissue tends to expand mainly through hyperplasia in women, but through hypertrophy in men (Tchoukalova et al. 2008). Accordingly, lower-body subcutaneous adipocytes of women tend to be larger than those of men with the same fat mass while no sex difference is observed in abdominal subcutaneous adipocyte size (Fried and Kral 1987; Mundi et al. 2010; Tchoukalova et al. 2008, 2010). A strong correlation is observed between abdominal subcutaneous adipocyte size and total body fat mass in lean to moderately obese individuals of both sexes, suggesting that the contribution of adipocyte hypertrophy to adipose tissue expansion may be similar in men and women (Tchoukalova et al. 2008). As a consequence, part of the higher subcutaneous fat mass values observed in women compared to men may be attributable to increased adipocyte number (Tchoukalova et al. 2008). Accordingly, a higher

adipocyte number is already observed in subcutaneous adipose tissue of adolescent girls suggesting sustained adipose tissue hyperplasia in young girls compared to boys (Chumlea et al. 1981). In adult women, expression of genes involved in pre-adipocyte differentiation is relatively higher in subcutaneous than in visceral adipose tissue (Drolet et al. 2008). Moreover, only subcutaneous expression of these genes tracked with adiposity measures, suggesting that in women, expansion of the subcutaneous adipose tissue depot relies more heavily on adipocyte hyperplasia than the visceral adipose tissue compartment, which may be predominantly hypertrophic (Drolet et al. 2008). This hypertrophic state is associated with alteration in adipocyte lipolysis and in the expression of genes implicated in adipose metabolism and inflammation (Michaud et al. 2014). Taken together, these observations may suggest that depot-specific differences in adipose tissue cellularity reflect the propensity of premenopausal women to store more lipids in lower-body compartments through adipocyte hyperplasia, while intra-abdominal adipose tissue depots of men (and postmenopausal women) are more prone to manage incoming lipids through adipocyte hypertrophy.

8.3.2 Lipoprotein Lipase and Lipid Uptake

Triglyceride-rich lipoprotein hydrolysis catalyzed by lipoprotein lipase (LPL) in circulation as well as adipocyte-mediated triglyceride synthesis are major determinants of the fatty acid flux and subsequent triglyceride storage in adipose tissue. These processes seem to be tightly associated with adipocyte size (Edens et al. 1993; Farnier et al. 2003). In women, gluteal, thigh, abdominal subcutaneous and visceral adipose tissue LPL activities have been positively associated with fat cells size in the corresponding depot (Votruba et al. 2007). Similarly, LPL activity increases along with adipocyte size in thigh, abdominal subcutaneous and visceral adipose tissues of men (Edens et al. 1993; Votruba et al. 2007). Studies including both sexes failed to observe differences between visceral and abdominal subcutaneous LPL activity (Fried et al. 1993; Panarotto et al. 2000). However, regional differences in LPL activity were observed in those examining sexes separately (Boivin et al. 2007; Marin et al. 1992a; Mauriege et al. 1995; Rebuffe-Scrive et al. 1989; Tchernof et al. 2006). Although these differences are partly explained by regional variations in cell size, adipose tissue depots of women and men also appear to have intrinsic differences in LPL activity. Indeed, higher LPL activity in subcutaneous than visceral adipose tissue is observed in women compared to men (Mauriege et al. 1995; Rebuffe-Scrive et al. 1989; Tchernof et al. 2006). Such findings are not surprising given that subcutaneous adipocytes are generally larger than visceral adipocytes in this sex. In men, adipose tissue LPL activity has been shown to be higher in visceral adipose tissue than subcutaneous adipose tissue (Boivin et al. 2007; Marin et al. 1992a; Rebuffe-Scrive et al. 1989), which contrasts with the similar adipocyte size in these fat compartments (Boivin et al. 2007; Edens et al. 1993; Fried and Kral 1987; Fried et al. 1993; Marin et al. 1992a; Rebuffe-Scrive et al. 1990). Thus,

sex-specific differences in LPL activity likely reflect the propensity of each adipose tissue depot to accumulate lipids in women and men.

Direct evidence on regional variations in lipid accumulation *in vivo* is limited. However, the use test meals with fatty acid tracers combined with adipose tissue sampling provided highly valuable information. In women, meal-derived fatty acid storage increased in proportion to the mass of lower body subcutaneous adipose tissue, whereas no association was observed between relative lipid uptake in abdominal subcutaneous fat and adiposity (Koutsari et al. 2008). With increasing adiposity, a preservation of the relative capacity to store fatty acids in adipose tissue from the thigh and femoral regions, but not from abdominal fat compartments may promote the development of the lower body fat partitioning phenotype in women. Conversely, the capacity of abdominal subcutaneous adipose tissue to assimilate fatty acids is higher compared to that of the femoral depot in men (Shadid et al. 2007). Moreover, a significant proportion of fatty acid uptake occurs in visceral adipose tissues of men during the postprandial period (Marin et al. 1996; Nguyen et al. 1996; Romanski et al. 2000). Indirect measurements of visceral adipose tissue lipid uptake revealed that this depot contributes more significantly to remove fatty acids from the circulation in men than in women (Nguyen et al. 1996; Romanski et al. 2000). These results are consistent with the fact that men have approximately twice the amount of visceral fat compared to women with similar overall adiposity values (Lemieux et al. 1994).

In addition to LPL activity and triglyceride synthesis, adipose tissue blood flow during the postprandial period is suggested as an important determinant of sex- and depot-related differences in lipid accumulation (McQuaid et al. 2011; Romanski et al. 2000). Indeed, increased blood flow is observed in lower body adipose tissue following meal ingestion in women, but not in men (Romanski et al. 2000). Consistent with these observations, triglyceride synthesis from glucose is lower in omental compared to abdominal subcutaneous adipose tissue in women (Edens et al. 1993; Maslowska et al. 1993), but is similar in both fat depots in men (Edens et al. 1993). These findings indicate that different mechanisms may be involved in the regulation of lipid accumulation in various fat compartments, which may consequently alter body fat distribution (Votruba and Jensen 2007). Direct meal fatty acid storage seems to contribute to the sex dimorphism in fat distribution (reviewed in (Santosa and Jensen 2014, 2015)). Interestingly, absolute FFA storage rates were found to be equal in small, medium and large adipocytes from abdomen and thigh, but the rates of storage expressed per cell number were higher in large than small adipocytes (Rajjo et al. 2014).

8.3.3 Lipolysis

Lipolysis is one of the major functions of adipose tissue by which the adipocyte supplies the body tissues with the required energy during fasting, starvation or exercise in the form of free fatty acids (reviewed in (White and Tchoukalova 2014)).

Net lipid accumulation in a given fat depot reflects the balance between triglyceride storage that is mainly regulated through LPL activity and the rates of lipolysis that is primarily controlled by insulin and catecholamines. Fat cell size is a major determinant of lipolytic responsiveness (Farnier et al. 2003; White and Tchoukalova 2014). On the other hand, the release of stored fatty acids from the femoral region seems to be resistant to adrenaline stimulation (Manolopoulos et al. 2012) suggesting a possible contribution of femoral adipose tissue to the prevention of ectopic fat deposition (Manolopoulos et al. 2012). Furthermore, high visceral fat accumulation is associated with high levels of free fatty acids (FFA) in the splanchnic circulation (Nielsen et al. 2004). Thus, in visceral obesity, omental and mesenteric adipose tissues may play a significant role in releasing not only fatty acids but also interleukin (IL)-6 to the liver (reviewed in (Jensen 2008). IL-6 is considered as an efficient stimulator of lipolysis in human (Van Hall et al. 2003).

FFA release differs not only as a function of regional body area but also depending on sex (Jensen 2007). *In vivo* studies have suggested, however, that regional differences in lipolysis seem to play a minor role in determining sex differences in body fat distribution, with greater contribution of regional meal FFA storage (reviewed in (Santosa and Jensen 2015)). In absolute values, women have higher lipolytic activity than men (Adler-Wailes et al. 2013; Schmidt et al. 2014). Moreover, healthy non obese women have higher rates of non-oxidative free fatty acid disposal than men (Koutsari et al. 2011b). Accordingly, women have higher FFA storage rates (palmitate) in upper- and lower-body subcutaneous fat depots than men (Koutsari et al. 2011a). The kinetics adipose tissue lipolysis also differs between obese male and female, suggesting higher FFA release among females (Adler-Wailes et al. 2013; Bush et al. 2014). Moreover, leg and splanchnic tissues are the main source of FFA in obese men and women compared to lean individuals.

In women, the greater rate of lipolysis is associated to lower activation of α2-adrenergic receptors (Schmidt et al. 2014). Yet in obesity, catecholamine-induced lipolysis is reversibly attenuated (Mikael et al. 2015). After menopause, increased lipid accumulation and enlargement of adipocytes in the visceral fat compartment is associated with higher lipolytic rates (Tchernof et al. 2004). In healthy men, acute exposure to cold increased the FFA release from WAT lipolysis associated with metabolic activation of brown adipose tissue (Blondin et al. 2015). Another interesting finding is that sleep restriction also increases the nocturnal and early morning FFA which may contribute to insulin resistance (Broussard et al. 2015).

Some animal studies contributed to a better understanding of sex differences in lipolysis. In a study of male and female mice, only female mice exhibited increases in adipose tissue forskolin-stimulated lipolysis, as shown by significantly higher glycerol release (Benz et al. 2012). In the same study, female mice exhibited efficient weight loss whereas, male mice exhibited higher body weight gain than females (Benz et al. 2012). Another study reported increase FFA concentration and adipose tissue lipolysis in female mice after training while FFA levels in male mice were decreased (Foryst-Ludwig et al. 2011).

8.3.4 Adipose Tissue Cytokine Release

In addition to its lipid storage function, adipose tissue is known to produce a number of cytokines, also termed adipokines, as well as many other factors involved in the regulation of several biological processes (Ahima and Flier 2000; Mohamed-Ali and Coppack 1998; Trayhurn and Wood 2005). White adipose tissue releases over 600 cytokines or adipokines involved in the regulation of multiple biological processes (Aguilar-Valles et al. 2015; Tchernof and Despres 2013). These cytokines are mainly secreted by adipocytes or preadipocytes, but also, especially in obesity, by immune cells invading the tissue (Cildir et al. 2013; Ferrante 2007; Lee and Lee 2014; Neels and Olefsky 2006; Trayhurn and Wood 2005). Chronic, low-grade inflammation caused by altered adipokine secretion may impair glucose and lipid metabolism and contribute to the altered cardiometabolic risk of individuals with visceral obesity (Ferrante 2007; Lee and Lee 2014; Trayhurn and Wood 2005). Mean adipocyte size and localization of fat as well as sex have been suggested as key determinants of inflammation and cytokine secretion (Drolet et al. 2009; Good et al. 2006; He et al. 2003; Hube and Hauner 1999; Skurk et al. 2007). Circulating levels of adiponectin, an adipocyte-derived adipokine with insulin sensitizing and anti-inflammatory properties, are inversely associated with visceral obesity (Whitehead et al. 2006). Adiponectin secretion by omental adipocytes is markedly reduced in visceral obese women, suggesting that this tissue is an important determinant of serum adiponectin levels in abdominally obese women (Drolet et al. 2009; Motoshima et al. 2002). There is a clear sex difference in adiponectin, with serum concentrations that are approximately 50% higher in women compared to men (Laughlin et al. 2006, 2007). Sex hormones are possibly involved in this difference, since the testosterone-to-estrogen ratio is positively related to serum adiponectin levels (Laughlin et al. 2006). However, expression and secretion of adiponectin in adipocytes are unaffected by sex steroid treatments suggesting that other mechanisms may explain this difference, including metabolic clearance, serum factors modulating adiponectin availability or sex-related differences in adipose tissue accumulation and distribution *per se* (Blouin et al. 2010; Horenburg et al. 2008).

Leptin, an adipocyte-secreted adipokine, plays a key role in the regulation energy intake and energy expenditure (Sinha and Caro 1998). Serum leptin concentrations are strongly associated with body fat mass (Sinha and Caro 1998). Leptin expression and secretion are also higher in subcutaneous than in visceral adipocytes (van Harmelen et al. 2002). Subcutaneous adipocyte size is positively correlated with plasma levels of leptin independent of adiposity (Lundgren et al. 2007). Thus, increased leptin levels in obese individuals are likely due to a combination of increased subcutaneous fat accumulation through hypertrophy and higher secretion rates (Lundgren et al. 2007; van Harmelen et al. 2002). A marked sex dimorphism has been reported for serum leptin levels. Independent of adiposity and body fat distribution, women have approximately three fold higher leptin levels than men (Laughlin et al. 2007). In both women and men, the testosterone-to-

estrogen ratio is inversely related to leptin (Laughlin et al. 2007). Testosterone also decreases the expression of leptin in mature adipocytes from both sexes (Horenburg et al. 2008). A direct action of estrogen on leptin expression is also possible (Machinal-Quelin et al. 2002).

In addition to these adipokines, adipose tissues secrete other factors involved in the regulation of metabolic pathways. Abdominally obese individuals display an altered expression and/or secretion pattern of some key cytokines/adipokines such as TNF-α, PAI-1 and IL-6 which can alter lipolysis, insulin sensitivity and fibrinolysis (Gnacinska et al. 2009). Moreover, macrophage infiltration in adipose tissue of obese individuals could be a major source of pro-inflammatory adipokines (Weisberg et al. 2003). The chronic, low-grade inflammation triggered and/or reflected by visceral adiposity may contribute to metabolic alterations observed in abdominally obese individuals, and subsequently to the increased risk of developing type 2 diabetes and cardiovascular disease.

8.4 Hormonal Determinants of Body Fat Distribution

We still know little about the etiological factors leading to preferential deposition of visceral fat in the presence of excess energy intake. The marked sex dimorphism clearly suggests that sex hormones play a key role in the regulation of body fat distribution. The involvement of sex hormones is confirmed in transsexuals who have been treated with sex hormones. Female-to-male transsexuals receiving intramuscular testosterone present a shift in body fat distribution from the gynoid to android pattern over the course of a few months to 3 years (Elbers et al. 1997, 1999a, 2003). Conversely, treatment with estrogens in male-to-female transsexuals significantly increases fat deposition in all subcutaneous fat depots while having a small effect on the size of the visceral fat compartment (Elbers et al. 1997, 1999a, 2003). As a result, male-to-female hormone treatments have beneficial effects on the cardiometabolic risk profile, whereas high testosterone doses in women have a detrimental effect (Elbers et al. 2003). These results suggest that the prevailing hormonal milieu may be an important determinant of body fat distribution in both women and men. The following section will address the role of steroid hormones in sex-specific adiposity patterns. A summary of steroid action on adipose tissue metabolism is included in Tables 8.1, 8.2 and 8.3.

8.4.1 Androgens

Androgens have been demonstrated to modulate adipose tissue distribution in both sexes (Allan and McLachlan 2010). In men, there is a bidirectional relationship between testosterone and obesity (Allan and McLachlan 2010). Low circulating levels of endogenous androgens are associated with abdominal/visceral obesity

Table 8.1 Summary of androgen actions on fat accumulation, metabolic profile and adipose tissue metabolism

Exogenous androgen administration				
Outcome	Model	Hormona exposure	Effect	References
Total and subcutaneous adiposity	Men	Testosterone tx	↘	Kenny et al. (2001); Snyder et al. (1999)
	Postmenopausal women	DHT tx	↘	Gruber et al. (1998)
	Female transsexuals	Testosterone tx	↘	Elbers et al. (1997, 1999a, 2003)
Abdominal/ Visceral fat accumulation	Men	Testosterone tx	↘	Lovejoy et al. (1995); Marin (1995); Schroeder et al. (2004); Woodhouse et al. (2004)
	Postmenopausal women	DHT tx	↘	Gruber et al. (1998)
	Female transsexuals	Testosterone tx	↗	Elbers et al. (1997, 1999b, 2003)
Body weight, BMI and waist circumference	Men	Testosterone tx	↘	Haider et al. (2014a, b); Saad et al. (2013); Saad et al. (2015, 2016); Yassin et al. (2016)
Insulin resistance	Men	Testosterone tx	↗/↘	Bonetti et al. (2008); Boyanov et al. (2003); Marin et al. (1992c, 1995)
	Female transsexuals	Testosterone tx	**NS**	Elbers et al. (2003); Gooren and Giltay (2008)
Atherogenic lipid profile	Men	Testosterone tx	↗/**NS**	Bonetti et al. (2008); Gruenewald and Matsumoto (2003); Snyder et al. (1999)
	Female transsexuals	Testosterone tx	↗	Elbers et al. (2003); Gooren and Giltay (2008)
Endogenous androgen levels				
Outcome	Model	Hormone exposure	Association	References
Total and subcutaneous adiposity	Men	Testosterone and DHT	–	Gapstur et al. (2002); Nielsen et al. (2007); Pasquali et al. (1991); Phillips et al. (2003)
	Women	Testosterone	–	De Pergola et al. (1994); Ivandic et al. (2002); Kaye et al. (1991)

(continued)

Table 8.1 (continued)

Exogenous androgen administration				
Outcome	Model	Hormona exposure	Effect	References
Visceral fat accumulation	Men	Andros-tenedione, testosterone, DHEA and DHT	–	Couillard et al. (2000); Gapstur et al. (2002); Khaw and Barrett-Connor (1992); Nielsen et al. (2007); Pasquali et al. (1991); Phillips et al. (2003); Pritchard et al. (1998); Seidell et al. (1990b); Tchernof et al. (1995a)
	Women	Testosterone	**+/–/NS**	Armellini et al. (1994); De Pergola et al. (1994); Evans et al. (1988); Ivandic et al. (2002); Kaye et al. (1991); Pedersen et al. (1995); Seidell et al. (1990b); Turcato et al. (1997)
Insulin resistance	Men	Testosterone	–	Pasquali et al. (1991); Phillips et al. (2003); Seidell et al. (1990a)
	Women	Testosterone	–	Ivandic et al. (2002)
Atherogenic lipid profile	Men	Testosterone	–	Haffner et al. (1996); Khaw and Barrett-Connor (1991)
Adipose tissue metabolism				
Outcome	Tissue or model	Hormone exposure	Effect	References
In vivo LPL activity and triglyceride accumulation	Visceral adipose tissue	Testosterone	↘	Marin et al. (1996)
	SC adipose tissue	Testosterone	**NS**	Marin et al. (1996); Rebuffe-Scrive et al. (1991)
In vitro LPL activity	SC and OM human adipose tissue	Testosterone and DHT	↘	Blouin et al. (2010)
In vivo catecholamine-stimulated lipolysis	Abdominal SC adipose tissue	Testosterone	↗	Rebuffe-Scrive et al. (1991)
	Femoral SC adipose tissue	Testosterone	**NS**	Rebuffe-Scrive et al. (1991)
In vitro catecholamine-stimulated lipolysis	Rat and abdominal OM and/or SC adipose tissue	Testosterone, DHT and DHEA-S	↗/↘	Anderson et al. (2002); Dicker et al. (2004); Hernandez-Morante et al. (2008); Xu and Bjorntorp (1990)

(continued)

Table 8.1 (continued)

Exogenous androgen administration				
Outcome	Model	Hormona exposure	Effect	References
Preadipocyte proliferation	3 T3-L1 and human primary preadipocytes	Testosterone, DHT and DHEA	**NS**/↘	Dieudonne et al. (2000); Fujioka et al. (2012); Monjo et al. (2005)
Preadipocyte differentiation	3 T3-L1 and rat primary preadipocytes	Testosterone and DHT	↘	Chazenbalk et al. (2013); Dieudonne et al. (2000); Singh et al. (2006)
	SC and OM human primary preadipocytes	Testosterone and DHT	↘	Blouin et al. (2010); Gupta et al. (2008)

↗: increased, ↘: decreased or NS: no significant change in outcome reported following hormone exposure
+: positive, −: negative or NS: no significant association between hormonal levels and outcome
tx: pharmacological treatment, DHT: dihydrotestosterone, LPL: lipoprotein lipase, SC: subcutaneous, OM: omental

Table 8.2 Summary of estrogen actions on adipose tissue metabolism

Adipose tissue metabolism				
Outcome	Tissue or model	Hormone exposure	Effect	References
In vivo LPL activity	Premenopausal women	Estradiol	↘	Price et al. (1998)
	Postmenopausal women	Estradiol	↗	Rebuffe-Scrive et al. (1987); Lindberg et al. (1990)
In vitro LPL activity	Human primary adipocytes	Estradiol	↗/↘	Palin et al. (2003); Tchernof et al. (2004)
In vivo basal lipolysis	Postmenopausal women	Estradiol	↘	Jensen et al. (1994)
In vitro basal lipolysis	Human primary adipocytes	Estradiol	↗	Palin et al. (2003)
	Human primary adipocytes	Menopause	↗	Tchernof et al. (2004)
In vivo catecholamine-stimulated lipolysis	Pre- and postmenopausal women	Estradiol	↗↘ (pre)/ ↘ (post)	Gavin et al. (2013); Gormsen et al. (2012); Lindberg et al. (1990)
In vitro catecholamine-stimulated lipolysis	Human primary adipocytes	Estradiol/ Menopause	**NS**	Rebuffe-Scrive et al. (1987)
Preadipocyte proliferation	Human and rat primary preadipocytes	Estradiol	↗	Anderson et al. (2001); Dieudonne et al. (2000)
Preadipocyte differentiation	Rat primary preadipocytes	Estradiol	**NS**	Dieudonne et al. (2000)

↗: increased, ↘: decreased or NS: no significant change in outcome reported following hormone exposure
LPL: lipoprotein lipase

Table 8.3 Summary of glucocorticoid actions on fat accumulation, metabolic profile and adipose tissue metabolism

Exogenous or chronic glucocorticoid exposure				
Outcome	Model	Hormone exposure	Effect	References
Total adiposity	Cushing's syndrome	Hypercortisolemia	↗	Carroll and Findling (2010)
Visceral fat accumulation	Cushing's syndrome	Hypercortisolemia	↗	Carroll and Findling (2010)
Insulin resistance	Cushing's syndrome	Hypercortisolemia	↗	Carroll and Findling (2010)
	Men and women	Glucocorticoid tx	↗	Schacke et al. (2002)
Atherogenic lipid profile	Cushing's syndrome	Hypercortisolemia	↗	Carroll and Findling (2010)
	Men and women	Glucocorticoid tx	↗	Schacke et al. (2002)
Endogenous glucocorticoid levels				
Outcome	Model	Hormone exposure	Association	References
Total and subcutaneous adiposity	Men and women	Urinary cortisol, fasting- and stress- induced cortisol	**+/−/NS**	Ljung et al. (1996); Marin et al. (1992c); Rosmond and Bjorntorp (1998); Strain et al. (1980); Walker et al. (2000)
Visceral fat accumulation	Men and women	Urinary cortisol, fasting- and stress- induced cortisol	**+/−/NS**	Ljung et al. (1996); Marin et al. (1992c); Rosmond and Bjorntorp (1998); Strain et al. (1980); Walker et al. (2000)
Insulin resistance	Men and women	Physiological elevation of cortisol levels	+	Divertie et al. (1991); Djurhuus et al. (2002); Macfarlane et al. (2008)
Atherogenic lipid profile	Men and women	Physiological elevation of cortisol levels	+	Divertie et al. (1991); Djurhuus et al. (2002); Macfarlane et al. (2008)
Adipose tissue metabolism				
Outcome	Tissue or model	Hormone exposure	Effect	References
In vitro LPL activity	Human fat samples and primary adipocytes	Cortisol	↗	Hauner et al. (1989); Ottosson et al. (1994, 1995)

(continued)

Table 8.3 (continued)

Exogenous or chronic glucocorticoid exposure				
Outcome	Model	Hormone exposure	Effect	References
In vivo basal lipolysis	Men and women	Cortisol	↗	Dinneen et al. (1993); Divertie et al. (1991)
In vitro basal lipolysis	Rat primary adipocytes	Dexamethasone	↗	Fain et al. (1965); Slavin et al. (1994); Xu et al. (2009)
In vitro catecholamine-stimulated lipolysis	Human fat samples and rat primary adipocytes	Dexamethasone, cortisol	↗/↘	Fain et al. (1965); Lacasa et al. (1988); Ottosson et al. (2000)
Preadipocyte proliferation	Rat and mice primary preadipocytes	Cortisone, cortisol, dexamethasone	↘	Gaillard et al. (1991); Gregoire et al. (1991)
Preadipocyte differentiation	3T3-L1, human and rat and mice primary preadipocytes	Cortisone, cortisol, dexamethasone	↗	Ailhaud et al. (1991); Gaillard et al. (1991); Gregoire et al. (1991); Hauner et al. (1989); Ottosson et al. (1995); Wolf (1999)
Local proinflammatory action and reticulum endoplasmic stress	3T3-L1 and mice primary preadipocytes	Dexamethasone	↗	Hoppmann et al. (2010); Ishii-Yonemoto et al. (2010); Yacoub Wasef et al. (2006)

↗: increased, ↘: decreased or NS: no significant change in outcome reported following hormone exposure
+: positive, −: negative or NS: no significant association between hormonal levels and outcome
tx: pharmacological treatment, LPL: lipoprotein lipase

assessed by waist circumference or computed tomography (Gapstur et al. 2002; Khaw and Barrett-Connor 1992; Nielsen et al. 2007; Pasquali et al. 1991; Phillips et al. 2003; Seidell et al. 1990b). On the one hand, changes in whole body and regional body fatness upon treatment with testosterone in men suggest a direct impact of the hormone on adiposity (see below). On the other, weight loss in severe obesity has been found to increase testosterone level (Allan and McLachlan 2010). Methodological limitations in the measurement of free-testosterone make it difficult to detect an association between body fat distribution and free androgen levels (Rosner et al. 2007; Vermeulen et al. 1999). However, plasma concentrations of sex hormone-binding globulin (SHBG), a determinant of testosterone bioavailability, are negatively associated with abdominal obesity in both men and women (Couillard et al. 2000; Gapstur et al. 2002; Garaulet et al. 2000; Khaw and Barrett-Connor

1992; Pasquali et al. 1991; Phillips et al. 2003; Tchernof et al. 1995a). Individuals with elevated plasma SHBG and testosterone levels are also generally characterized by a lower number of metabolic syndrome features (Blouin et al. 2005a; Hajamor et al. 2003; Laaksonen et al. 2003; Phillips et al. 2003). Circulating levels of dehydroepiandrosterone (DHEA), a precursor of active steroids in peripheral tissues, are also negatively associated with visceral obesity in some studies (Couillard et al. 2000; Pritchard et al. 1998; Tchernof et al. 1995b) as reviewed in (Tchernof and Labrie 2004).

The presence of steroid hormones in adipose tissue has been known for a long time (reviewed in (Belanger et al. 2002; Tchernof et al. 2015)). Omental adipose tissue levels of testosterone and dihydrotestosterone (DHT) were negatively associated with waist circumference in a sample of obese men (Belanger et al. 2006). Moreover, in the same study, androstenedione, testosterone and DHT levels were positively associated with adipocyte lipolytic responsiveness to catecholamine stimulation and this association was more pronounced in omental than in subcutaneous adipose tissue (Belanger et al. 2006). Accordingly, basal lipolysis in female-to-male transsexuals under testosterone treatment increased in abdominal but not in gluteal adipose tissue (Elbers et al. 1999b).

As opposed to androgen treatments in female-to-male transsexuals, supplementation with physiological doses of testosterone in men with initially low endogenous levels generally leads to a decrease in visceral fat accumulation (reviewed in (Blouin et al. 2010; Woodhouse et al. 2004)). Androgen supplementation also leads to increased insulin sensitivity (Boyanov et al. 2003; Marin et al. 1992c), while having neutral effects on the lipid profile (Gruenewald and Matsumoto 2003). In young men (age < 50 years) with hypogonadism, testosterone replacement lead to a number of beneficial effects not only on reproductive function but also by increasing skeletal muscle mass (Spitzer et al. 2013) and by reducing total body fat in both hypogonadal and aging men (Allan and McLachlan 2010). Saad et al. reported that testosterone therapy leads to weight loss and reduction in waist circumference in all three classes of obesity. Moreover, elderly men (age > 65 years) with late onset of hypogonadism seem to benefit from testosterone treatment as much as younger men (Saad et al. 2015, 2016). One possible effect of testosterone on body fat is the inhibition of preadipocyte differentiation which may explain the fat mass reduction in men treated with testosterone (Singh et al. 2006) and reviewed in (Zerradi et al. 2014). Haider et al., have shown that long term testosterone therapy up to 6 years leads to significant weight loss and improvement of type 2 diabetes and cardiometabolic risk factors in patients suffering from obesity, diabetes and testosterone deficiency (Haider et al. 2014b). In an observational study, administration of testosterone improved body weight and metabolic risk factors in hypogonadal men. However, withdrawal of testosterone reversed these effects, which returned again when testosterone therapy was resumed (Yassin et al. 2016). In men with testosterone deficiency, the effects of long term testosterone therapy on weight loss, BMI and waist circumference seems to be attributed to improvement of adipose tissue function, mitochondrial function, energy utilization, increased motivation and vigor which in turn improve the cardiometabolic

function and allow increases in physical activity (reviewed in (Traish 2014). On the other hand, androgen deprivation therapy (ADT), the first line of treatment and management of advanced prostate cancer in men represses the effects of androgen/androgen receptor signals. This type of treatment may be accompanied by obesity, insulin resistance, alterations of the lipid profile and cardiovascular complications (Yu et al. 2014).

Hence, within the physiological range, higher testosterone concentrations are associated with a favorable metabolic profile, either when considering endogenous levels or following physiological replacement in men with low baseline testosterone concentrations (Blouin et al. 2005b, 2008; Hajamor et al. 2003; Laaksonen et al. 2003; Phillips et al. 2003).

In women, based on the common observation of abdominal obesity in patients with the polycystic ovary syndrome (PCOS), investigators have often concluded that hyperandrogenism in women leads to abdominal obesity and hyperinsulinemia (Dunaif 1997). Advances in our understanding of PCOS reveal that the link between hyperandrogenism and abdominal obesity may be more complex than initially thought. For example, a careful analysis has shown that once differences in BMI are taken into account, there is no regional difference in patterns of fat distribution between PCOS cases and control women (Barber et al. 2008). Moreover, findings that insulin sensitizing treatments improve the ovarian and androgenic component of PCOS also led to a reconsideration of the basic causal relationship implying high androgens as the direct cause of visceral obesity in these patients.

On the other hand, testosterone therapy is effective in the treatment of female sexual dysfunction (Davis et al. 2016). In women with low testosterone levels who were subjected to testosterone replacement therapy in various doses for 24 weeks, no adverse effects in cardiometabolic risk factors were observed (Spitzer et al. 2013). Moreover, testosterone therapy up to 12 months has no adverse effect on female voice (Glaser et al. 2016). Long-term studies are required to establish the safety of testosterone therapy and its possible benefits in women (Davis et al. 2016). *In vitro* experiments show that androgen treatment of abdominal adipocytes or adipose tissue explants does not lead to increased adipogenesis or higher uptake of lipids as assessed by LPL activity (Blouin et al. 2010). In fact, androgens had the opposite effect as they inhibited these indirect measures of lipid storage, even at high doses (Blouin et al. 2010). An increasing body of evidence seems to suggest that prenatal androgenization of the fetus may be an important etiologic factor for PCOS and related metabolic alterations (reviewed in (Xita and Tsatsoulis 2006)).

Association studies on circulating androgens and body fat distribution are also equivocal in women. In some studies including non-PCOS women (Evans et al. 1988; Pedersen et al. 1995; Seidell et al. 1990b), visceral fat accumulation is associated with high total or free plasma testosterone levels. Others have found negative associations between plasma testosterone levels and visceral fat accumulation (Armellini et al. 1994; De Pergola et al. 1994; Turcato et al. 1997), while some observed no correlation (Ivandic et al. 2002; Kaye et al. 1991). As previously mentioned, low plasma SHBG levels have been consistently associated with abdominal

obesity and the metabolic syndrome in both sexes (De Pergola et al. 1994; Hajamor et al. 2003; Ivandic et al. 2002). However, as opposed to men, DHEA concentrations were not associated with body fat distribution in women (Tchernof and Labrie 2004). Although the clinical impact of androgen supplementation in women is not well characterized from the metabolic standpoint, one study reported an anti-adiposity effect of DHT administration (Gruber et al. 1998). This is supported by the finding that omental adipocyte hypertrophy was associated with low endogenous DHT level (Cote et al. 2012). Recently, another study demonstrated that DHEA and testosterone supplementation in women and men with low levels of these hormones has no effect on insulin suppression of systemic lipolysis (Espinosa De Ycaza et al. 2016).

Several studies have focused on androgenic action on adipose tissue function. Since treatment duration and concentration are important determinants of hormone action, data on androgen action remain slightly discordant. Consistent with the known inhibitory effect of androgens on lipid accumulation, testosterone reduces adipose tissue LPL activity in abdominal (Marin et al. 1996), but not in femoral subcutaneous adipose tissue depot (Marin et al. 1996; Rebuffe-Scrive et al. 1991). Testosterone supplementation also enhances norepinephrine-stimulated lipolysis in abdominal, but not in femoral subcutaneous adipose tissue (Rebuffe-Scrive et al. 1991). *In vitro* studies support the notion that catecholamine-stimulated lipolysis is enhanced by androgens in a dose-dependent and depot-specific manner (Anderson et al. 2002; Xu et al. 1991). Androgenic effects on preadipocyte proliferation appear to be relatively negligible (Anderson et al. 2002; Dieudonne et al. 2000; Monjo et al. 2005), but testosterone and DHT are important inhibitors of *in vitro* and *in vivo* preadipocyte differentiation (Blouin et al. 2009c; Dieudonne et al. 2000; Gupta et al. 2008; Lacasa et al. 1997; Singh et al. 2006; Tchernof and Labrie 2004). In most instances, androgen responsiveness is found to be more pronounced in visceral than subcutaneous adipose tissue (Dieudonne et al. 2000; Joyner et al. 2002; Lacasa et al. 1997; Rodriguez-Cuenca et al. 2005), although these findings are not unanimous (Blouin et al. 2010).

The notion of a specific and direct genomic action of androgens on body fat distribution is reinforced by observations of significant androgen receptor expression and binding in both preadipocytes and mature adipocytes (Dieudonne et al. 1998; Miller et al. 1990; Pedersen et al. 1996). Androgen receptor expression in adipose tissue is similar between women and men (Dieudonne et al. 1998; Joyner et al. 2002). However, visceral adipose tissue androgen receptor expression is higher in omental compared to subcutaneous adipose tissue in both sexes (Dieudonne et al. 1998; Joyner et al. 2002; Miller et al. 1990; Rodriguez-Cuenca et al. 2005). Increased androgen receptor expression was reported following induction of preadipocyte differentiation (Blouin et al. 2010; Dieudonne et al. 1998; Veilleux et al. 2009a). However, lower androgen receptor expression in mature adipocytes than preadipocytes has also been reported, particularly in visceral adipose tissue (Dieudonne et al. 1998). These observations suggest that mature adipocytes may be less responsive to androgen stimulation compared to preadipocytes. The study of androgen receptor knock-out mice supports an important role for androgens and its receptor in the modulation of body fat accumulation (Sato et al. 2003). These mice display a late-

onset visceral obesity phenotype triggered by decreased energy expenditure and defective lipolysis (Fan et al. 2005).

Local androgen synthesis and inactivation are now believed to be important determinants of androgen action in adipose tissue (Blouin et al. 2010; McIntosh et al. 1999). The aldo-ketoreductase 1C (AKR1C) enzymes may contribute to adipose tissue androgen metabolism as they are the most highly expressed steroidogenic enzymes in adipose tissue of both women and men (Blouin et al. 2005a, 2006). These enzymes have varying proportions of 20α-, 3α- and 17β-hydroxysteroid dehydrogenase activities (Zhang et al. 2000). In the context of androgenic action, the most interesting member of this family is AKR1C2 since it possesses a strong 3α-reductase activity. Thus, AKR1C2 has the ability to inactivate DHT into its inactive metabolite 5α-androstane-3α,17β-diol (3α-diol) (Zhang et al. 2000). Expression of AKR1C2 and 3α-HSD activity are higher in subcutaneous compared to visceral adipose tissue in both women and men (Blanchette et al. 2005; Blouin et al. 2003, 2005a, 2006). In both sexes, AKR1C enzyme expression and androgen inactivation rates of visceral adipose tissue are positively correlated with indices of obesity including BMI, fat cell size, and visceral adipose tissue area assessed by computed tomography (Blanchette et al. 2005; Blouin et al. 2003, 2005a, 2006; Wake et al. 2007). Preadipocyte differentiation is a strong stimulator of AKR1C2 expression, so that mature adipocytes show dramatically higher DHT inactivation rates than preadipocytes (Blouin et al. 2009a). This increase is believed to occur early in the preadipocyte differentiation process as the result of glucocorticoid stimulation (Blouin et al. 2009a). Increased androgen inactivation by AKRIC2, may lead to reduced local exposure of fat cells to active androgens. Accordingly, we have reported that down regulation of AKR1C2 expression potentiates the inhibitory effect of DHT on primary preadipocyte differentiation (Veilleux et al. 2012). Most interestingly, DHT inactivation was stimulated by glucocorticoids alone, which provides a new putative mechanism for the regulation of active androgen availability at the local level in each fat compartment. By stimulating DHT inactivation, glucocorticoids can remove part of the inhibitory effect of this hormone on adipocyte differentiation and create a permissive milieu for adipogenesis. Future studies may eventually establish the contribution of this mechanism to body fat distribution patterns in humans.

17β-HSD type 2 is another enzyme that may be involved in the modulation of androgen dynamics in human adipose tissue. This enzyme converts 17β-hydroxysteroids testosterone and estradiol to less active 17-ketosteroids androstendione (4-dione) and estrone respectively (Wetzel et al. 2011; Wu and von Eckardstein 2003). In adipose tissue, 17β-HSD type 2 is more highly expressed in the visceral than subcutaneous depot in both men and women (Blouin et al. 2009a; Fouad Mansour et al. 2015). Moreover, 17β-HSD type 2 is localized in the adipose tissue blood vessels and its activity in OM and SC adipose tissues was found to be positively correlated with BMI (Fouad Mansour et al. 2015). These findings suggest a possible role of this enzyme in human body fat distribution patterns.

On the other hand, 5α-reductase is a steroid-converting enzyme responsible for synthesis of DHT either directly from testosterone or indirectly through 5α-reduction

of 4-dione and further 17β-oxoreduction to DHT (Andersson and Russell 1990). Three 5α-reductase isoenzymes designated type 1, 2 and 3 that are generated from three different genes SRD5A1, SRD5A2 and SRD5A3 (Li et al. 2011). In adipose tissue, messenger RNA of SRD5A1 and SRD5A3 are detected but not SRD5A2 (Fouad Mansour et al. 2016). The expression of these genes is minimally influenced by induction of preadipocyte differentiation (Blouin et al. 2009a; Fouad Mansour et al. 2016). Adrenal androgen (4-dione) appears to be the main source of DHT formation in human OM and SC adipose tissues (Fouad Mansour et al. 2016). Moreover, the inhibitors of 5α-reductase (4-MA and finasteride) significantly abolish the inhibitory effects of 4-dione and testosterone on preadipocyte differentiation (Fouad Mansour et al. 2016) suggesting possible effect of 5α-reductase isoenzymes in the depot-specific regulation of preadipocyte differentiation.

Overall, active androgens, testosterone and possibly DHT seem to favor reduction of fat mass through inhibition of adipogenesis and lipogenesis and possible stimulation of lipolysis. Effects have been reported to vary according to the fat depot examined and as a function of the nature and dose of the androgen tested. Considering the clear androgenic impact on the distribution patterns of adipose tissue, local synthesis or inactivation of active androgens could logically have depot-specific effects on androgen availability and possibly accumulation of adipose tissue (reviewed in (Tchernof et al. 2015)).

8.4.2 Estrogens

Estrogens are involved in female sexual development and the reproductive cycle. In premenopausal women, estrogens are produced mainly in ovaries (Mattsson and Olsson 2007). However, in both women and men, estrogens are also generated through aromatization of androgens, locally, in several tissues, especially fat and muscle (Mattsson and Olsson 2007). This estrogen source is especially important in men and postmenopausal women (Labrie et al. 2003). The parallel sex dimorphisms in estrogen levels and body fat distribution as well as transsexual studies have highlighted the possibility that this hormone is involved in the regulation of regional fat deposition. Moreover, as mentioned, reduced estrogen levels after menopause have been associated with increased adiposity and visceral fat accumulation (Gambacciani et al. 1997; Guthrie et al. 2003, 2004; Keller et al. 2010; Lovejoy et al. 2008; Ryan et al. 2002).

While important central effects of estrogens have been described on energy balance (Brown and Clegg 2010; Richard 1986), other studies have reported direct estrogen action on adipose tissue metabolism (D'Eon et al. 2005). *In vivo*, exogenous estradiol administration decreases LPL activity in the lower body adipose tissue of premenopausal women (Price et al. 1998), but the opposite effect is observed in postmenopausal women (Rebuffe-Scrive et al. 1987). Hormone supplementation in estrogen-deficient postmenopausal women significantly decreased adipose tissue FFA release by 10–20% (Jensen et al. 1994). However, other studies have reported

no alteration of basal and catecholamine-stimulated lipolysis by estrogens in subcutaneous adipose tissue (Rebuffe-Scrive et al. 1987; Tchernof et al. 2004). However, higher LPL and basal lipolysis are observed in visceral adipose tissue samples of ovarian hormone-deficient women (Tchernof et al. 2004). *In vitro*, high concentrations of estradiol decreased LPL and increased hormone-sensitive lipase expression in subcutaneous mature adipocytes (Palin et al. 2003). However, the opposite is observed at low estrogen doses, suggesting that estrogens may have a biphasic action on adipose tissue lipogenic and lipolytic capacity (Palin et al. 2003). Studies have also reported that estrogens stimulate preadipocyte proliferation. This effect is greater in preadipocytes from women compared to preadipocytes from men, and responsiveness to estrogens is different in subcutaneous vs. visceral preadipocytes (Anderson et al. 2001; Dieudonne et al. 2000). Moreover, estrogenic status has been found to influence circulating levels of Acylation Stimulating Protein (ASP) as well as gene expression level of its receptor in adipose tissues (Rezvani et al. 2014). Overall, even if many aspects of estrogenic action remain to be clarified, most investigators agree that estradiol likely has a pivotal role in the regulation of adipose tissue function and metabolism.

Direct action of estrogens in adipose tissue is supported by the presence of both receptor isoforms: namely estrogen receptors α and β (Crandall et al. 1998; Dieudonne et al. 2004). ERα is the primary ER expressed in white adipose tissue; therefore ERα is likely to play an important role in adipose tissue biology (Faulds et al. 2012). In addition, studies on 3T3-L1 preadipocytes with transfected ERα showed decrease triglyceride accumulation and reduced expression of LPL (Homma et al. 2000). Sex- and depot-related differences in estrogen receptor levels have been reported but remain unclear (Blouin et al. 2009b; Dieudonne et al. 2004; Pedersen et al. 1991; Watson et al. 1993). Interestingly, deletion of the estrogen receptor α in male and female mice is associated with increased adiposity independent of food intake (Heine et al. 2000). Polymorphisms in the estrogen receptor α and β genes are associated with slightly higher body fat mass and visceral fat accumulation compared to women with the more frequent genotypes (Goulart et al. 2009; Nilsson et al. 2007; Okura et al. 2003).

Steroid-converting enzymes responsible for estradiol synthesis in adipose tissue contribute not only to local hormone availability, but also to estrogen dynamics in whole-body (reviewed in (Tchernof et al. 2015)). Several studies reported that P450 aromatase, which generates estradiol from testosterone, is expressed in adipose tissue (Blouin et al. 2009a; Cleland et al. 1983; Mackenzie et al. 2008). Estrogen also can be produced in adipose tissue from 4-dione by P450 aromatase. The aromatase-adipose tissue relationship was first highlighted in the 1970s when Edman and MacDonald (1978); MacDonald et al. (1978) reported a correlation between aromatase activity and body weight in pre- and postmenopausal women. Furthermore, the conversion of 4-dione to estrone was detected in the stromal cell fraction of human subcutaneous adipose tissue (Ackerman et al. 1981).

As demonstrated and reviewed by Simpson (2004), P450 aromatase gene expression is controlled by different promoters that are alternatively used, explaining that transcripts of this enzyme in different tissues differ by their 5′-termini. However, the

coding regions and the expressed proteins remain the same among the various tissues. Each promoter is stimulated by a specific pathway. In adipose tissue, promoter I.4 is regulated by inflammatory cytokines such as IL-6 and TNF-α (Simpson et al. 1997; Zhao et al. 1995b) as well as glucocorticoids (Zhao et al. 1995a). A plethora of scientific articles has now detected the critical role of aromatase for estrogen dynamics and pathological conditions like obesity and breast cancer (reviewed in (Simpson 2004)). Estrogens derived from adipose tissue may play a role in the stimulation of breast tumor growth because adipose tissue surrounding breast tumors expresses higher levels of aromatase. Accordingly, aromatase inhibitors are now designated as a treatment of choice in postmenopausal breast cancer (Rao and Cobleigh 2012). The independent impact of estrogens and the involvement of androgen aromatization in human fat distribution patterns remain to be clearly established.

8.4.3 Glucocorticoids

In addition to their involvement in the immune system, glucocorticoids regulate energy homeostasis especially under conditions of stress (Putignano et al. 2004). They promote hepatic glucose output as well as protein and lipid catabolism in muscle and adipose tissue (Putignano et al. 2004). Moreover, several studies underline the role of glucocorticoids in long term adipose tissue adaptation (Gregoire et al. 1991; Hauner et al. 1989). Active glucocorticoids can alter adipose tissue mass and distribution (Bujalska et al. 1999; Michailidou et al. 2007) by inhibiting cellular proliferation and promoting differentiation of preadipocytes to lipid-storing, mature adipocytes (Gregoire et al. 1991; Hauner et al. 1989). The impact of glucocorticoids on body fat distribution may also involve the regulation of adipose tissue blood flow. Specifically, hypercortisolaemia increases blood flow in gluteo-femoral more than in abdominal adipose tissue in the post-prandial state, which likely affects post-prandial fatty acid fluxes to each body fat compartment (Manolopoulos et al. 2015).

Excessive circulating glucocorticoid concentrations, as observed in Cushing's syndrome, create a pathologic phenotype of abdominal obesity, dyslipidemia, insulin resistance, and hypertension (Peeke and Chrousos 1995). In most cases, cortisol hypersecretion is pituitary-dependent (Cushing's disease) and involves the hypothalamo-pituitary-adrenal (HPA) axis (Beaulieu and Kelly 1990). While individuals with idiopathic abdominal obesity share several of the morphologic and metabolic features observed in Cushing's syndrome, alterations in the sensitivity and drive of the HPA axis have been shown to be much more subtle (Duclos et al. 2001; Marin et al. 1992b; Pasquali and Vicennati 2000). Moreover, common abdominal obese patients have circulating glucocorticoid levels that are similar to those of normal weight individuals (Peeke and Chrousos 1995; Westerbacka et al. 2003). Studies on urinary glucocorticoid metabolites reported increased metabolite excretion in obese compared to lean women and men. More specifically, these studies pointed toward enhanced glucocorticoid metabolism through 11β-reductase and

5α-reductase activities in abdominally obese individuals. These observations, combined with unaltered circulating glucocorticoid levels, support the hypothesis of increased peripheral cortisol metabolism in abdominally obese compared to lean individuals (Andrew et al. 1998; Seckl et al. 2004; Westerbacka et al. 2003).

Increased local cortisol synthesis in adipose tissue, without marked central HPA axis alterations, is now clearly recognized as an important etiologic factor of non-Cushing abdominal obesity (Masuzaki et al. 2001; Seckl and Walker 2001). Conversion of inactive cortisone to active cortisol (11β-oxoreductase activity) is catalyzed by type 1 11β-hydroxysteroid dehydrogenase (11β-HSD1). *In vitro*, inactivation of cortisol to cortisone (11β-deshydrogenase activity) may be catalyzed either by the type 1 or type 2 11β-HSD isoforms. However, 11β-oxoreductase activity is predominant for 11β-HSD1 *in vivo*. Thus, in adipose tissue, 11β-HSD1 is primarily a glucocorticoid-activating enzyme, while 11β-HSD2 activity protects cells from active glucocorticoid exposure (Bujalska et al. 1997; Engeli et al. 2004; Lee et al. 2008).

Local production of glucocorticoids by adipose tissue 11β-HSD1 has been clearly linked to the development of abdominal obesity in animal models (Kotelevtsev et al. 1997; Masuzaki and Flier 2003). 11β-HSD1 knock-out mice show attenuated hyperglycemia provoked by stress and by diet-induced obesity (Kotelevtsev et al. 1997). On the other hand, expression of 11β-HSD1 in intra-abdominal adipose tissue is possibly related to type 2 diabetes and obesity (reviewed in (do Nascimento et al. 2015)). Modest overexpression of the 11β-HSD1 gene in adipose tissue is sufficient to induce specific fat accumulation in the visceral fat compartments in mice. These experiments show that increased 11β-HSD1 expression is a direct cause of metabolic alterations such as dyslipidemia and insulin resistance, especially when mice are fed with a high-fat diet (Masuzaki and Flier 2003). This phenotype is also accompanied by increased adipocyte size especially in the visceral fat compartment, as well as increased FFA release (Masuzaki and Flier 2003). Authors of this elegant study concluded that excessive local cortisol production by 11β-HSD1 is a common molecular etiology for visceral obesity and the metabolic syndrome in rodents.

Other studies directly examined peripheral cortisol homeostasis and 11β-HSD1 expression in the context of human abdominal obesity. *In vivo*, one study reported low rates of glucocorticoid uptake and release by adipose tissue (Hughes et al. 2010). Thus, reduced exposure of adipose tissue to rapid circadian changes in circulating glucocorticoids reinforces the possible intracrine or paracrine impact of local cortisol generation (Hughes et al. 2010). Expression levels and *in vitro* activity of 11β-HSD1 are generally higher in visceral compared to subcutaneous adipose tissue (Bujalska et al. 1999; Michailidou et al. 2007; Veilleux et al. 2009b, 2010), although some studies which examined only mRNA expression, failed to observe regional differences (Desbriere et al. 2006; Paulsen et al. 2007; Tomlinson et al. 2002; Veilleux et al. 2010). 11β-HSD1 expression measures in human adipose tissue show higher 11β-HSD1 expression levels in both omental and subcutaneous adipose tissue in men compared to women (Paulsen et al. 2007). Activity and mRNA abundance of the enzyme in whole adipose tissue samples are increased in obese

compared to lean women and men (Desbriere et al. 2006; Kannisto et al. 2004; Lee et al. 2008; Lindsay et al. 2003; Michailidou et al. 2007; Paulsen et al. 2007; Veilleux et al. 2009b, 2010). The existence of positive correlations between 11β-HSD1 expression in subcutaneous adipose tissue and adiposity indices is clearly established (Desbriere et al. 2006; Kannisto et al. 2004; Lee et al. 2008; Lindsay et al. 2003; Paulsen et al. 2007; Rask et al. 2002; Veilleux et al. 2009b, 2010). A few studies which had access to human visceral adipose tissue show that 11β-HSD1 expression in visceral adipose tissue is positively associated with overall adiposity (Desbriere et al. 2006; Lee et al. 2008; Michailidou et al. 2007; Paulsen et al. 2007; Veilleux et al. 2009b, 2010). Abdominal fat accumulation indices are more closely related to 11β-HSD1 expression and oxoreductase activity in visceral adipose tissue than the same variables in subcutaneous adipose tissue (Michailidou et al. 2007; Veilleux et al. 2009b, 2010). As suggested by animal studies, relatively elevated 11β-HSD1 oxoreductase activity in visceral compared to subcutaneous adipose tissue is associated with increased visceral fat accumulation as well as with concomitant metabolic alterations, independent of overall obesity levels (Veilleux et al. 2009b, 2010).

Some factors designated as mediators of glucocorticoid action in adipose tissue have been identified. For example, Forkhead fox protein A3 (Foxa3) is involved in the regulation of selective epididymal fat depot enlargement and suppression of energy expenditure in a manner dependent on nutritional status and age (Ma et al. 2016). This factor is regulated by glucocorticoids in preadipocytes, adipocytes and adipose tissues, and also plays a role in the binding of glucocorticoid receptors to the promoter of target genes (Ma et al. 2016). Foxa3 appears as an amplifier glucocorticoid receptor signals in adipose tissue (Ma et al. 2016). As another example, dexamethasone increases lipocalin 2 gene expression in both omental and subcutaneous adipose tissues of premenopausal women but not in postmenopausal women. In turn, lipocalin 2 contributes to adipose tissue insulin resistance (Kamble et al. 2016). Finally, some metabolites of tryptophan may also mediate some effects of glucocorticoids. 5-hydroxytryptamine (5-HT) has been shown to mediate glucocorticoid-induced visceral adipose tissue insulin resistance in rats (Li et al. 2016). Interestingly, we recently reported that the circulating kynurenine/tryptophan ratio (kynurenine is the main metabolite of tryptophan) was among the metabolomic markers most closely related to obesity level in women (Boulet et al. 2015).

Other genes may be involved in the regulation of local adipose tissue cortisol levels in obese individuals. Expression of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), is detected in the stroma-vascular cell fraction of adipose tissue (Engeli et al. 2004; Lee et al. 2008). Expression of 11β-HSD2 in subcutaneous adipose tissue was negatively associated with BMI in one study (Engeli et al. 2004), but the physiological impact of this association on local concentrations of active glucocorticoids is lessen by the very low 11β-HSD2 expression levels observed both in subcutaneous and omental adipose tissue (Engeli et al. 2004; Hernandez-Morante et al. 2009; Veilleux et al. 2010). Other reports also identified another player for local adipose tissue glucocorticoid reactivation, namely hexose-6-phosphate dehydrogenase (H6PDH) (Bujalska et al. 2005; Rogoff et al. 2007).

H6PDH colocalizes and interacts with 11β-HSD1 by generating the NADPH cofactor needed for cortisone-oxoreductase activity (Zhang et al. 2009). However, visceral adipose tissue H6PDH expression levels are negatively associated with adiposity (Veilleux et al. 2010). Moreover, preadipocyte expression of the gene encoding this enzyme is reduced in obese individuals, although adipogenesis strongly induces its expression (Bujalska et al. 2005). While genetic studies in mice support a role for H6PDH activity in adipose tissue glucocorticoid exposure in mice (Bujalska et al. 2008b), its involvement in human body fat distribution is slightly less apparent based on available literature.

Glucocorticoid signal transduction in fat cells is mediated by glucocorticoid receptor α (GRα) in human adipose tissue (Boullu-Ciocca et al. 2003). Expression levels of this receptor are higher in omental than subcutaneous adipose tissue in most studies (Boullu-Ciocca et al. 2003; Bujalska et al. 2007; Hernandez-Morante et al. 2009; Michailidou et al. 2007; Veilleux et al. 2010). Associations between GRα mRNA expression and adiposity indices are reported in the literature but remain inconsistent (Boullu-Ciocca et al. 2003; Michailidou et al. 2007; Veilleux et al. 2010). These associations indirectly suggest that adipose tissue glucocorticoid action is reduced in obese individuals (Boullu-Ciocca et al. 2003). Inconclusive evidence on the role of 11β-HSD2 and H6PDH limits our ability to reach firm conclusions on their involvement in obesity and body fat distribution patterns. However, the lack of a clear demonstration of their role reinforces the hypothesis that visceral 11β-HSD1 expression may be a critical determinant of local active glucocorticoid levels and a major etiological factor for human abdominal-visceral obesity.

8.5 Clinical Implications

As already discussed in this chapter, excess accumulation of adipose tissue within the abdominal cavity is a critical determinant of the metabolic abnormalities of obesity. Consistent with this notion, weight loss therapy leading to a reduction in visceral adipose tissue mass has been shown to be associated with improvements in several cardiometabolic risk factors (Brochu et al. 2003; Despres and Lamarche 1993; Heilbronn et al. 2001; Kreisberg and Oberman 2003; Tchernof et al. 2002). In keeping with the high responsiveness of visceral adipocytes to positive lipolytic stimuli, a review of weight loss studies suggested that the visceral adipose tissue compartment may be preferentially mobilized in response to a negative energy balance in both sexes (Smith and Zachwieja 1999). In a 1-year lifestyle improvement program, there was significant reduction in body weight, fat mass and the visceral adipose tissue, the latter which was associated with improved insulin homeostasis and cardiometabolic risk in viscerally obese men (Borel et al. 2012a, b; Nazare et al. 2013). Thus, it appears that interventions producing a modest weight loss could lead to proportionally higher and clinically significant mobilization of visceral adipose tissue, which may in turn contribute to alleviate some of the abnormalities leading to type 2 diabetes and cardiovascular diseases.

Regarding the hormonal factors that contribute to visceral obesity, it seems that the correction of the relative androgen deficiency in men and ovarian hormone (estrogen) deficiency in women leads to improvement of the metabolic profile, at least partly through modulation of body fat distribution. However, substitutive hormonal treatments obviously need to be considered in the context of their effects or side-effects on other systems. For example, female hormone replacement therapy has been seriously reconsidered or even abandoned by many women following data indicating that the oral combination of equine estrogens and a progestin causes a 26% increase in the incidence of breast cancer at 5.2 years of follow-up with a negative impact on cardiovascular events (Rossouw et al. 2002). The link between androgen replacement and favorable body composition/fat distribution changes is increasingly recognized in hypogonadal, aging males (Bhasin et al. 2006). Further studies are required to determine whether other androgen replacement modes such as DHEA for example, could be suitable for metabolic improvements in men or women (Labrie et al. 2003, 2007). Inhibitors of local cortisol generation by 11β-HSD-1 are currently considered as a potentially important avenue for future drug development (Bujalska et al. 2008a; Gathercole and Stewart 2010). This was confirmed by the recent finding that cortisol enrichment was detected in overweight/obese diabetic individuals but not in the lean or overweight/obese non diabetic groups. These findings support the development of 11βHSD-1 inhibitors for type 2 diabetes (Dube et al. 2015). In a study in which the addition of one these inhibitors (INCB13739) to metformin therapy in patients with inadequate glycemic control was efficacious and well-tolerated, indicating that decreasing local cortisol exposure through 11βHSD-1 inhibition may improve hyperglycemia over 12 weeks in patients with type 2 diabetes (Rosenstock et al. 2010). Thus, inhibition of local cortisol generation may eventually offer a new potential approach to control abdominal obesity-related alterations and cardiometabolic risk factors in type 2 diabetes. More work is needed, however, as human adipose tissues seem to exhibit tachyphlaxis after repeated doses of 11β-HSD-1 inhibitor (Morentin Gutierrez et al. 2015).

8.5.1 Conclusion

The sex difference in body composition and fat distribution observed in humans transcends culture and time (Hoyenga and Hoyenga 1982). As we reviewed in this chapter, this sex dimorphism, along with specific characteristics of each fat compartment contributes to explain an important portion of the cardiometabolic risk associated with obesity. Evolutionary theories put forth to explain this dimorphism involve sexual selection and reproductive roles. Women would have evolved to maximize reproductive success through parental investment (gestation, breastfeeding) (Trivers 1972). The presence of larger, more stable body fat reserves in women is consistent with these reproductive roles, which are very demanding from the energetic standpoint. On the other hand, men would have evolved to maximize reproductive success through better motor performance and reproductive roles such

as searching, fighting and competing for mates (Dixson et al. 2005; Miller 1998; Trivers 1972). The presence of central, visceral fat depots which have a minor impact on the center of gravity (Pond 1992) and which are mobilized quickly in response to catecholamines is consistent with such activities. These characteristics apparently co-evolved in males and females both as courtship traits (e.g. breasts and buttocks in females, upper-body mass in males) and indicators of nutritional/reproductive status, in a context where males compete for females, who in turn select mates among the males that they attract (Miller 1998). Hence, the fat distribution dimorphism, which is unique to the human species (Pond 1992), appears as an extremely potent example of how interactions of sex- and gender-related traits through evolution may actually contribute to shape organic form.

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Chapter 9
Brown Adipose Tissue as a Therapeutic Target

Wouter D. van Marken Lichtenbelt and Emmani B.M. Nascimento

Abstract Roughly speaking, adipose tissue comes in two colors: white and brown. White adipose tissue (WAT) has the capability to store excess energy and it does so in one large lipid droplet. Brown adipose tissue (BAT) contains numerous small lipid droplets and many mitochondria enabling uncoupling in BAT ultimately resulting in heat production. BAT plays an important role in non-shivering thermogenesis. Increased non-shivering thermogenesis associates with increased energy expenditure (EE) and a negative energy balance. Increasing EE can be beneficial in obese humans subject, eventually resulting in weight loss if not compensated by increased energy intake. It was only in 2009 that active BAT was detected in adult humans. Therefore in this book chapter, we will discuss different aspects of BAT physiology in humans. Firstly, we will review the role of human BAT in non-shivering thermogenesis, lipid and glucose metabolism. Secondly, we will look at factors important for human BAT adipogenesis. Finally, we will assess different pharmacological interventions aimed to stimulate BAT activity in humans.

Keywords Brown adipose tissue • UCP1 • Glucose • Lipids • Non-shivering thermogenesis • Pharmacological activation

9.1 Introduction: Brown, Beige and White Adipose Tissue

Brown adipose tissue (BAT) is the stove of the body. When turned off, it produces no heat other than for maintenance. As soon as it is turned on by the sympathetic nervous system it starts using extra energy to produce heat. In many animal species, such as rodents, bats and large mammalian hibernators, this heat is used to maintain or raise the body core temperature by means of the so-called non-shivering thermogenesis (Cannon and Nedergaard 2004). In humans BAT is well known from babies, where it serves the same purpose as in animals. Although well known from older

W.D. van Marken Lichtenbelt (✉) • E.B.M. Nascimento
Maastricht University, Maastricht, The Netherlands
e-mail: markenlichtenbelt@maastrichtuniversity.nl

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anatomical studies (Huttunen et al. 1981; Heaton 1972), it was not until 2009 that dedicated cold exposure studies showed substantial amounts of functional BAT in adults (van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009; Saito et al. 2009). That is, during mild cold exposure BAT was made visible by nuclear medicine imaging technique FDG-PET/CT that shows increased amounts of glucose uptake (rate) (Fig. 9.1) (Nedergaard et al. 2007). The extra heat is produced by means of mitochondrial uncoupling. The mitochondria of BAT have a specific uncoupling protein uncoupling protein 1 (UCP1) that, when activated, short-circuits the electrochemical gradient across the inner membrane. This gradient normally is used for ATP synthesis. Due to uncoupling, the respiratory chain activity is stimulated and extra heat is released and extra fuel is needed mainly in the form of fatty acids from both internal and external stores, but also glucose is being taken up from the blood.

Brown adipocytes are distinct from white adipocytes in that they have multiple fat droplets instead of one large fat vacuole (for an overview of the characteristics of WAT and BAT, see Table 9.1). Even more distinct is the large amount of specialized mitochondria that together with an intense network of blood capillaries promote the

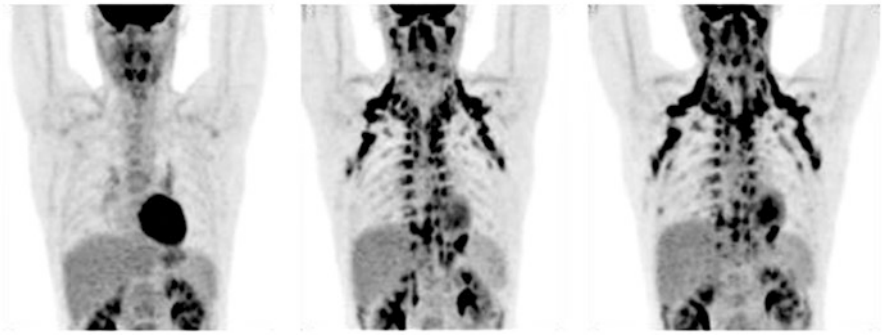

Fig. 9.1 Tissue specific glucose uptake in response to placebo (*left*), 200 mg mirabegron (*middle*) or cold (*right*) using FDG-PET/CT. Figure from Cypess et al. (2015)

Table 9.1 Main characteristics: white adipose tissue versus brown adipose tissue

	White adipose tissue	Brown adipose tissue
Function	Energy storage	Heat production
Purpose	Energy for periods of food shortage	Keep body temperature during cold
Processes	Lipolysis and lipogenesis	Mitochondrial uncoupling
Innervation	Some innervation by SNS and para-SNS	Highly innervated by the SNS
Histology	One large lipid droplet	Small lipid droplets and very many mitochondria
Blood flow	Low degree of perfusion	Densely perfused
Correlation with human obesity	Positively with obesity	Negatively with obesity

reddish flesh like appearance of the tissue. Finally, BAT is densely innervated by the SNS (Zingaretti et al. 2009). The main function of white adipose tissue (WAT) is fat (energy) storage. BAT's main function is heat production. From WAT there is a large amount of evidence that it also serves as an endocrine tissue. Recently, BAT is thought to display similar endocrine properties as well (Villarroya et al. 2013).

Besides the classical brown adipose cells, beige adipocytes also exist. The functional distinction between these two are not (yet) very exclusive. Beige (or brite i.e. brown in white) adipocytes develop as clusters of brown like cells in WAT in response to a range of stimuli (Vitali et al. 2012). These cells have similar properties compared to BAT, such as multilocular fat droplets, high mitochondrial content and SNS innervation. BAT specific genes, such as UCP1, also characterize them. Besides their similarities they are distinct to each other because of their embryonic development (Harms and Seale 2013). Most important differences are, first, classical brown adipocytes are derived from a $Myf5^{+}$ lineage that also form the precursors of muscle cells, while beige adipocytes derive from a $Myf5^{-}$ lineage sharing precursors of WAT (Seale et al. 2008). Secondly, brown adipocytes express high amounts of UCP1 and other thermogenic genes under basal conditions, while beige adipocytes only express these genes following stimulation of the beta-adrenergic receptor or peroxisome proliferator-activated receptor (PPAR)g (Petrovic et al. 2010). That means that brown adipocytes in culture systems increase their expression of thermogenic genes such as UCP1 without addition of classical activators, while beige cells need these activators in order to develop thermogenic properties (Klaus et al. 1995). Moreover, for the increase in brite adipocytes in WAT, sympathetic drive seems critical (Bartness and Ryu 2015). Once developed beige adipocytes may possess the same thermogenic properties as brown adipocytes, however from a biological point of view they may express a different set of genes, which can be activated by other stimuli. Both brown and beige adipocytes are relevant in humans because of the presence of these adipocytes in the deep neck region and supraclavicular human BAT region (Wu et al. 2012; Cypess et al. 2013).

9.2 Non-Shivering Thermogenesis

Non-shivering thermogenesis is a facultative component of energy expenditure (EE). Almost all energy that we derive from the food is eventually converted into heat (Rolfe and Brown 1997). Apart from the performance of external work, energy in the body is used in the form of ATP for all metabolic processes that have heat production as a final end product. The main components of energy expenditure are basal metabolic rate (BMR, resting in fasting conditions on average about 40% of total EE), diet induced thermogenesis (DIT, about 10% of total EE), and the highly variable component physical activity induced thermogenesis (PAEE, for an average active human about 50%). Resting metabolic rate (RMR) overlaps with BMR, but is measured under less strict conditions. Sleeping metabolic rate (SMR) is a few percent lower that BMR, the difference being attributed to arousal. A facultative

component of EE is NST, which is turned on in the cold. Part of DIT can also be facultative, i.e. in rodents it has been shown that unbalanced diets can increase energy expenditure to increase food intake in order to obtain enough valuable nutrients without gaining too much weight. Whether facultative DIT exists in humans is still under debate (Kozak 2010), although ingestion of a large meal increased DIT in humans and activated BAT (Vosselman et al. 2013).

Heat loss is by means of radiation, conduction, convection and evaporation. Under resting, thermoneutral conditions there is heat balance at a core body temperature of around 37 °C. Therefore, under resting, thermoneutral conditions no extra heat production is needed; however, if the environmental temperature is above or below the thermoneutral zone heat production increases (Kingma et al. 2012). This heat production is facultative and can be achieved by increasing the turnover of metabolic processes and induction of futile cycles, by muscle contraction (shivering), but also by the above-mentioned mitochondrial uncoupling in brown fat. Thus in the cell and mitochondria, energy substrates are broken down in processes like beta-oxidation, glycolysis and TCA cycle, ultimately leading to the building up of a proton gradient over the inner mitochondrial membrane, which is used for ATP generation (Mitchell 1966); mitochondrial uncoupling lowers the proton gradient without ATP formation, thereby reducing energy efficiency and indirectly stimulating heat production. Shivering can increase energy expenditure up to four times BMR (Benzinger 1969), but cannot be maintained for prolonged periods, as it is uncomfortable, decreases coordination and results in muscle fatigue. However, NST can be sustained and is insensible, and does not affect coordination. The maximal reported NST in humans is 40% of RMR (Vosselman et al. 2014), but is variable between individuals and in healthy lean subjects ranges from 0 to 30% of RMR (Dauncey 1981; Warwick and Busby 1990; van Ooijen et al. 2004).

In conclusion NST may be a way to increase energy expenditure and thereby promote a negative energy balance (van Marken Lichtenbelt and Schrauwen 2011). This can be accomplished by cold exposure, but also by pharmacological means (see below). In rodents BAT activation is responsible for NST and possibly also in the facultative part of DIT (Cannon and Nedergaard 2004). The contribution of BAT to NST in humans is not yet clear. Recently, other health aspects of the cold induced increase of energy expenditure have been identified, that are linked to glucose and lipid metabolism.

As mentioned above there is a large individual variation in NST (Warwick and Busby 1990; van Ooijen et al. 2004; Celi et al. 2010), that can partly be explained by differences in body composition (Wijers et al. 2010) and seasonal changes (van Ooijen et al. 2004). It is well documented in animal studies that the capacity of NST is variable, depending on the seasons and weather (temperature) conditions. Cold acclimation in rodents replaces shivering thermogenesis completely by NST (Davis et al. 1960). One of the first studies on cold acclimation in humans was from Davis (1961) who showed that comparable to the rodent studies shivering can be replaced by NST, although not completely (Davis 1961). A few years ago we showed that 10 day cold acclimation for 6 h per day at 14–15 °C increased NST van der Lans et al. (2013) and Yoneshiro et al. showed that 6 weeks acclimation for 2 h per day at

19 °C also affected NST and even led to a reduction in body fat (Yoneshiro et al. 2013). Finally, a recent study showed that 20 min per day extreme cold (4 °C) exposure for 4 weeks also increased NST capacity (Peterson et al. 2016).

9.3 The Role of BAT in Non-shivering Thermogenesis

The imaging technology FDG-PET/CT is used in nuclear medicine to localize tumors. FDG is a radioactively ^{18}F labeled deoxyglucose that is trapped in tissues that take up glucose, such as tumor tissue. PET can visualize and quantify glucose uptake, while the CT scan provides anatomical and tissue specific information. Since active BAT also takes up significant amounts of glucose it can be detected by FDG-PET. It was first thought to be tense muscle (Barrington and Maisey 1996), but with the advance of CT technique it was identified as fat (Hany et al. 2002; Yeung et al. 2003). Several studies from the field of nuclear medicine reported BAT in cancer patients. In this clinical field BAT (then named USA fat) was considered an artifact obscuring the image quality in search for tumors. It was Nedergaard et al. in 2007 who reviewed these nuclear medicine retrospective studies in the context of metabolic implications for human brown fat physiology (Nedergaard et al. 2007). In 2009, several research groups independently identified functional brown fat in adult humans after performance of dedicated cold exposure experiments (van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009; Saito et al. 2009) together with retrospective patient studies (Cypess et al. 2009).

In parallel to the observed individual variation in NST capacity, there appears to be a significant individual variation in BAT presence and activity. This can partly be explained by the amount of body fat (or BMI), age, level of exercise, season, and amount of cold acclimation. Indeed BAT is negatively related to BMI and body fat percentage (van Marken Lichtenbelt et al. 2009; Vijgen et al. 2011), to age (Yoneshiro et al. 2011; Hanssen et al. 2016), and the level of exercise training (Vosselman et al. 2015). BAT is lower in summer compared to winter (Yoneshiro et al. 2016) and related to outside temperatures preceding the measurement day (Vosselman et al. 2013). Recent studies on cold acclimation ranging from 10 days to 2 months and 10 °C to 19 °C show an increase in BAT that went hand in hand with and increase in NST in lean adults (Yoneshiro et al. 2013; van der Lans et al. 2013), increased oxidative capacity of BAT (Blondin et al. 2014), and increased BAT and NST in obese subjects (Hanssen et al. 2016).

Although cold activated BAT activity is related to NST (Fig. 9.2), it does not show the actual contribution of BAT to NST. Therefore heat production of BAT has to be measured. FDG measures glucose uptake (rate), but BAT mainly uses fatty acids as fuel. This is indicated by the change in CT values, so-called Hounsfield values, that show that the water content of the BAT region increases during cold exposure (Ouellet et al. 2012). Several studies investigated fatty acid utilization by using a labeled fatty acid (FTHA) (Blondin et al. 2015; Din et al. 2016). These studies also show substantial FA utilization, but cannot quantify the contribution of stored FAs. Another way to

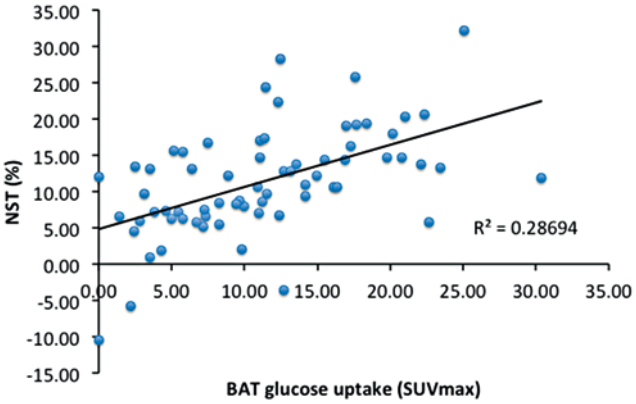

Fig. 9.2 Non-shivering thermogenesis (NST) in relation to cold induced BAT glucose uptake by FDG-PET/CT. Data from five studies combined (van Marken Lichtenbelt, unpublished)

study BAT activity is measuring blood perfusion using labeled water containing the oxygen isotope ^{15}O. This isotope has a very short half-life of approximately 2 min. Therefore measurements must take place in close vicinity to the production by a cyclotron. Two studies showed BAT perfusion in response to cold and/or insulin (Yoneshiro et al. 2016; Orava et al. 2011). Oxidative metabolism of BAT in response to cold has been shown using PET with ^{11}C–acetate (Ouellet et al. 2012).

Finally two studies used the isotope ^{15}O, showing a contribution of only 1% of NST (Din et al. 2016; Muzik et al. 2012). Din et al. conclude that BAT-specific EE and oxygen consumption was higher during cold stimulus (approximately 50%); similarly, whole-body EE was higher during cold stimulus (range 2–47%). However, there was no association in BAT-specific EE and whole-body EE. BAT-specific EE was found to be a minor contributor in cold induced whole-body thermogenesis (1% of total whole-body elevation in EE). The latter is in accord with the study of Muzik et al. (2012) that BAT EE amounted to 20 kcal/day during moderate cold stress, even in subjects with relatively large BAT depots. However, the cooling protocols were not very well controlled (Muzik et al. 2012) or started cooling at very low temperature (6 °C; (Din et al. 2016)) which may induce a reflex response, and cooling was not individually attuned as is needed to accomplish maximal NST. More studies are needed to actual measure the BAT activity. Another problem is that only the most active parts of BAT depots can be measured by PET/CT and therefore the contribution of brite cells in other depots cannot be taken into account. This is because of the dispersion of these brite cells in different WAT depots. Even if BAT cannot contribute for 100% of the cold induced thermogenesis, it may still have a significant function in the cross talk between the different tissues and organs responsible for cold induced energy expenditure and glucose and lipid metabolism.

In conclusion, BAT is present in adult humans, is a metabolically flexible tissue, just as observed in many animal species. BAT presence and activity goes hand in hand with

NST, and changes in BAT activity go hand in hand with changes in NST. Indeed, BAT is significantly related to NST. However, it should be noted that the relative amount of BAT seems to be less than for many rodent species, where BAT is responsible for completely replacing shivering in extreme cold conditions and thereby can contributes to a majority of the total energy budget. Although the precise quantification of BAT contribution to whole body metabolism is still not determined, it can be concluded that the amount of BAT in adult humans is small relative to many rodent studies.

9.4 Role of BAT in Lipid and Glucose Metabolism

9.4.1 Lipid Metabolism

Triglyceride-derived FAs are the main fuel for BAT thermogenesis as shown in many animal studies and there is no indication that this is different in humans. In both animal and human brown adipocytes fats are stored in vacuoles. Activation of BAT results in a fast induction of intracellular lipolysis, induced by activation of adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL), resulting in release of FA from the TG-filled lipid droplets. FA are directed to the mitochondria where they either allosterically activate UCP1 present in the inner membrane of the mitochondrion or can undergo oxidation (Cannon and Nedergaard 2004). During activation both lipid intracellular stores are used as well as FAs derived from the blood. After activation of BAT the TG stores need to be replenished. This is accomplished by uptake of glucose followed by *de novo* lipogenesis, uptake of albumin-bound free FA, or uptake of TG-derived FA from very-low-density lipoproteins and chylomicrons in the plasma.

Animal studies indicate that BAT activation can have significant effects on energy expenditure and lipid clearance. Indeed Bartelt et al. clearly showed that in mice cold exposure drastically accelerated plasma clearance of TGs as a result of increased uptake into BAT (Bartelt et al. 2011). In the same publication the authors show that cold exposure corrected hyperlipidemia and improved deleterious effects of insulin resistance. The contribution of BAT to TG metabolism in humans is not yet clear. Most studies use the glucose analog FDG as the tracer to study BAT activity. However, as mentioned above, the use of FA by human BAT is made likely by the change in Hounsfield units using CT scan. During cold exposure Hounsfield Units increase, indicating that internal lipid stores are used (Ouellet et al. 2012; Baba et al. 2010; Hanssen et al. 2015). It is likely that human BAT also utilizes FA from circulating lipoproteins, though this has not been investigated yet. Thus, a fast initial combustion of intracellular TG upon acute BAT activation may well explain why short-term cold exposure does not result in acute lowering of plasma TG in human subjects. Probably, prolonged BAT activation will result in lowering of plasma TG levels in human subjects as a consequence of increased clearance from the plasma towards BAT. The actual contribution of human BAT to this TG clearance is similarly unknown.

9.4.2 Glucose Metabolism

Although fatty acids are the main fuel for active BAT, glucose uptake is significantly enhanced when BAT is activated. Indeed, murine studies have shown that BAT expresses both glucose transporter 4 (GLUT-4) and 1 (GLUT-1) (Cannon and Nedergaard 2004). This suggests that both insulin-dependent and insulin-independent pathway for glucose uptake in BAT. Glucose is mainly used for anaplerotic pathways, *de novo* lipogenesis, and ATP generation. Several animal studies indicate that plasma clearance of glucose by BAT can substantially contribute to whole-body glucose metabolism. BAT transplantation experiments in mice showed that glucose tolerance improved due to higher uptake of glucose by the tissue (Stanford et al. 2013). Furthermore, long-term BAT activation by means of beta3-adrenergic activation lowered plasma glucose (Wang et al. 2013).

In humans, glucose uptake is significantly increased in cold or adrenaline activated BAT (van Marken Lichtenbelt et al. 2009; Virtanen et al. 2009; Cypess et al. 2015) (Fig. 9.1). Interestingly, there is also five-fold increase in insulin-stimulated glucose uptake, and gene expression of GLUT-4 was relatively higher in BAT compared to WAT (Orava et al. 2010). This shows a role for GLUT-4 in human BAT and that human BAT may be insulin sensitive. Whether glucose uptake is also mediated by GLUT-1 is yet unknown. Despite the significant glucose uptake of activated human BAT, the question remains whether active BAT is sufficient to have an impact on whole-body glucose metabolism in humans. An association study in which different plasma parameters were measured in healthy humans with and without BAT (BAT status was determined via FDG /PET-CT), showed that BAT was a significantly independent determinant of circulating glucose and HbA1c, suggesting that BAT could impact glucose metabolism (Matsushita et al. 2014). However, whether long-term BAT activation indeed results in improvement of glucose metabolism in obese subjects with impaired glucose tolerance remains to be determined and is an interesting and relevant topic for future studies.

Recently, it was shown that after 10 days of cold acclimation in humans with T2D insulin sensitivity increased on average by 43% (Hanssen et al. 2015). However, the amount of active BAT, determined by FDG-PET/CT, although increased significantly remained very low. It was concluded that skeletal muscle was involved in the improvement of insulin sensitivity. On the other hand FDG may not have been the right tracer to use in T2D, since BAT is insulin sensitive (Orava et al. 2011) and therefore T2D may affect insulin sensitivity of BAT. It may be that more active BAT is present in patients with type 2 diabetes than can be detected by FDG-PET/CT imaging following cold exposure.

9.5 Targets for Pharmacological Activation

The presence of brown fat in humans has invigorated the scientific community to find novel ways to metabolically profit from this tissue. Increased BAT activity is associated with greater whole body energy expenditure and potentially prevent

metabolic-related disease (e.g. obesity, but potentially type 2 diabetes and cardiovascular disease (Thoonen et al. 2016)). To date, cold exposure is the best method to increase BAT activity in both rodents and humans. However other pharmacological means of activation BAT may be useful. Studies have been conducted to better understand necessary factors for BAT adipogenesis. Over the years these efforts yielded various genes/proteins proven to be important in the process of human and murine adipogenesis of BAT (see for review (Townsend and Tseng 2015)). Some of these could now be modified in vivo by a pharmacological intervention, and several examples be discussed, below.

9.5.1 Peroxisome Proliferator-Activated Receptor Gamma (PPARg)

PPARg is a master regulator of adipocyte differentiation. FAs are endogenous ligands for this receptor resulting in transactivation of target genes with the help of retinoid X receptor (RXR). PPARg can be targeted by specific class of agonists called thiazolidinediones (TZDs). These agonists are able to increase mitochondrial biogenesis and UCP1 expression (Petrovic et al. 2008; Bartesaghi et al. 2015) by the deacetylase SIRT1 (Qiang et al. 2012). TZDs have been used in the treatment of type 2 diabetes, however the withdrawal of troglitazone and the observed side effects of this class of drugs which include increased risk for hepatitis, edema and cardiovascular events have decreased their popularity. When more specific PPARg agonists have been developed displaying less adverse effects, it would be interesting to establish the effects of these agonists on human BAT activity.

9.5.2 Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1 Alpha (PGC1a)

Mitochondrial function, more specifically mitochondrial uncoupling is a key trait of BAT metabolism. The presence of UCP1 in the mitochondria ensures the heat production capacity of this tissue. Therefore it makes sense that stimulating mitochondrial function in brown adipocytes could promote BAT activity. Nowadays it has been established that PGC1a plays a crucial role in mitochondrial biogenesis in various tissues (Austin and St-Pierre 2012), however it was originally discovered in brown adipocytes as a PPARg binding partner (Puigserver et al. 1998). In BAT, PGC1a regulates expression of thermogenic genes following cold stimulation (Uldry et al. 2006), thus having more of a role in activation of brown adipocytes. Targeting PGC1a is an interesting approach, however no specific agonists have been developed to target PGC1a in human BAT. Indirect activation through sirtuin 1 (SIRT1) and/or AMP mediated protein kinase (AMPK) (for review see (Canto and Auwerx 2009)) is potentially more feasible, because of their pivotal role in the regulation of PGC1a expression which could enhance mitochondrial function.

9.5.3 PR Domain-Containing Protein 16 (PRDM16)

PRDM16 was identified as a factor that regulates adipogensis of BAT. Expression of this histone methyltransferase and zinc-finger-containing protein is higher in BAT compared to WAT (Seale et al. 2007). Expression of this gene results in upregulation of a thermogenic program, while repressing the classic WAT transcriptional program (Kajimura et al. 2008). This is in part accomplished by enhanced transcriptional function of PPARg and PGC1a (Sears et al. 1996; Hondares et al. 2011; Iida et al. 2015). PRDM16 shows promise in activating BAT, however no pharmacological means have been identified. PRDM16 is under control for miRNA133 (Yin et al. 2013; Liu et al. 2013), which could be a novel way to increase PRDM16 to increase BAT activity.

9.5.4 Bone Morphogenetic Protein (BMP)

BMPs belong to the transforming growth factor beta family of which the BMP subfamily comprises 12 family members. For signal transduction BMPs bind two types of serine-threonine kinase receptors: type I and type II receptors. Following binding of BMPs to its receptor, that results phosphorylation and recruitment of different types of SMAD proteins. These proteins can be divided into receptor-regulated SMADs (R-SMAD), common-mediator SMADs (co-SMAD) and inhibitory SMADs (I-SMAD). Aside from bone osteogenetic properties, BMPs also have a pivotal role in adipogenesis of BAT (Kang et al. 2009). BMP family members BMP4, BMP7 and BMP8b exert different effects on rodent and human BAT at the level of lineage commitment of precursor cells towards adipocyte, differentiation of precursor cells and terminally differentiated lipid-accumulating adipocytes (see (Grgurevic et al. 2016) for review). For instance BMP7 is important for in murine BAT, since its knock out decreases BAT presence concomitant with lack of UCP1 expression (Saini et al. 2015). In human white adipocytes BMP7 stimulates UCP1 expression, which is not always the case in human brown adipocytes (Xue et al. 2015). The lack of increase of UCP1 in brown adipocytes is not a problem per se, since UCP1 abundance is significantly higher in brown adipocytes compared to white adipocytes. Family member BMP4 is more associated with adipogenesis of in WAT (Gustafson and Smith 2012), but more recently it has been demonstrated that BMP4 stimulates the expression of UCP1 in human white adipocytes resulting in a more a brown phenotype (Elsen et al. 2014). In humans expensive treatment with recombinant BMP2 and BMP7 has been performed in order to enhance union of damaged bone. A major drawback of BMP action is that is not only restricted to BAT. Thus in order to maximally benefit from BMPs, specific human pharmacological intervention needs to be directed towards adipose tissue.

Table 9.2 Pharmacological interventions in humans directed to stimulate BAT activity

Pharmacological intervention	Targeted receptor	Outcome on BAT activity	Reference
Isoprenaline	Beta3-adrenergic receptor	=	Vosselman et al. (2012)
Ephedrine	Beta3-adrenergic receptor	=	Cypess et al. (2012)
Ephedrine (lean vs. obese)	Beta3-adrenergic receptor	↑ (only in lean)	Carey et al. (2013)
Ephedrine (chronic)	Beta3-adrenergic receptor	↓	Carey et al. (2015)
CL316243	Beta3-adrenergic receptor	=	Weyer et al. (1998)
L796568	Beta3-adrenergic receptor	=	Larsen et al. (2002)
TAK677	Beta3-adrenergic receptor	=	(Redman et al. 2007)
Mirabegron	Beta3-adrenergic receptor	↑	Cypess et al. (2015)
Levothyroxine	Thyroid hormone receptor	↑	Broeders et al. (2016)
Capsinoids	Transient receptor potential channels	= (increased cold-induced whole body EE)	Yoneshiro et al. (2013)
CDCA	TGR5	↑	Broeders et al. (2015)

Adapted from Pfeifer and Hoffmann (2015)

9.6 Pharmacological Activation of BAT

Although it is important to understand the function of the above-mentioned genes and their related signal transduction pathways, the ultimate goal is to increase EE (through BAT) in humans. Numerous strategies have been attempted or are currently under investigation examining BAT activity and/or increased EE (for review see (Pfeifer and Hoffmann 2015)). However here we specifically focus on pharmacological means to stimulate BAT activity in humans as summarized in Table 9.2, which will be discussed in more detail below.

9.6.1 Beta-Adrenergic Receptor Agonists

The beta-adrenergic receptor (bAR) family is present throughout the body comprising b1AR, b2AR, and b3AR. bAR is a G-protein coupled receptor. Cold stimulation releases NE from the sympathetic neuron resulting in greater activity of these receptors in brown adipocytes. This also stimulates adenylate cyclase and the

release of second messenger cAMP resulting in activation of PKA. Activation of PKA promotes the thermogenic gene program and lipolysis of intracellular TGs thereby freeing FAs. These FAs can aid in mitochondrial uncoupling or activate UCP1 present in the mitochondria of brown adipocytes. BAT expresses all three family members, however BAT function is mostly dependent on b3AR. Administration of bAR agonists in humans has yielded different results. bAR agonist isoprenaline (Vosselman et al. 2012), ephedrine (Cypess et al. 2012), CL316243 (Weyer et al. 1998), L796568 (Larsen et al. 2002) and TAK677 (Redman et al. 2007) were without any apparent effects on BAT activity. Total energy expenditure was increased, but it was concluded that BAT did not play a significant role at the prescribed dose. Possibly higher dose would be more effective, but could be rendered irrelevant because of clinical side effects. However recently bAR agonist mirabegron (Fig. 9.1) was able to stimulate BAT activity and increase energy expenditure (Cypess et al. 2015). However, in the same study, increased heart rate and blood pressure was observed due to expression of bAR in the human heart in combination with the relatively high dose of mirabegron used (200 mg). Although stimulation of the bAR can increase BAT activity, the major issue with activation of the bAR at the whole body level remains that stimulation is not only restricted to BAT. In order to make use of bAR agonists, they would need to become specific for BAT.

9.6.2 Thyroid Hormone

Thyroid hormones are produced in the thyroid gland. The inactive hormone thyroxine (T4) is converted by deiodinase 2 (D2) to active (triiodothyronine) T3 in BAT which expresses the thyroid hormone receptor so binding can stimulate BAT activity. T3 is commonly used as a component in the in vitro adipogenesis differentiation cocktail and also stimulates UCP1 expression (Bianco et al. 1988). Thyroid hormones can have a significant effect on energy expenditure as demonstrated by the manifestation of altered energy expenditure in hypo- or hyperthyroidism. Hyperthyroidism is associated with decreased thyroid stimulating hormone (TSH) because the active thyroid gland has no need for stimulation. Another hallmark of hyperthyroidism is elevated free T4. Hyperthyroid patients display increased BAT activity compared to euthyroid patients (Lahesmaa et al. 2014). Removal of the thyroid gland results in hypothyroidism which is associated with low free T4 (Broeders et al. 2016). Supplementation of hypothyroid patients with a synthetic levothyroxine stimulates whole body energy expenditure and increases BAT activity (Broeders et al. 2016). This shows that in humans thyroid hormone plays a significant role in BAT activity. However in order to modify BAT activity by this pathway careful monitoring is required which would not be feasible for the general population. As an alternative targetting D2 activity could provide new strategies to activate BAT.

9.6.3 Capsinoids

Capsaicin is a food ingredient found in red pepper and is known to stimulate EE in humans. Capsaicin is able to exert its effect by a vanilloid subtype 1 of transient receptor potential channel (TRPV1). Due to the sharp and pungent nature of capsaicin, research focus has shifted towards nonpungent analogues of capsaicin called capsinoids. Ingestion of a single dose of capsinoids stimulated EE in humans (Yoneshiro et al. 2013). However more interestingly, EE was only increased in human subjects with detectable BAT, while no effects were observed in those without detectable BAT (Yoneshiro et al. 2013). These findings demonstrate a pivotal role for BAT in the mode of action of capsinoids, although no measurements of BAT activity have been performed in the afore-mentioned study. The link between BAT activity and TRP channels needs further research because various food ingredients (for review see (Saito and Yoneshiro 2013)) can stimulate different TRP channels, which could result in alternative ways of increased BAT activity.

9.6.4 Bile Acids

Bile acids are known for their role in lipid metabolism and cholesterol catabolism. They can also function as signaling molecules mainly using two receptors thereby providing regulation of metabolism: i.e. the nuclear receptor farnesoid X receptor (FXR) and G-protein coupled receptor TGR5 (for review see (Vitek and Haluzik 2016)). Their effect is dependent on the presence of the expressed receptor and the affinity of the specific bile acid for the receptor. Activation of the FXR pathway via downstream target FGF19 has been proposed as a mechanism to regulate glucose and lipid metabolism (Miyata et al. 2012). Activation of TGR5 through bile acids increases levels of cAMP resulting in activation of D2 (Watanabe et al. 2006). As mentioned before, D2 is responsible for the conversion of inactive T4 to active T3, which stimulates BAT. Indeed bile acids increase in vitro energy expenditure through skeletal muscle and/or BAT (Watanabe et al. 2006). However it was recently shown that short-term treatment with bile acids also stimulates EE and BAT activity in human subjects. In vitro studies using primary cultured human brown adipocytes, demonstrated that bile acids or a TGR5 agonist were able to stimulate mitochondrial respiration (as a measure of cellular EE), while this increase in mitochondrial respiration was absent when a FXR agonist was used. Together these results revealed a significant contribution of TGR5 in bile acid mediated BAT activity in humans (Broeders et al. 2015). Further research directed towards long-term treatment with bile acids will provide us with answers regarding increased BAT activity to stimulated metabolism.

In conclusion, BAT can be stimulated pharmacologically in humans. Increased BAT has huge potential to counteract metabolic disease such as obesity, type 2 diabetes and cardiovascular disease.

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Chapter 10
Dietary Determinants of Fat Mass and Body Composition

María A. Zulet, María J. Moreno-Aliaga, and J. Alfredo Martínez

Abstract Body weight and fat content as well as enery metabolism depends on several factors such as food intake, nutrient-associated turnover, thermogenesis, and physical activity. These elements underlie complex interrelated feedback mechanisms, which are affected by personal genetic traits. A number of investigations have evidenced that not all calorie may count equal and that some specific biofactors occurring in foods may affect energy efficiency and fat deposition. Thus, the role of protein and specific amino acids, the glycemic load of different carbohydrates and foods, the type of fats, as well as the involvement of some food components with bioactive functions affecting the energy equation are being ascertained, since they can influence body composition and adiposity. Indeed, moderately high protein intake, carbohydrate with low glycemic index, n-3 fatty acids, calcium, and some thermogenic substances and antioxidants have been found to possibly contribute to reduce the body fat content. Many of these findings have been supported not only through epidemiological studies, but also by animal and cell investigations as well as through controlled nutritional interventions in humans. A better understanding of the putative involved mechanisms concerning the effects of individual fatty acids such as conjugated linoleic acid, eicosapentaenoic acid, and docosahexaenoic acid in body composition maintenance, as well as the identification of new bioactive compounds affecting lipid turnover and energy metabolism will open the way for a better control and management of fat deposition in different stages of the life cycle, since some of them are able to control relevant metabolic pathways at the molecular level, which will contribute to precision nutrition.

M.A. Zulet • M.J. Moreno-Aliaga • J. Alfredo Martínez (✉)
Department of Nutrition, Food Science and Physiology, Centre for Nutrition Research, University of Navarra, Pamplona, Spain

IDISNA Navarra's Health Research Institute, Pamplona, Spain

CIBERobn Physiopathology of Obesity and Nutrition, Centre of Biomedical Research Network, ISCIII, Madrid, Spain
e-mail: jalfmtz@unav.es

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10.1 Introduction

Overweight and obesity are linked to more deaths worldwide than underweight. In 2014, more than 1.9 billion adults, 18 years and older, were overweight being over 600 million obese (WHO 2016). Obesity is associated to several chronic morbidities, including Type 2 diabetes, dyslipidaemia, and hypertension, which are major components of the metabolic syndrome (MetS) (Bruce and Byrne 2009). Obesity, defined as abnormal or excessive fat mass accumulation, is a complex disease, caused by an interaction of a myriad of genetic, dietary, lifestyle, and environmental factors. A substantial body of evidence suggest that body composition is not only a matter of the amount of calories ingested, but that the macronutrient distribution and micronutrient content and other dietary factors within the diet are critical contributors to fat mass and body weight regulation. Adipose tissues play crucial roles in the development of obesity, with white adipose tissue (WAT) functioning as an energy storage organ, while brown adipose tissue (BAT) as an energy consumption organ. Several studies have suggested the importance of WAT metabolism and WAT-derived factors in the development of obesity and systemic insulin resistance, being a key event in the pathophysiology of the MetS (Lafontan 2014). In fact, during the last two decades, it has been demonstrated that WAT is an important secretory organ, which produces a number of molecules that putatively play critical roles in fuel homeostasis and contributes to maintain metabolic control. These bioactive molecules, generally termed "adipokines" are involved in the physiological regulation of fat storage, adipogenesis, energy metabolism, food intake, and also play an important role in metabolic disorders (Galic et al. 2010). Indeed, obesity and accompanying comorbidities are associated with an altered function of the WAT adipocytes, especially concerning the synthesis and secretion of adipokines (Klöting and Blüher 2014; Jung and Choi 2014). Several studies have also suggested emerging roles of BAT in the physiological regulation of metabolism, such as control of triglyceride clearance and glucose homeostasis (Kajimura et al. 2015). Moreover, BAT produces factors such as fibroblast growth factor 21 (FGF-21), which could modulate the function of other metabolic organs (Villarroya et al. 2013). During the last years a new type of adipose tissue has been described and named as beige or "brite" (brown-in-white) adipose tissue (Wu et al. 2012). Increasing BAT/beige activity has been proposed as a promising therapeutic strategy to treat obesity (Yoneshiro and Saito 2015). The present chapter reviews the scientific evidence for the effects of several dietary factors on fat mass and body weight regulation (Fig. 10.1), as well as on specific features of the MetS in humans. Moreover, the ability of dietary fat and specific fatty acids as well as of other bioactive food components to regulate adipocyte metabolism and secretory functions is also considered.

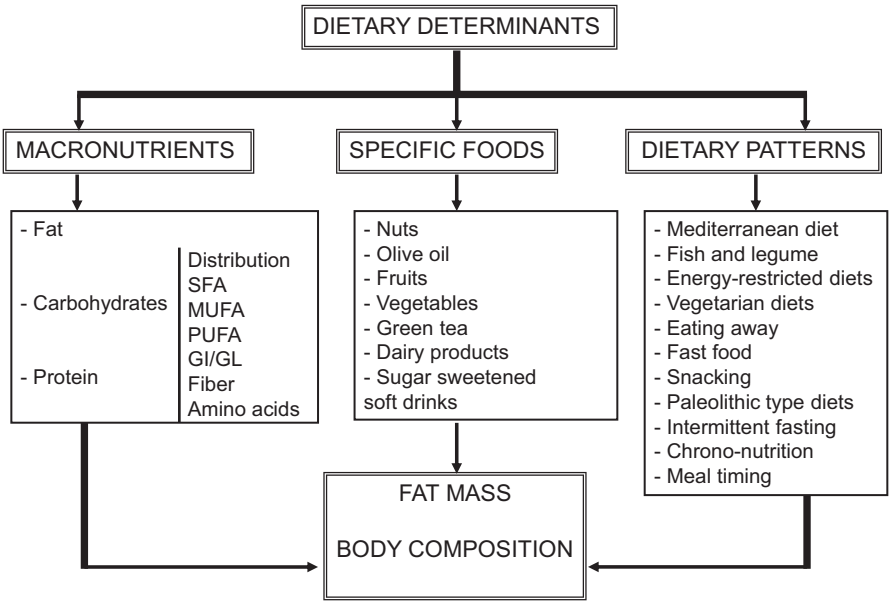

Fig. 10.1 Dietary determinants of fat mass and body composition. *SFA* saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *GI* glycemic index, *GL* glycemic load

10.2 Macronutrient Distribution and Energy Density as Determinants of Fat Mass and Body Composition

Restriction of energy intake is the primary method of producing a negative energy balance leading to weight loss. However, owing to the different metabolic roles of proteins, carbohydrates and lipids in energy homeostasis, diets of similar overall energy content but with different macronutrient distribution can differentially affect metabolism, appetite and thermogenesis (Martinez et al. 2014a). In this way, the macronutrient distribution of energy has been commonly set at 15% protein, <30% lipids, and 50–60% carbohydrates in diets designed to reduce body weight and fat mass by restricting the energy content. Although this recommendation seemed to be effective for decreasing energy density, lowering and promoting weight and fat loss in the short term (Abete et al. 2010), currently other dietary distribution patterns are being taken into account in order to increase the adherence and to achieve benefits for long-time periods (Table 10.1).

Table 10.1 Studies evaluating the role of dietary determinants on fat mass and body composition

Dietary determinant	Subjects/period	Findings	Reference
Macronutrient distribution			
– *Atkins*	n=77–79 per group women, BMI: 27–40 kg/m^2, 1 year of intervention	– Weight loss was greater in the Atkins diet group compared with the other diet groups at 12 months	Gardner et al. (2007)
30%Protein/65%Lipids/5%CHO		– Weight loss:	
– *Zone*		Atkins, −4.7 kg	
30%Protein/30%Lipids/40%CHO		Zone, −1.6 kg	
– *LEARN*		LEARN, −2.6 kg	
15%Protein/30%Lipids/55%CHO		Ornish, −2.2 kg	
– *Ornish*			
Very-high-CHO			
– *Conventional*			
15%Protein/30%Lipids/55%CHO	n= 130 men/women, BMI: 32.6±0.8 kg/m^2, 4 months weight loss and 8 months weight maintenance	– At 4 months, protein group had lost 22% more fat mass than the CHO but weight loss did not differ between groups	Layman et al. (2009a)
Energy restriction (−500 kcal/d)			
– *Moderately high protein*			
30%Protein/30%Lipids/40%CHO		– At 12 months, protein group had greater improvement in body composition; however, weight loss did not differ between groups	
Energy restriction (−500 kcal/d)			
– *Four diets with different macronutrient distribution:*			
– 15%Protein/20%Lipids/65%CHO	n= 811 men/women	– Weight losses ranged from 2.9 to 3.6 kg (3.0–3.6 kg for 15% and 25% protein diets, respectively; 3.3 for both 20 and 40% fat diets and 2.9–3.4 for 65–35% carbohydrates diets, respectively)	Sacks et al. (2009)

– 25%Protein/20%Lipids/55%CHO	Overweight		POUNDS LOST study
– 15%Protein/40%Lipids/45%CHO	2 years		
– 25%Protein/40%Lipids/35%CHO			
– *Conventional*			
15%Protein/30%Lipids/55%CHO	n=19 men, BMI: 34±2 kg/m^2, 8 weeks	– Moderate-protein diet produced a greater weight loss (−8.3±1.2% *vs.* −5.5±2.5%, P = 0.012) than the control diet	Abete et al. (Abete et al. 2009a, b)
Energy restriction (−30%)			
– *Moderate Protein*			
30%Protein/30%Lipids/40%CHO			
Energy restriction (−30%)			
– *Low-fat diet*			
15%Protein/20–25%Lipids/60–65%CHO	n=771, BMI ≥ 30 kg/m^2, 10 weeks	– The low-fat diet produced similar mean weight loss as the high-fat diet, but resulted in more subjects losing >10% of initial body weight and fewer dropouts	Petersen et al. (2006)
Energy restriction (−600 Kcal/d)			NUGENOB study
– *High fat diet*			
15%Protein/40–45%Lipids/40–55%CHO			
Energy restriction (−600 Kcal/d)			
RESMENA project			
– *AHA*	n= 93 men/women, MetS, 6-months (2-month nutritional-learning intervention period + a 4-month self-control period)	– Only the RESMENA group exhibited a significant decrease in body weight (−1.7%; p=0.018), BMI (−1·7%; p= 0·019), waist circumference (- 1.8%; p=0.021), waist:hip ratio (- 1·4%; P= 0·035) and android fat mass (- 6.9%; P= 0·008)	de la Iglesia et al. (2014)

(continued)

Table 10.1 (continued)

Dietary determinant	Subjects/period	Findings	Reference
15%Protein/30%Lipids/55%CHO			RESMENA project
Energy restriction (−30%)			
– *RESMENA diet*			
30%Protein/30%Lipids/40%CHO			
Energy restriction (−30%)			
Protein and Glycemic index			
DIOGENES study:			
1 of 5 *ad libitum* diets:	n= 827 children boys/girls, 5 to 18 years,6 months	– Neither GI nor protein had an isolated effect on body composition	Papadaki et al. (2010)
– *Low protein/Low glycemic index (LP/LGI)*		– LP/HGI combination increased body fat, whereas the HP/LGI combination was protective against obesity in this sample of children	DIOGENES study
– *Low protein/High GI (LP/HGI)*			
– *High protein/Low glycemic index (HP/LGI)*			
– *High protein/High glycemic index (HP/HGI)*			
– *Control diet*			
All diets were low in fat (25–30 % of energy) while protein content was 10–15% energy in the LP and 23–28% in the HP groups			
DIOGENES study:	n= 256 adults, BMI ≥ 27 kg/m^2, 12 months	– Subjects on the HP diets regained less weight than subjects on the LP diets. The difference in weight regain after 1 year was 2.0 (0.4, 3.6) kg (P=0.017) (completers analysis, N=139) or 2.8 (1.4, 4.1) kg (P<0.001) (intention-to-treat analysis, N=256). No consistent effect of GI on weight regain was found	Aller et al. (2014)

LP/LGI			DIOGENES study
LP/HGI			
HP/LGI			
HP/HGI			
Control diet			
– *Higher-GI:*	n=32 men/women, BMI: 32.5±4.3 kg/m^2, 8 weeks	– Lower-GI diet showed a significantly higher weight loss than their counterparts (−5.3±2.6% *vs.* −7.5±2.9%)	Abete et al. (2008)
15%Protein/30%Lipids/55%CHO		– One year after the nutritional intervention weight regain was only statistically significant in the higher-GI group	
– *Lower-GI:*			
15%Protein/30%Lipids/55%CHO			
Prebiotic consumption			
SUN project:	n=8569 men/women, middle-aged university graduates, initial BMI <25, 9 years	– Risk of overweight was 15% lower in participants in the highest quartile of fructan consumption (≥2.3 g/d). Subjects in the highest quartile of galacto-oligosaccharides consumption (≥0.45 g/d) had 17% lower risk of overweight	Perez-Cornago et al. (2014)
A 136-item semi-quantitative food frequency questionnaire (FFQ) previously validated			SUN project
Conjugated linoleic acid			
– *Placebo*:	n=60 men/women, BMI: 25–35 kg/m^2, 12 weeks	– A significantly higher reduction in body fat mass was found in the conjugated linoleic acid groups compared with the placebo group (p: = 0.03)	Blankson et al. (2000)
Olive oil		– No additional effect on fat mass was achieved with doses > 3.4 g CLA/d	

(continued)

Table 10.1 (continued)

Dietary determinant	Subjects/period	Findings	Reference
– *CLA groups*:		– No significant differences among the groups were observed in lean body mass and body mass index	
1.7, 3.4, 5.1 and 6.8 g/d			
50%c9,t11-50%t10,c12			
– *Placebo:*	n=62 prepubertal children aged 6–10 males/females, BMI at or above the 85th percentile (overweight or obese), 7 ±0.5 months	– The increase in BMI and the percentage change in body fat in the CLA group were smaller than that in the placebo group	Racine et al. (2010)
Sunflower oil in chocolate milk		– Abdominal and peripheral fat as a percentage of total body weight decreased and differed significantly from that in the placebo group	
– *CLA groups*			
3 g/d of 80% CLA			
50%c9,t11-50%t10,c12			
In chocolate milk			
Dairy products			
In addition to their usual diet:	n=45 men/women, BMI: 23.5±3.6 kg/m^2, Normotensive	– Weight did not change significantly after the low-fat dairy intervention	Alonso et al. (2009)
– *Whole-fat dairy supplementation:*		– Whole-fat dairy increased weight significantly compared to low-fat dairy (1.2 kg)	
5 servings/day of whole-fat milk/ yogurt			
– *Low-fat dairy supplementation:*	Two 8-week periods with a 4-week washout period between both interventions		

5 servings/day of low-fat milk/ yogurt			
SUN project:	n=8516 men/women	– A high (>7 servings/week) consumption of total and whole-fat yogurt was associated with lower incidence of overweight/obesity in comparison with low consumption (0–2 servings/week). This inverse association was stronger among participants with higher fruit consumption	Martinez-Gonzalez et al. (2014)
A 136-item semi-quantitative food frequency questionnaire (FFQ) previously validated	Middle-aged university graduates		SUN project
	Initial BMI <25		
	6.6 years		
Fish			
Energy-restricted diets (−30% energy expenditure):	n=324 men/women, BMI 27.5–32.5 kg/m^2, 8 weeks	– In men, the inclusion of either lean or fatty fish, or fish oil as part of an energy-restricted diet resulted in approximately 1 kg more weight loss after 4 weeks, than did a similar diet without seafood or supplement of marine origin	Thorsdottir et al. (2007)
– *Conventional*		– The diets did not differ in their effect on weight loss in women	The SEAFOODplus YOUNG study
Sunflower oil capsules, no seafood		– The weight-loss from midpoint to endpoint was 0.45 (0.41–0.49) times the observed weight loss from baseline to midpoint	
– *Lean fish*			
(3 x 150 g portions of cod/week)			

(continued)

Table 10.1 (continued)

Dietary determinant	Subjects/period	Findings	Reference
– *Fatty fish*			
(3 x 150 g portions of salmon/ week)			
– *Fish oil* (DHA/EPA capsules, no seafood)			
Legume			
– *Control*	n=32 obese men/women, BMI: 32.0±5.3 kg/m^2, 8 weeks	– All obese subjects lost weight, especially those individuals who followed the legumes-enriched diet as compared to the control (−7.7±3 *vs.* −5.3 ±2.7%; p = 0.023)	Crujeiras et al. (2007)
15%Protein/30%Lipids/55%CHO; Energy-restricted diet (−30% energy expenditure)			
– *Legume (4 meals/week)*			
15%Protein/30%Lipids/55%CHO; Energy-restricted diet (−30% energy expenditure)			
– *Control*	n=39 overweight/obese, 12 weeks	– The body weight, BMI, WC were only significantly reduced in soy fiber group after 12 weeks	Hu et al. (2013)
Biscuits for breakfast			
– *Supplemented*			
Biscuits + soy fiber for breakfast			
Fruit			
– *Low fruit diet:*	n=15 women, BMI: 34.9±2.9 kg/m^2, 8 weeks	– All volunteers lost body weight, which was accompanied by marked decreases in BMI	Crujeiras et al. (2006)

5% energy from fructose; 15%Protein/30%Lipids/55%CHO; energy-restricted diet (−600 kcal/d)		– No differences were observed between diets concerning weight loss (low fruit diet −6.9 ± 2% *vs* high fruit diet −6.6 ± 2%) and body fat reduction (low fruit diet −11.7 ± 4.8% *vs* high fruit diet −13.3 ± 6.4%)	
– *High fruit diet:*			
15% energy from fructose; 15%Protein/30%Lipids/55%CHO; energy-restricted diet (−600 kcal/d)			
Sugar-sweetened beverages			
All diets were *ad libitum* and sugar *provided 25% of energy:*	n=15–17 men/women, BMI: 27–31 kg/m^2, 10 weeks	– Both groups of subjects exhibited significant increases of body weight, fat mass, and waist circumference	Stanhope et al. (2009)
– *Fructose-sweetened beverage*		– Subcutaneous adipose tissue volume was significantly increased in subjects consuming glucose	
– *Glucose-sweetened beverage*		– Both total abdominal fat and visceral adipose tissue volume were significantly increased in subjects consuming fructose	
Prospective cohort study:	n=2371 children, 13 months, 2, 3, 4 and 6 years	– Higher sugar-containing beverages intake at 13 months was associated with higher BMI up to age 6 years in girls but not in boys. Our results imply that the unfavorable effects of SCB intake start early in life and that dietary advice regarding limiting SCB intake should already be given early in life	Leermakers et al. (2015)
Food Frequency Questionnaire with validation against 24-h recalls			

(continued)

Table 10.1 (continued)

Dietary determinant	Subjects/period	Findings	Reference
Mediterranean diet			
All diets were *ad libitum*:	n=1224, subjects at high risk for cardiovascular disease, 1 year	– One-year prevalence of high waist circumference was significantly reduced in the MD + nuts group compared with the control group	Bes-Rastrollo et al. (2006b)
– *Low-fat diet*		– No significant 1-year changes in body weight were observed	PREDIMED study
– *MD + 1 L/week virgin olive oil*			
– *MD + 30g/d mixed nuts*			
– *MD restricted-calorie*	n= 322, moderately obese subjects, 2 years	– All diets resulted in weight loss: low-fat 3.3 kg, MD 4.6 kg, and low-carbohydrate 5.5 kg (p=0.03 for low-fat *vs* low-carbohydrate; p=0.05 for comparison among groups)	Shai et al. (2008)
– *Low-fat diet restricted-calorie*			DIRECT study
– *Low-carbohydrate diet non-restricted calorie*			
A cross-sectional study:	n=4388 (73.2 women)	– The adherence to the mediterranean dietary pattern was a protective factor for obesity (OR = 0.717, 95%CI: 0.555–0.922) and visceral abdominal tissue excess (OR = 0.717, 95%CI: 0.530–0.971)	Bertoli et al. (2015)
Mediterranean dietary score from a validated 14-item questionnaire			

10.2.1 Energy from Fat

The percentage of energy from fat in diets has been widely thought to be an important determinant of body fat, and several arguments support the hypothesis that a high percentage of energy from fat in the diet may lead to greater body fat: (1) dietary fat is the most energy-dense macronutrient in the diet, (2) fats give flavor and palatability to foods, which could increase their consumption, (3) fat produces a lower thermogenic effect than carbohydrate and thus may be utilized more efficiently, and (4) fat has a relatively low satiety value. However, overweight rates have continue to increase despite decreasing intakes of fat in many countries, which suggests that factors other than dietary fat may play a role in the increasing prevalence of obesity (Willett 1998). Thus, in the Nurses' Health Study, an 8-year follow-up of 41,518 women, the results showed that, overall, percent of calories from fat had only a weak positive association with weight gain. However, the percentage of calories from animal, saturated, and *trans* fat had stronger associations, while monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) were not associated (Field et al. 2007). Equally, data from 89,432 men and women from six cohorts of the EPIC (European Prospective Investigation into Cancer and Nutrition) study were analyzed to assess the association between baseline fat intake (amount and type of total, saturated, PUFA, and MUFA fats) and annual weight change. The results showed no significant association between the amount or type of dietary fat and subsequent weight change in this large prospective study, which do not support the use of low-fat diets to prevent weight gain (Forouhi et al. 2009). In this context, Melanson et al. (2009) systematically reviewed the literature from 1993 to 2009 with respect to the relationship between dietary fat and fatty acid intake and body weight and composition, diabetes, and MetS. With regards to obesity, they concluded that larger intervention studies suggest that lower dietary fat is associated with weight loss; however, the studies were not designed to specifically examine dietary fat and weight, and thus differences in intervention intensity make it impossible to draw definitive conclusions from these results. Currently it has been published as a systematic review and meta-analysis to summarise the large body of evidence from randomized controlled trials (RCTs) to determine whether low-fat diets contribute to greater weight loss than participants' usual diet, low-carbohydrate diets, and other higher-fat dietary interventions (Tobias et al. 2015). The results suggest that the long-term effect of a low-fat diet on body weight depends on the intensity of the intervention in the comparison group. Thus, when compared with dietary interventions of similar intensity, evidence from RCTs does not support low-fat diets over other dietary interventions for long-term weight loss.

10.2.2 Low-Carbohydrate Diets

Low-carbohydrate diets have been thought as an alternative to a low-fat diet for producing weight loss and fat losses (Martinez et al. 2014a). Thus, Gardner et al. (2007) carried out a comparison of the four weight-loss diets representing a

spectrum of low to high carbohydrate intake: Atkins (very low in carbohydrate), Zone (low in carbohydrate), LEARN Lifestyle, Exercise, Attitudes, Relationships, and Nutrition; low in fat, high in carbohydrate, based on national guidelines, and Ornish (very high in carbohydrate). In this review study, premenopausal overweight and obese women assigned to follow the Atkins diet, which had the lowest carbohydrate intake, lost more weight and experienced more favorable overall metabolic effects at 12 months than women assigned to follow the Zone, Ornish, or LEARN diets (Gardner et al. 2007). Additionally, several studies on short-term carbohydrate restriction have shown significant improvements in lipid profile and glycemic control, and greater weight loss or even in the absence of weight loss (Feinman and Volek 2006; Nordmann et al. 2006; Liu et al. 2013; Tay et al. 2014). In other investigation, long-term adherence (up to 22 months) to a carbohydrate-restricted diet, with less than 20% of energy intake coming from carbohydrates, appeared to be effective in obese people with type 2 diabetes, as evidenced by the absence of negative cardiovascular outcomes (Nielsen and Joensson 2006). Equally, the effect of long-term (>1 year) consumption of a low-carbohydrate high-fat diet does not induce deleterious metabolic effects and does not increase the risk for cardiovascular disease as evidenced by maintenance of adequate glycemic control and relatively low values for conventional cardiovascular risk factors (Grieb et al. 2008). On the other hand, a low-carbohydrate diet based on the consumption of low-glycemic index (GI) vegetables with unrestricted consumption of fat and protein vs. a low-fat diet consisted of limited energy intake (1200–1800 kcal/day; £ 30% calories from fat), both diets successfully achieved weight loss. Moreover, low-carbohydrate diet was associated with favourable changes in cardiovascular disease risk factors after 2 years (Foster et al. 2010). Likewise, a recent systematic review and meta-analysis has reported that in weight loss trials, low-carbohydrate interventions led to significantly greater weight loss than did low-fat interventions (Tobias et al. 2015). This conclusion is reinforced in other systematic literature review via PubMed (1966–2014) showing RCTs with ≥8 weeks follow up comparing low carbohydrate (≤120 g carbohydrates/day) and low fat diet (≤30% energy from fat/day). Compared with low fat diets, low carbohydrate patterns were associated with significantly greater reduction in weight and significantly lower predicted risk of atherosclerotic cardiovascular disease events. These results suggest that evaluations of dietary guidelines should consider low carbohydrate diets as effective and safe intervention for weight management in the overweight and obese, although long-term effects require further investigation (Sackner-Bernstein et al. 2015). However, the effects of low-carbohydrate diets on body weight, cardiovascular risk and diabetes are yet under investigation, reporting varying results (Dyson 2015; Hernández Alcantara et al. 2015; Gardner et al. 2016; Mansoor et al. 2016). In fact, a limitation in some studies is that it could not be determinated whether the benefits were attributable specifically to the low carbohydrate intake or are due to other aspects of the diet (e.g., high protein intake, specific dietary fat, satiety). Additional information is necessary about the impact of very low carbohydrate diets during weight maintenance due to some undesirable effects reported such as increased levels of ketone bodies, high losses of body water, headache, constipation, and lipid abnormalities

(Abete et al. 2010). For example, future studies should investigate the minimum level of ketosis required to achieve appetite suppression during ketogenic weight loss diets, as this could enable inclusion of a greater variety of healthy carbohydrate-containing foods into the diet (Gibson et al. 2015).

10.2.3 Moderate/High-Protein Diets and Rich in Leucine

Scientific literature suggest that an elevated protein intake plays a key role in weight loss and weight maintenance through: (1) increased satiety to a greater extent than carbohydrate and fat, (2) increased thermogenesis, it has been estimated to account for 5–10% of daily energy expenditure, much greater than that of carbohydrate and lipids and (3) enhanced glycemic control (Brehm and D'Alessio 2008; Westerterp-Plantenga et al. 2008). Many favourable results have been published with respect to body-weight loss after high-protein, low-carbohydrate, high-fat diets. In this sense, weight losses of 4.5–12.0 kg compared with 2.5–6.5 after control diets in 2–6 months have been reported. Overall, diets with increased protein and reduced carbohydrates have reported beneficial effects on weight loss and other metabolic syndrome features after a short and long-term follow-up period (de la Iglesia et al. 2014; Aller et al. 2014). Thus, it has been published that frequent chicken consumption, within a controlled diet with a moderately high content in protein (30% energy), produced a slight but statistically significant weight reduction mainly due to the loss of fat mass, while fat-free mass remained unchanged during the 10 weeks of intervention as well as lipid, glucose, and selected inflammation and oxidative stress biomarkers (Navas-Carretero et al. 2011a). In this way, the Diogenes project (Diet, Obesity and Genes), a randomized clinical trial, conducted to investigate the effect of protein and glycemic index on weight loss maintenance in overweight or obese adults in eight centers across Europe, has reported that the subjects on the high protein diets regained less weight than subjects on the low protein diets after 1 year (Aller et al. 2014). In addition, other studies have published that obese and metabolic syndrome patients under energy-restricted diets based on moderate-high protein (30%) showed greater body composition improvement during long-term maintenance respect to conventional-protein (15%) energy-restricted diets (Layman et al. 2009; de la Iglesia et al. 2014). Furthermore, the inclusion of structured meal replacements at breakfast, morning snack and afternoon snack (low glycemic index products with moderately high protein content) produced a significant reduction in body weight and fat-mass loss in type-2 diabetes patients after 4 weeks (Navas-Carretero et al. 2011b). Concerning the type of protein, the evidence suggests that lower animal protein intakes may be important for maintenance of healthy body weight since that protein derived from animal sources might be associated with increased risk of both global and abdominal obesity among presumably healthy adults (Alkerwi et al. 2015). Moreover, animal protein and meat protein, but neither vegetable- nor fish-derived proteins were found to influence inflammation, a mechanism linked to obesity, in obese subjects under a moderate high protein energy-restriction diet

(Lopez-Legarrea et al. 2014). In this way, it has been reported that proteins from animal sources was an independent determinant of betatrophin levels, a therapeutic target in metabolic disorders associated to adiposity, in metabolic syndrome patients (Crujeiras et al. 2015).

On the other hand, it was suggested that the metabolic improvements related to moderate high-protein intake could be mediated by specific amino acids such as the branched-chain amino acid leucine (Petzke et al. 2014). In this way, leucine is a well-known activator of the mammalian target of rapamycin (mTOR). Because mTOR signaling regulates several aspects of metabolism, the potential therapeutic application of leucine as a dietary supplement for treating obesity has been investigated. Thus, higher-protein diets rich in leucine are the key to stimulating protein synthesis in skeletal muscle and staving off muscle loss (Jitomir and Willoughby 2008). In relation to food control, a recent review shows that there is no robust evidence indicating that oral leucine supplementation can reduce food intake (Pedroso et al. 2015). Notably, central leucine injection appears to decrease food intake; however, this effect is not well reproduced when leucine is provided as a dietary supplement, which puts into question its therapeutic application. Furthermore, the physiological conditions in which leucine can activate mTOR and therefore affect metabolism need to be clarified. In addition other authors raise the question of whether a dietary pattern approach that provides obese adults with foods rich in plant protein and micronutrients could achieve a similar impact on weight loss and muscle function, with equal or better potential for long-term maintenance, and without the risk of adverse health consequences (Bernstein et al. 2015).

In summary, there is increased evidence supporting the benefits of moderate high-protein diets. These benefits also lie in protein's ability to protect skeletal muscle during caloric restriction. The latest insight into protein research reveals putative bioactive effects derived of single amino acids such as branched-chain amino acids, but the underlying mechanisms of these beneficial effects are not fully understood and there is no enough information to justify its supplementation for treating obesity. Overall, investigations in humans in relation these findings are necessary since maintaining healthy muscles during energy restriction is essential for maximizing fat loss and ultimately long-term energy expenditure. In addition, key issues must be resolved regarding the long-term compliance and safety of chronic high-protein intake as well as about the influence of protein type on global health, but a role in weight maintenance after slimming is clear.

10.2.4 Portion Size and Energy-Density

In addition to macronutrient distribution, properties of foods such as portion size and energy density (Kcal/g) have robust effects on energy intake. Large portion size is often accompanied by a higher total energy content, and thus could contribute to weight gain and fat deposition (Rolls et al. 2006a). Furthermore, energy-dense foods, usually high in fat and sugar but low in fiber and water, tend to be less

satiating and high palatable, which could stimulate overeating (Du and Feskens 2010). Equally, large portion sizes increased bite size and eating rate in overweight women (Almiron-Roig et al. 2015). Thus, reductions in both portion size and energy density can help to moderate energy intake without increased hunger (Ello-Martin et al. 2005; Rolls et al. 2006b). In this context, it has been proposed that a good alternative may be to reduce dietary energy density by the addition of water-rich foods, which is associated with substantial weight loss even though participants eat greater amounts of food (Rolls 2010; Chang et al. 2010). Thus, limiting portions of high energy-dense foods would not only improve diet quality but could also lower energy intake. The effectiveness of this strategy will depend on altering the current food environment so that lower-energy density choices are easily accessible, appealing and affordable. In relation to food portion size and dietary quality, it has been published that lower dietary energy density and saturated fat intakes, and higher dietary fibre intakes, were observed on the days that larger portions of fruit and boiled potatoes were consumed and higher dietary energy density and lower micronutrient intakes were recorder on the days larger portions of sugar-sweetened beverages (SSB) were consumed (Lyons et al. 2015). Concening childhood obesity, the portion size and energy density of food are two major determinants of children's energy intake (Pourshahidi et al. 2014). Consequently, it is needed to counteract their deleterous impact and to report detailed information about appropriate average portion size ranges and suggest a practical food plan for feeding preschool children, providing adequate nutrient intakes within energy requirements (More and Emmett 2015).

10.3 Type of Dietary Fat Intake and Adiposity

Several studies have demonstrated that the type of fat is more important than the amount consumed in terms of body weight and adiposity regulation (Willett 1998; Field et al. 2007; Soriguer et al. 2010; Eguaras et al. 2015). Thus, incidence of obesity was lower in persons who consumed olive oil than those who consumed sunflower oil (Soriguer et al. 2009). Concerning the use of vegetables oils for cooking and the risk of overweight/obesity or weight gain, a systematic review shows that although higher consumption of fried foods is probably related to a higher risk of weight gain, the type of oil may perhaps modify this association (Sayon-Orea et al. 2015b). Interestingly, it has been proposed that, at any given level of dietary fat intake (percentage fat calories), those whose diets have a relatively low ratio of saturate fatty acids to unsaturates will be leaner than those whose diets have a higher saturate/unsaturate ratio. Particularly, long-chain saturated fatty acids (SFAs), in excess, have a more negative impact on insulin sensitivity than do unsaturated fats, and these findings are important since that insulin resistance is linked to obesity (McCarty 2010). In relation to trans-fatty acids, in a recent study, associations between adipose tissue levels of trans-fatty acids as a marker for intake and adiposity have been evaluated (Smit et al. 2010). It has been also reported that individual

trans-fatty isomers have divergent effects on adiposity. In this study, the main finding is the consistent adverse association between industrial 18:2 t and all measures of adiposity analyzed (body mass index (BMI), waist circumference (WC), and skinfold thickness). Other prospective and intervention studies are necessary to further clarify this issue. Likewise, regardless of an association with adiposity, removal of partially hydrogenated oils from the diet is important in order to reduce metabolic complications (Smit et al. 2010). In this sense, it has been published that although interesterified fat production is a feasible and economically viable solution for replacing dietary trans-fatty acids, outstanding questions must be answered regarding the effects of interesterification on modifying certain aspects of lipid and glucose metabolism, inflammatory responses, hemostatic parameters, and satiety (Mensink et al. 2016). On the other hand, it has been reported that the fatty acid composition in maternal diet and in breastmilk during lactation may be a factor in the development of childhood overweight later in life (Hatsu et al. 2008). Mothers who consumed at least 4.5 g of trans-fatty acids/day were 5.8 times more likely to have body fat greater than or equal to 30% than those subjects consuming less, and their infants were over 2 times more likely to have body fat greater than or equal to 24% (Anderson et al. 2010).

However, MUFA and PUFA fat consumption have been associated with healthy effects on some metabolic disorders. With respect to adiposity, an isocaloric MUFA-rich diet prevents central fat redistribution induced by a carbohydrate- rich diet in insulin-resistant subjects (Paniagua et al. 2007). Also, in a randomized crossover study in overweight men (28 days in each arm), substitution of dietary saturated with unsaturated fat, predominantly MUFA, produced a small, but significant loss of body weight and fat mass from both trunk and limbs, without a signifi cant change in total energy or fat intake (40% of total energy). Sources of fat for the SFA-rich diet were milk, butter, cream, cheese, and fatty meat, while fat in the MUFA-rich diet was provided from olive oil, nuts, and avocados (Piers et al. 2003). In addition, a moderate-fat diet rich in MUFA represents for some people a considerably more palatable alternative than the usual low-fat approaches for promoting healthy eating and weight loss in the diabetics and obese individuals, thus making easier the adherence and the long-term compliance of participants (Martínez-González and Bes-Rastrollo 2006). Overall, the beneficial effects of MUFAs are provided by the traditional Mediterranean food pattern and, specifically, by olive oil and most nuts, which are reviewed later in this chapter.

On the other hand, the consumption of n-3 PUFA, eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) has been linked to a reduced cardiovascular risk and to reduced fasting glucose levels, providing a protective effect against the development of type 2 diabetes (Krebs et al. 2006; Moosheer et al. 2014; Lorente-Cebrián et al. 2013). Moreover, n-3 dietary fat intake has been shown to play an important role in the treatment of MetS (Robinson et al. 2007; Jiménez-Gómez et al. 2010) as well as in other co-morbidities accompaining MetS such as non-alcoholic fatty liver disease (Nobili et al. 2016) and depressive symptons (Perez-Cornago et al. 2014). There is also continuing debate as to whether or not n-3 PUFA contribute to weight loss and body composition modulation (Kunesová et al. 2006; Lorente-Cebrián

et al. 2013). In this context, the intake of n-3 PUFA has evidenced to influence the fatty acid composition of membrane phospholipids, thus modulating several metabolic processes that take place in the adipocyte (Martínez-Fernández et al. 2015). Lipid management at the cellular level influences the degree of the development of disease and comorbidities in obesity. In this sense, higher plasma levels of total n-3 PUFA have been associated with a healthier BMI, waist, and hip circumference (Micallef et al. 2009). These findings suggest that n-3 PUFA may play an important role in weight status and abdominal adiposity. In fact, a moderate dose of n-3 PUFAs for 2 months reduced adiposity and atherogenic markers without a deterioration of insulin sensitivity in subjects with type 2 diabetes (Kabir et al. 2007). In children, the plasma levels of long-chain PUFAs (LC-PUFAs) were associated with the degree of obesity (Scaglioni et al. 2006) as well as a higher intake of PUFAs was associated with lower total and visceral adiposity and higher lean mass (Cardel et al. 2015). In addition, central obesity was positively associated with n-6 PUFA and inversely associated with MUFA and n-3 PUFA in adipose tissue samples obtained in an obese population from a Mediterranean area (Garaulet et al. 2001). Moreover, it has been suggested beneficial effects of omega-3 fatty acids on satiety (Parra et al. 2008; Buckley and Howe 2010). Taking together these results, fatty fish, fish oils, omega-3 fatty acid-rich foods, and omega-3 supplements could be included in weight loss and weight maintenance programs as well as be incorporated into the dietary habits of healthy subjects separately or combined with other potential bioactive compounds such as α-lipoic acid (Abete et al. 2011; Huerta et al. 2015). However, more clinical trials are necessary to recommend the most effective dosages and formulas (type of n-3 LC-PUFA, EPA/DHA ratio) for specific metabolic disturbances (Lorente-Cebrián et al. 2013).

In addition to the type of dietary fat, the effect of fatty acid chain length on body fat has been also studied. Thus, in contrast to the consumption of long-chain triacylglycerols (LCT), the intake of medium (MCT) has shown to reduce body weight, BMI, WC, body fat, and subcutaneous and visceral fat more greatly (Han et al. 2007; Xue et al. 2009; Zhang et al. 2010). In this context, it has been concluded that substitution of MCT for LCT in a targeted energy balance diet may prevent long-term weight gain and improve body composition via increased energy expenditure and fat oxidation (St-Onge et al. 2003).

With this background, several nutritional interventions have been focused about dietary fat quality. In this context, the PREDIMED study (Prevention with Mediterranean Diet) a randomized, multicenter, cardiovascular primary prevention trial conducted in Spain from October 2003 to December 2010, compared three dietary interventions: two Mediterranean diets, one supplemented with extra-virgin olive oil and the other supplemented with mixed nuts, versus a control (low-fat) diet (Martínez-González et al. 2012; Estruch et al. 2014). This project has reported, among other beneficial effects, that the Mediterranean diets counteracted the harmful effect of abdominal adiposity regarding the risk of CVD events, which might be attributed in part to the type of fat considering Mediterranean diets (Eguaras et al. 2015). In addition, the RESMENA (Metabolic Syndrome Reduction in Navarra) diet, a personalized weight loss dietary strategy

designed for reducing metabolic syndrome was also based on mediterranean pattern (Zulet et al. 2011). In this case, the quality of the fat was focused on extra-virgin olive oil and n-3 fatty acids. The results showed that RESMENA diet was as effective as American Heart Association (AHA) pattern for reducing body weight, adiposity and other features of metabolic syndrome after two nutritional-learning intervention period. Moreover, only the RESMENA participants exhibited a significant decrease in body weight, BMI, waist circumference, waist:hip ratio and android fat mass after an additional 4-month self-control period (Lopez-Legarrea et al. 2013; de la Iglesia et al. 2014).

However, it should take into account that the results obtained of integral dietary strategies depends on the quality and quantity of other macronutrients, not only fat, the consumption of other natural bioactive compounds such as antioxidants, anti-inflammatory occurring in the dietary pattern as well as due to the inclusion of lifestyle change. Likewise, current trends lead to decreased consumption of food supplying high trans and SFA contents, while foods containing PUFA or MUFA tend to increase.

In addition, several studies have been conducted to elucidate the impact of maternal n-3 PUFA supplementation during pregnancy on childhood adiposity. In this sense, further high-quality trials are needed since that there is currently no enough evidence to support that n-3 LCPUFA supplementation during pregnancy and/or lactation favourably affects child adiposity (Stratakis et al. 2014)

10.3.1 Conjugated Linoleic Acid and Body Composition

The term conjugated linoleic acid (CLA) concerns a group of isomers of linoleic acid, which are characterized by having conjugated double bonds in several positions and conformations (Zulet et al. 2005). CLA is found naturally in beef, lamb, and dairy products, but since CLA seems to have beneficial effects on various health-related issues, many investigations have been conducted to elucidate the effects of dietary supplementation with CLA (Agueda et al. 2009; Kennedy et al. 2010). Thus, some published results support a possible beneficial role, producing a significant reduction on body fat while maintaining or increasing the lean mass in humans adults (Blankson et al. 2000; Smedman and Vessby 2001; Gaullier et al. 2004, 2007; Watras et al. 2006; Syvertsen et al. 2007; Sneddon et al. 2008; Dilzer and Park 2012; Kim et al. 2016a). In this context, some studies have not found beneficial effects with regards to body composition after the CLA supplement in adults (Zambell et al. 2000; Risérus et al. 2002; Whigham et al. 2004; Desroches et al. 2005; Taylor et al. 2006; Steck et al. 2007; Norris et al. 2009; Venkatramanan et al. 2010; Sluijs et al. 2010). Concerning the lean mass, some investigations indicate a significant increase in fat free mass after the CLA supplementation (Kamphuis et al. 2003; Gaullier et al. 2004, 2007; Kim et al. 2016b), while in the majority of investigations, significant effects have not been found (Blankson et al. 2000; Mougios et al. 2001; Tricon et al. 2004; Gaullier et al. 2005a, b; Larsen et al. 2006;

Syvertsen et al. 2007; Steck et al. 2007). Also, the results remain contradictory, especially concerning the effect of CLA on weight outcomes. Gaullier et al. (2004) therefore observed a significant difference in body weight after CLA supplementation for 12 months. In a subsequent study published in the same subjects, Gaullier et al. (2005b) found that weight loss of the first 12 months was maintained for the following year with CLA supplementation. Also, CLA supplementation among overweight adults significantly reduced body fat over 6 months and prevented weight gain during the holiday season (Watras et al. 2007). Other researchers have reported body weight reduction in overweight/obese men receiving 4.5 g/d of the CLA isomeric mixture respect to safflower oil in a 4-week double-blind study (Pfeuffer et al. 2011). By contrast, others found no weight loss in the subjects who were given this fatty acid (Tricon et al. 2004). In fact, in the study by Kamphuis et al. (2003), which measured the recovered weight after a low-calorie diet, there was a greater weight gain in the group receiving CLA, compared with the control group. Daily CLA supplementation (3.4 g) for 1 year did not prevent weight or fat mass regain in a healthy obese population (Larsen et al. 2006). On the other hand, in the literature there are scarce studies showing CLA's efficacy with regards to change in fat and BMI in children. Racine et al. (2010) published that CLA supplementation decreased body fatness in children who were overweight or obese but at the moment there is not sufficient evidence about the safety and efficacy of CLA supplementation in children.

In conclusion, the discrepancies in the results on the effect of CLA on body composition, may be related to several factors, including the doses used, adherence to treatment, type and/or proportion of isomers, duration of the study, methodology (anthropometry, hydrodensitometry, bioimpedance, DXA, Infrared), physiopathalogical situations (normal weight, overweight, obese, diabetes, MetS, healthy), in addition to lifestyle (physical exercise or not), among others factors (Table 10.2). Several scientific review articles completed these findings and assessed the pros and cons of CLA consumption (Dilzer and Park 2012; Benjamin et al. 2015; Kim et al. 2016a)

10.4 Influence of Glycemic Index, Glycemic Load and Fiber on Body Fat and Composition

Various dietary factors seem to play a critical role in body weight regulation, among them GI, which is the area under the 2-h blood glucose response curve (AUC) after the ingestion of a fixed amount of carbohydrates, and glycemic load (GL), which is the product of the GI × the amount of available consumed carbohydrate divided by 100. However, the results remain a subject of debate. The ARCA Project, a cross-sectional survey of 3734 obese Italian children carried out in the southern Italy, evaluated the association between dietary GI, BMI, and body fat distribution in school children. The results showed that GI was an independent determinant of both

Table 10.2 Effects of CLA administration on body composition in human intervention studies

Subjects	Duration	Doses	Placebo	Fat mass	Fat-free mass	Method	Reference
$n = 60$, BMI: 25–35 kg/m^2,overweight and obese, men and women	12 Weeks	1.7–6.8 g/day	Olive oil	↓	n.s.	DXA	Blankson et al. (2000)
$n = 17$, BMI: about 22 kg/m^2, normoweight, Women	64 Days	3 g/day	Sunflower oil	n.s.	n.s.	Impedance and DXA	Zambell et al. (2000)
$n = 54$, BMI: 27.5–29.9 kg/m^2 , overweight, men and women	13 Weeks	1.8 or 3.6 g/day	Oleic acid	n.s.	↑	Hydrodensitometry and deuterium dilution	Kamphuis et al. (2003)
$n = 122$, BMI: 28–35 kg/m^2 , overweight and obese, men and women	1 Year	3.4 g/day	Olive oil	n.s.	n.s.	DXA	Larsen et al. (2006)
$n = 118$, BMI: 28–32 kg/m^2 , overweight and obese, men and women	6 Months	3.4 g/day	Olive oil	↓	↑	DXA	Gaullier et al. (2007)
$n = 55$, BMI: >30 kg/m^2 , obese, postmenopausal, type 2 DM, women	36 Weeks crossover: 16 weeks/16 weeks, 4-week washout	6.4 g/day	Safflower oil	↓	n.s.	DXA	Norris et al. (2009)
$n = 22$, overweight, men and women	24 Weeks	3 g/day	Oleic acid	n.s.	n.s.	DXA	Malpuech-Brugère et al. (2010)

DXA dual-energy X-ray absorptiometry, *DM* diabetes mellitus, *n.s.* no significant effect, ↓ decrease, ↑ increase

BMI and waist z -scores. In particular, GI was the sole nutritional independent determinant of WC marker of abdominal obesity (Barba et al. 2012). Equally, associations among dietary glycemic index, glycemic load, and subsequent changes of weight and WC were investigated in five European countries. However, data show that associations of GI and GL with subsequent changes of weight and WC were heterogeneous across centers (Du et al. 2009). Another cross-sectional study on 8195 Spanish adults showed that after adjusting for energy, GL was associated with reduced BMI in this Mediterranean population, while GI was not associated with BMI (Mendez et al. 2009). In line with these studies, data from a total of 1124 patients from Italy showed that GI and GL were inversely associated related to BMI, but no consistent associations were found with waist-to-hip ratio (Rossi et al. 2010). Thus, possibly, the heterogeneity of carbohydrate type and intakes in various populations may in part explain these differences.

On the other hand, intervention studies in humans focusing on GI or GL also show contradictory results and need more investigation. The comparison of highcarbohydrate (55%) and high-protein (25%) diets varying GI content on weight loss and body composition was carried out in a total of 129 overweight or obese young adults during 12 weeks (McMillan-Price et al. 2006). The findings of this study show that a conventional diet of high carbohydrate/high GI was associated with the slowest rate of weight loss. Moreover, subjects instructed to follow a high-carbohydrate/low-GI or a high-protein/high-GI diets were twice as likely to achieve weight loss of 5% (McMillan-Price et al. 2006).

Overall, data from clinical trials suggest that low-GI diets based on high amounts of fruits, vegetables, legumes, and whole grains are better than conventional diets for weight and fat loss (Abete et al. 2008). In this sense, patients who followed a low-GI diet based on legume intake during an 8-week energy-restricted period registered higher weight loss (7% of the initial body weight) than those included in a conventional diet (5% of the initial body weight), and the reduction in body weight was directly associated with fiber intake. Interestingly, 1 year after the nutritional intervention, weight regain was only statistically significant in the higher GI group.

Simultaneous to GI and GL, fiber intake has been investigated with regards to obesity. In fact, an energy-dense, low-fiber, high-fat diet is associated with higher fat mass and greater odds of excess adiposity in childhood (Johnson et al. 2008). The SUN project (Seguimiento Universidad de Navarra (University of Navarra Follow-up)) found that the inverse association between fruit/vegetable consumption and weight gain in the previous 5 years was more evident among those with a high intake of total fiber (Bes-Rastrollo et al. 2006a). Moreover, in this investigation the benefit of total fiber was more evident among those with a high consumption of fruits and vegetables. Furthermore, results of 48,631 men and women from five countries participating in the EPIC study suggest that a diet with low GI and energy density may prevent visceral adiposity, defined as the prospective changes in the WC for a given BMI (WC(BMI)). Thus, men and women with higher energy density and GI diets showed significant increases in their WC(BMI), compared to those with lower energy density and GI. Among women, lower fiber intake, higher GL, and higher alcohol consumption also predicted a higher different WC(BMI) (Romaguera et al.

2010a, b; Estruch et al. 2016). Moreover, a cross-sectional study of 3931 Japanese women showed an independent negative association between dietary fiber intake and BMI, while GI and GL showed an independent positive association with BMI (Murakami et al. 2007).

Additionally, it has been published that the type of fiber may play different roles in body composition. Data from a prospective cohort study with 89,432 European participants support a beneficial role of higher intake of dietary fiber, especially cereal fiber in prevention of body-weight and WC gain (Du et al. 2010). Moreover, the inclusion of whole-grain ready-to-eat oat cereal (3 g/day oat beta-glucan), as part of a dietary program for weight loss, had favorable effects on fasting lipid levels and WC in adults with overweight and obesity more than a dietary program including low-fiber control foods (Maki et al. 2010). In this context, public health professionals could drive their efforts towards the promotion of even healthier ready-to-eat cereals when issuing advice on weight management (Kosti et al. 2010). However, a recent report focused on the efficacy of dietary fiber and supplements on weight loss in interventional studies shows that while a number of human trials have shown weight reduction with diets rich in dietary fiber or dietary fiber supplements, other studies failed to show any effect (Papathanasopoulos and Camilleri 2010). In this way, other study evaluated the association between the carbohydrate quality index and weight change in a mediterranean cohort of 8741 participants, showing a significant inverse association with the incidence of overweight/obesity (Santiago et al. 2015).

With regards to the effect of protein and GI on body composition, the European project DIOGENES (Diet, Obesity, and Genes) is the first dietary study in which the effect of both protein and GI content in children from different European countries were examined (Saris and Harper 2005; Larsen et al. 2010a, b). A points-based system was used to manipulate dietary protein and carbohydrate (Moore et al. 2010). This randomized dietary intervention study adds that neither GI nor protein had an isolated effect on body composition. However, the low-protein/high-GI combination increased body fat, whereas the high-protein/low-GI combination was protective against childhood obesity. All diets were low in fat (25–30% of energy), while protein content was 10–15% energy in the low-protein and 23–28% in the high-protein groups (Papadaki et al. 2010). In addition, in a study comparing the effects on body composition of two energy restricted diets (−30%E) in metabolic syndrome patients after 6 months (RESMENA Project), the group following a diet with a macronutrient distribution of 30% proteins, 30% lipids, 40% carbohydrates and characterized by a low glycemic load, exhibited a significant decrease in body weight (−1·7%), BMI (−1·7%), waist circumference (−1·8%), waist:hip ratio (−1·4%) and android fat mass (−6·9%), but no significant differences were found respect to the AHA strategy. The fibre from legumes, fruits and vegetables was the dietary component that apparently most contributed to the improvement on anthropometry measurements (de la Iglesia et al. 2014).

In conclusion, further research about the role of GI and GL and type of fiber in the prevention and management of obesity is needed. However, overall data suggest that low-GI diets based on high amounts of fruits, vegetables, legumes, and whole

grains are a good strategy to lose weight loss and improve body composition concerning adiposity. Moreover, other aspects such as the effects on lipid and glucose metabolism should be considered in these investigations. Moreover, the carbohydrate intake guidelines related to obesity prevention should be focused on improving the carbohydrate quality index of the diet (Santiago et al. 2015).

10.5 Antioxidants Intake as a Useful Strategy in the Regulation of Body Composition and Fat Depots

In the last years, growing scientific investigations have reinforced the hypothesis that obesity might be an inflammatory disorder (Zulet et al. 2007; Tai and Ding 2010; Serhan 2017). In addition, oxidative stress has also been proposed as a potential inductor of inflammatory status and susceptibility to obesity and related disorders (Pérez-Matute et al. 2009). In this context, several studies have been conducted to assess the potential relationships between dietary antioxidant intake and inflammation oxidative stress and inflammation interactions in human obesity (Bondia-Pons et al. 2012). Thus, a negative association between sialic acid and selenium intake, a recognized antioxidant trace element, has been reported in healthy young subjects, reinforcing the view of selenium as a potential anti-inflammatory nutrient (Zulet et al. 2009). Moreover, in 100 health subjects circulating C3, an inflammatory marker, showed a positive association with several adiposity markers such as BMI, WC, waist-to-height ratio, body fat mass, whereas nail selenium was a statistically significant negative predictor of C3 concentrations (Puchau et al. 2009a). Concerning vitamin C, plasma ascorbic acid was associated with fat distribution independent of BMI in 19,068 British men and women in the EPIC Norfolk cohort study (Canoy et al. 2005). Later, in another cross-sectional trial including 35 men and 83 women with BMI of 30.4 ± 0.6 kg/m^2, plasma vitamin C was inversely related to BMI, percentage of body fat, and WC, particularly in women (Johnston et al. 2007). Thus, it has been proposed that not only calories count in weight gain and body fat mass changes, but so does the antioxidant status (Campión et al. 2008). Other antioxidants such as vitamin A intake were related, not only with the total antioxidant intake, but also with several anthropometrical (weight, BMI, WC, and waist-to-hip ratio) and biochemical measurements linked to MetS manifestations and other features related to oxidative stress in healthy young adults (Zulet et al. 2008). In a study carried out to assess the potential relationships between the dietary total antioxidant capacity (TAC), as a measure of antioxidant intake, and obesity-related features in children and adolescents, TAC showed positive associations with fiber, folic acid, magnesium, and vitamins A, C, and E. In this investigation, BMI, standard deviation score of BMI, and total body fat were inversely associated with dietary TAC only in obese subjects (Puchau et al. 2010a). In addition, potential associations have been observed among dietary TAC and several early MetS manifestations in healthy young adults (Puchau et al. 2010b) as well as in patients suffering from metabolic syndrome (de la Iglesia et al. 2013; Lopez-Legarrea et al. 2013).

Equally, energy density and other relevant nutritional quality indexes were also inversely associated with dietary TAC (Puchau et al. 2009b). In summary, the role of oxidative stress and inflammation in several chronic diseases is receiving attention due to identified links with chronic diseases such as obesity.

In this way, antioxidant intake consumption has been suggested to protect against oxidative damage and related inflammatory complications (Coelho et al. 2013; Abdali et al. 2015). In addition to vitamins and minerals, current investigations are being focused on polyphenols since that have been considered among the bioactive molecules to get beneficial effects by exerting antioxidant activity. Polyphenols are a class of naturally-occurring phytochemicals, some such as catechins, anthocynines, resveratrol and curcumin have been shown to modulate physiological and molecular pathways that are involved in energy metabolism, adiposity, and obesity (Meydani and Hasan 2010). Due to their body fat-lowering effects are being considered as potentially important anti-obesogenic ingredients (Barbosa et al. 2008; Boqué et al. 2013). At present, the interest for the inclusion of polyphenols in the development of functional foods for the prevention and/or treatment of obesity and co-morbidities is growing but the results are still inconclusive (Ibero-Baraibar et al. 2014a, b).

Overall, consumption of foods containing antioxidants, such as fruits, vegetables, green tea, nuts, olive oil, grapes, and the follow-up of a Mediterranean dietary pattern as well as the inclusion of functional foods considering bioactive compounds with antioxidant properties as polyphenols could be a useful strategy in the regulation of body composition and the maintenance of the fat depot, as well as in the improvement of metabolic diseases related to obesity. The effects on adiposity, weight, and body composition of other foods containing antioxidants such as nuts, olive oil, fruits, vegetables, legumes, green tea, etc. are reviewed in this chapter.

10.6 Specific Foods Consumption in Relation to Fat Mass and Body Composition

10.6.1 Nuts and Olive Oil

The SUN is a prospective cohort study designed to establish associations between diet and the occurrence of several diseases and chronic conditions including obesity. In this context, it has been found that a high amount of olive oil (a MUFA-rich source), consumption is not associated with higher weight gain or a significantly higher risk of developing overweight or obesity in the context of the Mediterranean food pattern (Bes-Rastrollo et al. 2006b). In addition, frequent nut consumption has been associated with a reduced risk of weight gain. Nuts are an integral part of the Mediterranean food pattern, which includes a substantial intake of fat (up to 35–40% of total energy intake). Particularly, nuts are high in unsaturated FA, especially oleic acid (MUFA) and linoleic acid (PUFA), which can vary their content according to

types of nuts (Mattes and Dreher 2010). In addition, nuts are a good source of plant protein (arginine), fiber, copper and magnesium and also supply significant amounts of tocopherols, squalene, and phytosterols that are relevant compounds of antioxidant properties. These results support the recommendation of nut consumption as an important component of a cardioprotective diet and also allay fears of possible weight gain (Bes-Rastrollo et al. 2007). In the same way, the Nurses' Health Study II found that the highest consumption of nuts was not associated with increased weight gain during followup 8 in middle-aged healthy women. Instead, it was associated with a slightly lower risk of weight gain and obesity. The results of this study suggest that incorporating nuts into diets does not lead to greater weight gain and may help weight control (Bes-Rastrollo et al. 2009). Participants in the PREDIMED (Prevención con Dieta Mediterránea) study (a multicenter, three-arm, randomized clinical trial to determine the efficacy of the MedDiet on the primary prevention of cardiovascular disease) were following a Mediterranean-style diet with high intake of virgin olive oil or high intake of nuts, or a conventional low-fat diet. Thus, a Mediterranean diet (MD), especially rich in virgin olive oil, was associated with higher levels of plasma antioxidant capacity. In addition, plasma TAC was related to a reduction in body weight after 3 years of intervention in a high cardiovascular risk population with a Mediterranean-style diet rich in virgin olive oil (Razquin et al. 2009). Also, nut consumption was inversely associated with adiposity independent of other lifestyle variables. It was predicted that BMI and WC decreased by 0.78 kg/m^2 and 2.1 cm, respectively, for each serving of 30 g of nuts (Casas-Agustench et al. 2011). In addition, olive oil and walnut breakfasts reduced the postprandial inflammatory response in mononuclear cells compared with a butter breakfast in healthy men (Jiménez-Gómez et al. 2009). Taking into account these findings, it is important to emphasize the recommendation of olive oil and nuts as a substitute for other energy-dense snacks that lack nutritional value to facilitate beneficial changes in dietary habits (Bes-Rastrollo et al. 2007). Moreover, nuts are useful adjuvants to prevent, delay or ameliorate a number of chronic conditions accompaining obesity, such as risk of cardiovascular disease and mortality, cancers and depression (Grosso and Estruch 2016).

10.6.2 Fruits and Vegetables

Natural compounds highest in antioxidants and fiber are those coming from foods such as fruits, vegetables, legumes, olive oil, red wine, green tea, and some nuts. Thus, fruits and vegetables are usually included in dietary guidelines to combat obesity. They are fiber rich, low in energy density, and high in vitamins, and contain a variety of compounds with antioxidant capacity in plasma (AOP), such as vitamins C and E, carotenoids, flavonoids, and polyphenols, which may produce benefi cial actions (Barbosa et al. 2008; Badimon et al. 2010). In a recent study, subjects within the highest tertile of energy-adjusted fruit and vegetable consumption showed significantly lower values of BMI, WC, systolic, and diastolic blood pressure, as

compared with those of the lowest tertile, as well as lower mRNA expression in peripheral blood mononuclear cells of some relevant proinfl ammatory markers (Hermsdorff et al. 2010). Interestingly, fiber and dietary TAC also were statistically higher in those individuals included in the highest tertile of fruit and vegetable consumption (Hermsdorff et al. 2010). In a subsample of the RESMENA study the beneficial effects observed on oxidative stress in patients suffering from metabolic syndrome with hyperglycemia were associated to dietary TAC and fruit consumption (de la Iglesia et al. 2013). Additionally, dietary TAC was the most contributing factor involved in body weight and obesity related markers reduction (Lopez-Legarrea et al. 2013). Thus, dietary patterns including foods with a high antioxidant capacity, such as fruits, could be recommended to improve body composition in addition to impaired oxidative status accompanying obesity. Furthermore, the high-water content of fruits allows individuals to eat satisfying portions of food while decreasing energy intake (Rolls 2009).

10.6.3 Green Tea/Coffee

Several trials have evaluated the effect of green tea on body weight and weight maintenance among obese subjects. A systematic review and meta-analysis including 15 studies (n = 1243 patients) has been published and describes that the administration of green tea catechins with caffeine is associated with statistically significant reductions in BMI, body weight, and WC; however, the clinical significance of these reductions is modest at best (Phung et al. 2010). Since green tea (epigallocatechin gallate (EGCG) + caffeine) and protein were shown to improve body weight maintenance after weight loss, a study analyzed the effect of a Green tea–caffeine mixture added to a high-protein diet on weight maintenance after body weight loss in moderately obese subjects. The results showed that the green tea–caffeine mixture, as well as the high-protein diet, improved weight maintenance independently, while a possible synergistic effect failed to appear (Hursel and Westerterp-Plantenga 2009). In this way, a novel green tea meal replacement formula produced more weight loss and had a greater reduced total body fat mass tan control group (Tsai et al. 2009). Otherwise, patients with type 2 diabetes receiving catechin-rich beverage for 12 weeks reduced WC greater than control group. Moreover, adiponectin, which is negatively correlated with visceral adiposity, increased significantly only in the catechin group (Nagao et al. 2009). Thus, tools for obesity management including catechin-rich beverages have been proposed as strategies to improve body fat and composition (Westerterp-Plantenga 2010; Hursel and Westerterp-Plantenga 2013).

In relation to mechanisms of action, two main components, caffeine and catechin content, are associated with energy expenditure separately (Türközü and Tek 2017). In this sense, a cross-sectional study evaluated the relationships among daily consumption of coffee and tea with components and prevalence of metabolic syndrome. The authors concluded that high coffee consumption was negatively associated with waist circumference, hypertension, and triglycerides, whereas tea consumption with

central obesity and fasting plasma glucose in women, but not in men (Grosso et al. 2015). Additionally, caffeine intake has been related to successful weight loss maintenance (Icken et al. 2016). Thus, weight loss maintainers (n=494) reported to consume significantly more cups of coffee and caffeinated beverages compared with the participants in the general population simple (n=2129).

In addition to green tea catechins especially EGCG, other dietary polyphenols have been described as potential nutritional strategies for the prevention of obesity and associated inflammation and oxidative stress, such as resveratrol, curcumin and cocoa flavanols (Wang et al. 2014; Ibero-Baraibar et al. 2014a). However, polyphenols have a low bioavailability in humans and more importantly, translational studies from animal observations to human clinical trials are needed to reconcile discrepancies and confirm the anti-obesity benefits of these polyphenols and foods rich in these bioactive compounds.

10.6.4 Dairy Products

From an experimental perspective, current evidence supporting the role of dairy in weight loss is rather conflicting. In a young nonhypertensive population, dietary supplementation with whole-fat dairy products, compared to low-fat dairy, was associated with weight gain (Alonso et al. 2009). On the other hand, a longitudinal study in 53 preschool children observed that higher intakes of calcium and dairy products were correlated with a lower total body fat (Carruth and Skinner 2001). Similarly, cohorts of 12,829 children (9–14 years old) were studied to determine the association between milk, calcium, dairy fat, and weight gain. Results suggested that children with a milk consumption of greater than three glasses per day were more likely to gain weight. As weight gain is the result of excess caloric intake, the authors hypothesized that this weight gain effect was the result of the additional energy associated with intake of large quantities of milk rather than the dairy product per se (Berkey et al. 2005). However, Zemel et al. (2008) have reported that individuals consuming at least three servings of dairy products per day had greater fat oxidation and were able to consume significantly more energy without greater weight gain in comparison to individuals consuming minimal amounts ($\leq$1 serve/day) during periods of weight maintenance. Thus, recommended levels of dairy products may be used during weight maintenance without contributing to weight gain compared to diets low in dairy products (Zemel 2004; Zemel et al. 2008). In the SUN study central adiposity was inversely associated with high yogurt consumption and the combination of high consumption of both yogurt and fruit was inversely associated with the development of MetS over 6 years of follow-up (Martinez-Gonzalez et al. 2014; Sayon-Orea et al. 2015a). In addition, in the PREDIMED study the consumption of whole-fat yogurt was associated with higher probability for reversion of abdominal obesity (Santiago et al. 2016).

Calcium is often identified as one of the key components that may explain observed effects of dairy products on health (Christensen et al. 2009). It was sug-

gested that the prevalence of obesity (or weight gain) in women could be reduced by 60–80% by the simple stratagem of ensuring population-wide calcium intakes at the currently recommended levels (Heaney 2003). Nevertheless, supplementation with dietary calcium (1500 mg/day) for 2 years had no statistically or clinically significant effects on weight in overweight and obese adults (Yanovski et al. 2009). In young overweight children, it has been suggested that in addition to lifestyle changes, an isocaloric dairy-rich diet (>800 mg calcium/day) may be a well-accepted regimen and can be a safe and practical strategy for weight control (Kelishadi et al. 2009). In contrast, other intervention studies have demonstrated greater weight loss in obese adults when consuming diets high in dairy products providing 1200–1300 mg calcium from dairy products in comparison to calcium supplementation alone (800 mg calcium) (Zemel et al. 2004). This finding suggests that the observed dairy product-mediated effects are the likely result of a complex matrix of nutrients and bioactive components contained within the whole dairy food in addition to calcium.

Recently, current knowledge on dairy food consumption and obesity-related chronic illness have been reviewed, and it has been proposed that future research might discriminate between types of dairy foods and focus on the synergy provided by the food matrix, rather than simply the component parts of the food (Warensjo et al. 2010). Moreover, not all dairy foods appear to be the same, and their effects may be different for different stages of metabolic dysfunction. Additionally, a dairy supplemented diet produced significant and substantial suppression of the oxidative stress and inflammatory biomarkers associated with overweight and obesity (Zemel et al. 2010). In this context, nowadays there is growing interest about the potential role of the intestinal microbiota in programming health and disease (Goulet 2015). Gut microbiota seems to be an important and promising target in the prevention and treatment of obesity and its related metabolic disturbances (Boroni Moreira et al. 2012; Chakraborti 2015). In this way, lactobacillus and Bifidobacterium that come from dairy products, including yogurt, are suggested to positively modulate the gut microbiota and may help to prevent or treat some diseases (DiBaise et al. 2012).

10.6.5 Sugar Sweetened Soft Drinks and Water

There is increasing concern that intake of free sugars – particularly in the form of sugar-sweetened beverages – increases overall energy intake and may reduce the intake of foods containing more nutritionally adequate calories, leading to an unhealthy diet, weight gain and increased risk of noncommunicable diseases (World Health Organization 2016). Thus, greater sweetened beverage consumption was positively associated with adiposity (Fiorito et al. 2009; Barrio-Lopez et al. 2013; Ambrosini et al. 2013) showing that dietary advice limiting SSB intake should already be given early in life (Leermakers et al. 2015).

With regards to the type of sugar intake, the predominant added sugars are fructose-containing sugars, sucrose and high fructose corn syrup (HFCS). Mechanistically, it is plausible that fructose consumption causes increased energy intake and reduced energy expenditure due to its failure to stimulate leptin production (Stanhope 2016). In a study of 10 weeks, the consumption of 25% of energy requirements from fructose increased visceral adiposity and lipids and decreased insulin sensitivity in older, overweight and obese men and women (Stanhope et al. 2009). Yet, a recent investigation have reported that consumption of added sugars, either from sucrose or HFCS at 10 and 20% of calories consumed (normal population consumption levels) in a 10 week trial, showed no significant changes in any group with respect to weight, adiposity, or abdominal adiposity and no adverse effects on triglycerides, LDL, or blood pressure in a randomized, controlled trial of free living individuals lasting 10-week (Lowndes et al. 2014). In addition, there is also little data to determine whether the way in which added sugar is consumed, as beverage or as solid food, affects its potential to promote weight gain. Because beverages are less satiating than solid foods, consumption of energy-containing beverages may increase energy intake and lead to weight gain. Likewise, energy provided by beverages should be compensated by reduced consumption of other foods in the diet (Dennis et al. 2009). The field needs higher-quality experimental studies in humans, with relevant direct comparisons between sweetened beverages and their sweetened solid-food alternatives (Pereira 2014).

Based on the entire body of evidence, WHO generated the following recommendations for free sugars intake in adults and children (World Health Organization 2015): 1) WHO recommends a reduced intake of free sugars throughout the life-course (strong recommendation); 2) In both adults and children, WHO recommends reducing the intake of free sugars to less than 10% of total energy intake (strong recommendation); 3) WHO suggests a further reduction of the intake of free sugars to below 5% of total energy intake (conditional recommendation). In addition, the current eating patterns in the United States (Dietary Guidelines for Americans 2015–2020) recommend consume less than 10% of calories per day from added sugars. However, it has been reported that compliance require strict dietary compliance and may not be sustainable for many Americans and general public (Erickson and Slavin 2015). Furthermore, public health recommendations about a reduction of excessive sugar intake has involved consumption of artificial sweeteners in a wide range of products. However, simple substitution of artificial sweeteners for sugars in humans may not produce the intended consequences. Thus, it is critical to provide more evidence about the impacts of artificial sweeteners on health and disease (Shearer and Swithers 2016).

On the other hand, findings from clinical trials, along with those from epidemiologic and intervention studies, suggest that water has a potentially important role to play in reducing energy intake, and consequently in obesity prevention. One of the most consistent sets of findings was related to adults drinking sugar-sweetened beverages versus water before a single meal. In these comparisons, total energy intakes were 7.8% higher when SSBs were consumed (Daniels and Popkin 2010). Respect

to energy intake, consuming 500 ml water prior to each main meal fullness leads to greater weight loss than a hypocaloric diet alone in middle-aged and older adults. This may be due in part to an acute reduction in meal energy intake following water ingestion (Dennis et al. 2010). However, drinking water has heterogeneous effects on energy intake, energy expenditure, fat oxidation (FO) and weight change in RCTs involving adults and/or children. A recent qualitative review shows 134 randomized controlled trials reporting negative, null and positive effects of drinking water on energy intake, energy expenditure, fat oxidation and weight change (Stookey 2016). Heterogeneity across studies is associated with different study conditions. The main conclusions are: (1) drinking water instead of caloric beverages decreases energy intake when food intake is ad libitum; (2) drinking water increases energy expenditure in metabolically-inflexible, obese individuals; (3) drinking water increases fat oxidation when blood carbohydrate and/or insulin concentrations are not elevated and when it is consumed instead of caloric beverages or in volumes that alter hydration status.

Taking together, there are promising results for promoting water as a replacement beverage. But, longer-term randomized controlled trials and more interventions with strong compliance-monitoring designs are needed to fully understand the benefits of drinking water as a replacement for a range of caloric and non-nutritive beverages. Future research that examines beverage habits and weight should address factors such as portion sizes, lifestyle, dieting behaviors, etc. in order to determine if/what specific conditions optimize drinking water interventions for weight management.

10.7 Dietary Patterns Including Specific Foods and Body Composition

10.7.1 Mediterranean Diet

The traditional MD, as studied in the 1950s–1960s in the South of Europe, is characterized by moderate energy intake, low animal fat, high olive oil, high cereals, high legumes, nuts and vegetables, and regular and moderate wine consumption (Hermsdorff et al. 2009). Moreover, numerous epidemiological studies have supported the concept that adherence to the traditional MD is beneficial for health and particularly protects against cardiovascular disease (Lairon 2007; Sotos-Prieto et al. 2010). More recent evidence indicates that MD has a favorable effect on type 2 diabetes and adiposity. However, the beneficial impact of the traditional MD on adiposity is still under debate (Babio et al. 2009). In relation to adiposity, data of different studies suggest that adherence to the MD is inversely associated with BMI and obesity e.g., in Spanish men and women (Schröder et al. 2004). Equally, a MD with low consumption of liquid sweets and refined cereals was negatively associated with adiposity in adults from rural Lebanon (Issa et al. 2011).

In the EPIC-PANACEA project (European Prospective Investigation into Cancer and Nutrition-Physical Activity, Nutrition, Alcohol Consumption, Cessation of Smoking, Eating Out of Home, and Obesity), the association between the degree of adherence to the modified-Mediterranean Diet Score and BMI or WC was studied in a total of 497,308 individuals from ten European countries. Despite the observed heterogeneity among regions, results of this study suggest that adherence to a modified MD, high in foods of vegetable origin and unsaturated fatty acids, is associated with lower abdominal adiposity measured by WC in European men and women (Romaguera et al. 2009). Further investigations within the EPIC-PANACEA study show that individuals with a high adherence to the MD are 10% less likely to develop overweight or obesity than those individuals with a low adherence. The authors concluded that the low meat content of the MD seemed to account for most of its positive effect against weight gain since an increase in meat intake of 250 g/day (e.g., one steak at approximately 450 kcal) would lead to a 2-kg higher weight gain after 5 years. Positive associations were observed for red meat, poultry, and processed meat (Vergnaud et al. 2010). Overall, data shows that promoting the MD as a model of healthy eating may help to prevent weight gain and the development of obesity (Romaguera et al. 2010a, b). Other study carried out in a large simple of Italian adults (n=4388, 73.2% women) confirms the inverse association between the mediterranean dietary pattern, BMI and WC and adds that the association with abdominal obesity as detected by WC is due to an association with visceral and not with subcutaneous abdominal tissue (Bertoli et al. 2015). Also, a systematic review of RCTs has noted that the MD resulted in greater weight loss than the low-fat diet at $\geq$ 12 months (range of mean values: −4.1 to −10.1 kg vs. 2.9 to −5.0 kg), but produced similar weight loss as other comparative diets (range of mean values: −4.1 to −10.1 kg vs. -4.7 to −7.7 kg) in overweight or obese individuals trying to lose weight. Moreover, the Mediterranean diet was generally similar to comparison diets at improving other cardiovascular risk factor levels, including blood pressure and lipid levels (Mancini et al. 2015). In relation to diet during pregnancy and childhood overweight, a study has reported that adherence to the MD was associated with lower WC, a marker of abdominal obesity, at 4 years of age (Fernández-Barrés et al. 2016).

In this way, several components of MD have been inversely related with BMI or WC. Among numerous foodstuffs characterizing the MD, virgin olive oil has been shown to display beneficial effects on a wide range of risk factors as has been reported in PREDIMED study. In this chapter, the main dietary components of MD with influence on body composition are discussed. In conclusion, the MD is a healthy eating pattern with protective effects on chronic diseases, such as obesity, cardiovascular disease and associated disorders, possibly because it is negatively associated with BMI and visceral adiposity. Moreover, there is growing evidence suggesting that the MD could serve as an anti-inflammatory and antioxidant dietary pattern, which may be useful in the development of dietary approaches for dietary counseling and the prevention of obesity (de la Iglesia et al. 2013; Marques-Rocha et al. 2016).

10.7.2 Fish-Based Energy-Restricted Diet

The SEAFOODplus-YOUNG project is a randomized controlled trial of energy restricted diet varying in fish and fish oil content and followed for 8 weeks. Subjects (324 participants, 20–40 years of age, BMI 27.5–32.5 kg/m^2, from Iceland, Spain, and Ireland) were randomized to one of four energy-restricted diets (−30% relative to estimated requirements): salmon (150 g 3 times/week, resulting in a daily consumption of 2.1 g of omega-3 LC-PUFAs), cod (150 g 3 times/week, 0.3 g of omega-3 LC-PUFAs/day), fi sh oil capsules (1.3 g of omega-3 LC-PUFAs/day), or control (sunflower oil capsules, no seafood). The important finding of the such study is that in young, overweight men, the inclusion of either lean or fatty fish, or fish oil as part of an energy-restricted diet resulted in approximately 1 kg more weight loss after 4 weeks, than did a similar diet without seafood or supplement of marine origin. Therefore, the addition of seafood to a nutritionally balanced energy restricted diet may boost weight loss (Thorsdottir et al. 2007). Later, it has been published that fatty seafood, particularly salmon intake exerted positive additional benefits on insulin resistance, diastolic blood pressure and inflammatory markers, leading to greater benefits than those achieved with a weight loss intervention alone in overweight and obese European young adults (Ramel et al. 2008, 2010a, b). Moreover, consumption of fatty seafood can modulate fasting insulin, ghrelin, and leptin during an 8-week intervention, and these effects are partly gender specific and partly explained by weight loss (Ramel et al. 2009a, b). Additionally, the inclusion of lean fish to an energy-restricted diet for 8-weeks resulted in significantly more weight loss than an isocaloric diet without seafood in young overweight or obese individuals. Overall, there was on average 1.7 kg significantly more weight loss among subjects consuming 150 g cod 5 times a week compared to the control group receiving no seafood as well as significant reductions inBMI and WC (Ramel et al. 2009b). Thus, the results of these investigations show that following an energy-restricted diet containing lean or fatty fish or fish oil supplements result in more beneficial effects on adiposity and associated metabolic disorders than an isocaloric energyrestricted diet without marine food, which may be a useful strategy to lose weight and manage adiposity. However, other authors have not found additional benefits derived from a healthy low calorie diet after advocate to consume two fish meals per week in a 12 mo weight loss trial (Tapsell et al. 2013).

10.7.3 Legume-Based Energy-Restricted Diet

It is evident that the inclusion of fruits, vegetables, and legumes increases the consumption of fiber, antioxidants, low glycemic index carbohydrates, and minerals that produce a crucial effect on body composition. Thus, legumes are foods containing important nutritional and functional factors that may play a crucial role in health maintenance and disease treatment, such as vegetable protein, fiber,

oligosaccharides, phytochemicals, minerals (e.g., potassium), and other bioactive compounds, such as saponins and polyphenols (Duranti 2006). In this context, nutritional intervention studies including three or more legume servings per week have found not only weight loss improvements, but also important benefits in the inflammatory and antioxidant status of participants as well as improvement of some metabolic features. Thus, an 8-week energy restriction (−30% energy expenditure) study in obese subjects that included legume consumption (four servings per week) showed that those patients following the legume diet lost more weight and additionally showed a reduction in lipid peroxidation and total cholesterol as compared to a control hypocaloric diet (Crujeiras et al. 2007). Similarly, it has been published that the specific consumption of legumes within a hypocaloric diet could activate mitochondrial oxidation, which could involve additional benefits to those associated with the weight reduction (Abete et al. 2009a). Additionally, the consumption of legumes (four servings per week) within a hypocaloric diet resulted in a specific reduction in proinflammatory markers, such as CRP and C3 and a clinically significant improvement of some metabolic features (lipid profile and BP) in overweight/obese subjects, which were in some cases independent from weight loss (Hermsdorff et al. 2011). In this way, a trial was conducted to determine the association of consuming beans on nutrient intakes and physiological parameters using the National Health and Examination Survey 1999–2002. The results showed that those consuming beans had a lower body weight and a smaller waist size relative to nonconsumers. Additionally, consumers of beans had a 23% reduced risk of increased waist and a 22% reduced risk of being obese. Also, baked bean consumption was associated with a lower systolic blood pressure (Papanikolaou and Fulgoni 2008). In this regard, a high-fibre bean-rich was as effective as a low-carbohydrate diet for weight loss, while only the bean-rich diet lowered atherogenic lipids (Tonstad et al. 2014). In fact, the inclusion of legumes in a fat lowering program appears important since a number of data support additional benefits to weight loss of legume consumption. This outcome could be attributed to the dietary quality, lower fat, higher bioactive compounds intake, higher vegetable protein supply, lower GI, as well as higher satiety favoring dietary compliance.

10.7.4 Vegetarian Diets/Plant-Based Diet

A vegetarian diet is defined as one that does not include meat (including fowl) or seafood, or products containing those foods (Craig et al. 2009). Epidemiological studies indicate that vegetarian diets are associated with a lower BMI and a lower prevalence of obesity in adults and children. A meta-analysis in 2001 of 36 studies in women and 24 studies in men using references from Messina and Messina's publication "The dietician's guide to vegetarian diets," showed no marked differences in height between vegetarians and nonvegetarians; however, vegetarians had significantly lower weight (−7.7 kg for men and −3.3 kg for women; $P < 0.0001$and $P = 0.007$, respectively) and a two-point lower BMI (Sabaté and Wien 2010). Similarly,

compared with nonvegetarians, vegetarian children are leaner, and their BMI difference becomes greater during adolescence. Thus, 215 adolescents consuming predominantly vegetarian foods showed significantly better scores on markers of cardiovascular health, including, BMI, WC, cholesterol/high density lipoprotein ratio, and low density lipoprotein. Adolescents consuming nuts more than once per week, also showed lower scores for BMI and serum glucose irrespective of their vegetarian status (Grant et al. 2008). It has further been reported that a dietary pattern characterized by a high intake of dark-green and deep-yellow vegetables was related to low fat mass and high bone mass, while high processed-meat intake was related to high bone mass and high fried-food intake to high fat mass in young children. Thus, beginning at preschool age, diets rich in dark-green and deep-yellow vegetables and low in fried foods may lead to healthy fat and bone mass accrual in young children (Wosje et al. 2010). Thus, traditional messages to reduce calories and fat are important, and follow-up of well-planned vegetarian diets can assist individuals to maintain weight and improve body composition, due to several factors, such as lower caloric density, the avoidance of foods containing SFA, as well as higher variety of components with healthy benefits as complex carbohydrate, fiber, antioxidants, PUFA fat-to-saturated fat ratio, water content, among others (Rodríguez et al. 2005; Grant et al. 2008; Tanumihardjo et al. 2009). Moreover, well-planned vegetarian diets may be appropriate for individuals during all stages of the life cycle, including pregnancy, lactation, infancy, childhood, and adolescence, and for athletes (Craig et al. 2009).

Nowadays, there is consistent evidence from clinical trials showing that the prescription of balanced vegetarian (including vegan) diets reduces mean body weight in study groups, suggesting that they may be helpful for prevention and management of weight-related conditions (Barnard et al. 2015). Likewise, additional research studies are needed to identify the best means of introducing plant-based diets and maximizing adherence.

10.7.5 Eating Away, Fast Food, and Snacking

Eating away from home and particularly fast food consumption have been shown to contribute to weight gain. Increased geographic access to fast food outlets and other restaurants may contribute to higher levels of obesity, especially in individuals who rely largely on the local environment for their food purchases. Car owners show higher BMIs than non-car owners. However, individuals who do not own cars and reside in areas with a high concentration of fast food outlets have higher BMIs tan non-car owners who live in areas with no fast food outlets. Higher restaurant density is associated with higher BMI among local residents. The local fast food environment has a stronger association with BMI for local residents who do not have access to cars (Inagami et al. 2009). In this sense, public health efforts to limit access to fast food among nearby residents could have beneficial effects on child obesity since students who resided within one-tenth or one-quarter of a mile from a fast food

restaurant had significantly higher values of BMI (Mellor et al. 2011). Additionally, students with fast-food restaurants near (within 1½ mile of their schools): (1) consumed fewer servings of fruits and vegetables, (2) consumed more servings of soda, and (3) were more likely to be overweight or obese than youths whose schools were not near fast-food restaurants (Davis and Carpenter 2009). In the EPIC study, energy intake at restaurants was higher than intake at work in southern Europe, whereas in northern Europe, eating at work appeared to contribute more to the mean daily intake than eating at restaurants. Cross-sectionally, eating at restaurants was found to be positively associated with BMI only among men (Naska et al. 2011). Public health policies should thus be implemented to help people make healthier food choices away from home (Bezerra et al. 2015). In addition, snacking is also considered an important factor in the development of obesity (Sánchez-Villegas et al. 2002). Furthermore, the results of SUN study support the hypothesis that self-reported between-meal snacking can be a potential risk factor for obesity (Bes-Rastrollo et al. 2010). However, healthy snacks consumption was associated with decreased prevalence of overweight, general obesity, and abdominal obesity in adolescents (Azadbakht et al. 2015). Equally, eating at home, involvement in meal preparation, higher eating frequency and slower eating rate were habits of weight loss maintainers (Karfopoulou et al. 2016).

In summary, exposure to poor-quality food and nutritional environments has important effects on adolescent eating patterns and overweight. Policy interventions limiting the proximity of fast-food restaurants to schools could help reduce adolescent overweightness as well as public health policies should be implemented to help people make healthier food choices away from home. The lifestyle habits of successful maintainers of weight loss provide target behaviors to improve obesity treatment.

10.7.6 Other Current Dietary Patterns

10.7.6.1 Paleolithic Type Diets

Paleolithic nutrition has attracted substantial public attention lately because of its putative health benefits. The nutritional patterns of our ancestors from the Paleolithic era (2.6 million to ~10,000 years ago) differed considerably from current standards. Diets based on meats, fish, fruits, vegetables and nuts and excluding processed foods, dairy products and refined grains, the so-called Paleolithic (Paleo-) type diets. Paleo diets typically are also lower in sodium and very much higher in potassium, antioxidants, micronutrients and fiber and with a much lower diet acid content (Masharani et al. 2015). In this way, it has been suggested they could prevent or reverse metabolically related Western disease such as cardiovascular disease, diabetes and metabolic syndrome (Lindeberg et al. 2007; Jönsson et al. 2009). A systematic review and meta-analysis including four RCTs that involved 159 participants has published that the paleolithic diet resulted in greater short-term improvements

in metabolic syndrome components than did guideline-based control diets (Manheimer et al. 2015), particularly resulted in greater short-term improvements than did the control diets (random-effects model) for waist circumference (mean difference: −2.38 cm; 95% CI: −4.73, −0.04 cm). Moreover, it has been reported that paleolithic diet is more satiating per calorie than a Mediterranean-like diet (Jönsson et al. 2010) and that a diabetes diet in patients with type 2 diabetes (Jönsson et al. 2013).

10.7.6.2 Intermittent Fasting

Intermittent fasting is a broad term that encompasses a variety of programs that manipulate the timing of eating occasions by utilizing short-term fasts in order to improve body composition and overall health. Intermittent fasting protocols can be grouped into alternate-day fasting, whole-day fasting, and time-restricted feeding (Tinsley and La Bounty 2015). This approach has been reported to promote weight loss, fat mass and be effective for cardio-protection (Varady et al. 2013; Eshghinia and Mohammadzadeh 2013; Tinsley and La Bounty 2015). However, these regimes seem linked to hunger, which can be a limiting factor for maintaining food restriction. In addition, the long-term effect of chronic food restriction in humans is not yet clear. Future studies should examine long-term effects with larger sample sizes before definitive conclusions can be reached.

10.7.6.3 Chrono-Nutrition/Timing of Meal

Together with portion size and enery density, the inclusion of satiety-enhancing foods into meals could be a beneficial strategy to help moderate energy intake for weight management (Forde et al. 2015). It is needed not only to find foods that enhance satiety but also to determine how best to incorporate these foods into diets to effectively reduce energy intake (Williams et al. 2014). In this way, temporal distribution of food intake play an important role on weight management. The interest for the timing of feeding and weight regulation is growing, emphasizing that the timing of food intake itself may have a significant role in obesity. These evidences are linked to the intime interplay between nutrition, metabolism, and the circadian clock (Asher and Sassone-Corsi 2015). Hence, the term "chrono-nutrition" refers to food administration in coordination with the body's daily rhythms. This concept reflects the basic idea that, in addition to the amount and content of food, the time of ingestion is also critical for the well-being of an organism (Asher and Sassone-Corsi 2015). In fact, several studies have evaluated the implications of time allocation for meals and timing of macronutrient consumption during the day (Alves et al. 2014; Sofer et al. 2015). In this regard, a longitudinal study showed that the timing of the main meal was predictive of weight loss during a 20-week dietary intervention and that this effect was independent from total 24-h caloric intake (Garaulet et al. 2013). The authors found that late lunch eaters lost less weight and displayed a slower weight-loss rate during the 20 weeks of

treatment than early eaters. Equally, it has been reported that avoidance of snacking between main meals can be included among the preventive approaches to reduce the risk of metabolic syndrome development, especially when snacks contain foods of poor nutritional quality (Pimenta et al. 2015). However, a well-planned dietary strategy characterised by a higher meal frequency, consisting of seven meals/d (including breakfast, lunch, dinner and two snacks in the morning and two snacks in the afternoon) was as effective or more than AHA dietary guideliness (3–5 meals/d) for metabolic syndrome management (de la Iglesia et al. 2014).

Although portion control is important for weight management, the message to 'eat less' of all foods may not be the best approach. A more effective strategy may be to encourage people to increase the proportion of foods low in energy density in their diets while limiting portions of high-energy-dense foods (Rolls 2014). In addition, a clearer understanding of the mechanisms underlying the portion size effect as well as satiety are critical steps toward developing more effective obesity interventions (English et al. 2015). Moreover, regular eating habits might facilitate weight balance, while unplanned snacking as well as consuming the major part of the energy intake at the end of the day seem to be unfavourable (Berg and Forslund 2015).

Overall, effective dietary interventions that promote long-term adherence and healthy beneficial effects on body composition and related metabolic markers are required. In general, nutritional interventions strategies need to be palatable and satiating promote loss of fat and preserve lean body mass, ensure long-term safety, be simple to administer and monitor and have widespread public health utility (Johnstone 2015).

10.8 Regulation of Adipose Tissue Functions by Dietary Factors

Many investigations during the last decades have focused on the study of the mechanisms underlying the beneficial effects of bioactive food on obesity and the MetS (Milagro et al. 2013). Thus, the anti-obesity effects of some dietary nutrients and non-nutrient factors have been related to its ability to reduce food intake (Becskei et al. 2009). Furthermore, metabolic key organs including adipose tissue, liver, intestine, and skeletal muscle have been also shown to be targets of nutrients and bioactive food components. For example, green tea, green tea catechins, and EGCG have demonstrated in cell culture and animal models of obesity to reduce lipogenesis, fat mass, body weight, fat absorption, plasma levels of triglycerides, free fatty acids, cholesterol, glucose, insulin, and leptin, as well as to increase beta-oxidation and thermogenesis (Wolfram et al. 2006; Huang et al. 2014).

Health effects of food compounds have been related mostly to specific interactions on molecular level, such as the regulation of gene expression by modulating the activity of transcription factors (Siriwardhana et al. 2013). In this context, several studies support that the ability of n-3 PUFA supplements in treating hypertri-

glyceridemia (Goldberg and Sabharwal 2008) could be associated to reduce lipogenic enzyme expression (Pérez-Echarri et al. 2009a), probably via down-regulation of sterol regulatory element binding protein 1c (Howell et al. 2009). In this section, we focus in reviewing the regulation of WAT metabolism and secretory function by some bioactive food components.

Inhibition of adipocyte differentiation represents a key strategy to reduce fat mass. In this context, it has been proposed that the omega-3 DHA may exert anti-obesity effects by inhibiting differentiation to adipocytes (Kim et al. 2006). Lipoic acid, a very important micronutrient with antioxidant and anti-obesity properties in rodents (Prieto-Hontoria et al. 2009; Shay et al. 2009) and probably in humans (Carbonelli et al. 2010; Koh et al. 2011; Huerta et al. 2015), also inhibits adipocyte differentiation by down-regulating pro-adipogenic transcription factors (Cho et al. 2003). The green tea polyphenol EGCG is also able to reduce adipocyte differentiation in 3T3-L1 adipocytes (Lin et al. 2005). Resveratrol also inhibits adipogenesis and induces apoptosis in 3T3-L1 adipocytes (Rayalam et al. 2008). Targeting apoptosis in adipose tissue has been also proposed as an approach for reducing adiposity. In this way, several studies have demonstrated the ability of different nutrients and bioactive food components to induce apoptosis in fat cells including DHA, CLA, and EGCG (Lin et al. 2005; Kim et al. 2006; Fischer-Posovszky et al. 2007).

Moreover, inhibition of WAT lipogenesis and stimulation of lipolysis have been hypothesized to underlie the anti-obesity actions of some dietary components. Thus, n-3 PUFAs have been proposed as modulators of lipogenic enzymes, such as stearoyl-CoA desaturase (Scd)-1 and fatty acid synthase (Fas) (Martínez-Fernández et al. 2015). Regarding lipolysis, Lee et al. (2008) suggested that EPA increases lipolysis through upregulation of the lipolytic gene expression in 3T3-L1 adipocytes. However, other studies have shown that EPA directly inhibits tumor necrosis factor-induced lipolysis (Price and Tisdale 1998; Lorente-Cebrián et al. 2012). Some authors also observed that trans −10, cis −12 CLA increases adipocyte lipolysis (Chung et al. 2005), while others suggest that the body fat-lowering effect of CLA is not due to this process (Simón et al. 2005). The body-fat lowering properties of resveratrol have been in part attributed to the inhibition of de novo lipogenesis and adipose tissue fatty acid uptake and, although resveratrol per se seems to be unable to induce lipolysis, it promotes β-adrenergic agonist-induced lipid mobilization (Aguirre et al. 2014). Recent studies have shown the ability of lipoic acid to inhibit lipogenesis by down-regulating key lipogenic enzymes, through the activation of AMPK signaling pathway in human subcutaneous adipocytes from overweight/obese subjects (Fernández-Galilea et al. 2014). Lipoic acid has also lipolytic properties, mainly mediated by phosphorylation of hormone sensitive lipase (HSL) through cAMP-mediated activation of protein kinase A (Fernández-Galilea et al. 2012), which could also account for the anti-obesity properties of this molecule.

Defective mitochondrial function of adipose tissue has been related with the progression of obesity and type 2 diabetes, and promotion of mitochondrial function and biogenesis has been proposed as a therapeutic strategy to overcome these metabolic disorders (Heinonen et al. 2015). Thus, n-3 PUFAs have been shown to upregulate mitocondrial biogenesis and induce fatty acid beta-oxidation in white fat in

mice, associated with a threefold stimulation of the expression of genes encoding regulatory factors for mitochondrial biogenesis and oxidative metabolism such as peroxisome proliferator activated receptor gamma coactivator 1-alpha and nuclear respiratory factor-1 (Flachs et al. 2005). Moreover, resveratrol can also induce adipose tissue mitochondrial biogénesis (Beaudoin et al. 2013). The combination of relatively low doses of lipoic acid and acetyl-l-carnitine can also improve mitochondrial function in 3T3-L1 murine adipocytes (Shen et al. 2008). Interestingly, a recent study have shown that lipoic acid is also able to increase mitochondrial biogenesis in adipocytes from overweight/obese subjects, upregulating Nrf1 and Tfam by SIRT1-induced deacetylation and, therefore, activation of PGC-1α (Fernández-Galilea et al. 2015).

Adenine monophosphate-activated protein kinase (AMPK) is an important regulator of energy metabolism. In WAT, AMPK activation inhibits fatty acid synthesis, whilst promotes free fatty acid oxidation (Hardie 2008). Interestingly, AMPK is also able to promote mitochondrial biogenesis by activating PGC-1α (Wan et al. 2014). Regarding the regulation of AMPK by nutrients, it has been recently demonstrated that EPA strongly stimulates AMPK phosphorylation in 3T3-L1 adipocytes (Lorente-Cebrián et al. 2009). Moreover, two additional trials have described the ability of n-3 PUFAs to activate AMPK in vivo (González-Périz et al. 2009; Kopecky et al. 2009). CLA also activates AMPK and reduces adiposity in mice adipocytes (Jiang et al. 2009). The metabolic effects of both resveratrol and lipoic acid in WAT have been also related to the stmulation of AMPK (Wang et al. 2015; Fernández-Galilea et al. 2015).

Development of brown-like/beige adipocytes within WAT has been proposed as an strategy to reduce obesity and related-metabolic disorders (Kajimura et al. 2015; Sidossis and Kajimura 2015). A study has suggested the ability of EPA to promote browning of inguinal fat adipocytes, via recruiting brite adipocytes (Zhao and Chen 2014). Furthermore, it has been proposed that lipoic acid can induce brown-like features in subcutaneous adipocytes from overweight/obese subjects, based on the findings that mitochondria from lipoic acid-treated adipocytes exhibited some morphological characteristics of brown mitochondria, in parallel with the up-regulation of some brown/beige adipocytes markers such as PRDM16, uncouplin protein 1 (UCP1), cell death-inducing DFFA-like effector a (Cidea) and T-box 1 (Tbx1) (Fernández-Galilea et al. 2015). Resveratrol is also able of inducing brown-like adipocyte formation in inguinal WAT via AMPKα1 activation and it has been stated that its beneficial antiobesity effects may be partly due to the browning of WAT (Wang et al. 2015).

Nutrients and dietary factors have also been demonstrated to regulate the production of bioactive adipokines (including leptin, adiponectin, and visfatin) that directly regulate body composition, energy metabolism, and insulin sensitivity (Moreno-Aliaga et al. 2010). Leptin is an adipokine involved in the regulation of food intake, energy expenditure, body fat storage, and insulin signaling (Marti et al. 1999). It has been shown that meals high in fructose caused lower leptin concentrations than meals containing the same amount of glucose (Teff et al. 2004). Moreover, several studies from different laboratories have evidenced the ability of dietary n-3 PUFA

to modulate leptin gene expression and secretion both in vitro and in vivo (Pérez-Matute et al. 2005, 2007a, b, c). Thus, in vitro studies with EPA showed the ability of this fatty acid to stimulate in a concentration-dependent manner leptin mRNA expression and secretion in 3T3-L1 cells (Murata et al. 2000) and in primary rat adipocytes (Pérez-Matute et al. 2005). In contrast, an inhibition of leptin secretion has been described after treatment of cultured adipocytes with arachidonic acid, linoleic acid, CLA (Pérez-Matute et al. 2003, 2007a, b) and lipoic acid (Prieto-Hontoria et al. 2011).

Circulating levels of adiponectin have been positively associated with whole-body insulin sensitivity (Yamauchi et al. 2001). Several trials have suggested that the insulin-sensitizing properties of dietary fish oils could be related to their ability to increase circulating adiponectin both in rodents (Flachs et al. 2006; Neschen et al. 2006; González-Périz et al. 2009) and humans (Itoh et al. 2007). In contrast, several studies have observed that CLA decreases adiponectin production in mice (Ohashi et al. 2004; Poirier et al. 2005), an effect opposite to what would be expected with a reduction in fat mass. In fact, a direct inhibitory effect of CLA on the ability of adipocytes to produce this adipokine has been reported (Pérez-Matute et al. 2007a, b, c). However, other researchers have described an increase, or no changes, in adiponectin after the supplementation of the diet with CLA, in rodents and humans, respectively (Noto et al. 2007; Norris et al. 2009). Other trials have described that the administration of green tea extract leads to a marked increase in the level of adiponectin and high-density lipoprotein-cholesterol, together with a significant reduction in low-density lipoprotein-cholesterol and triglyceride in obese women (Hsu et al. 2008). Also, resveratrol and lipoic acid have been shown to upregulate adiponectin levels in adipose tissue (Wang et al. 2011; Prieto-Hontoria et al. 2013)

Visfatin and apelin are two adipokines involved in the pathogenesis of obesity and related disorders (Beltowski 2006; Bertrand et al. 2015). Conflicting results have been described regarding the role played by visfatin in obesity, insulin resistance, and inflammation (Garten et al. 2015). Several studies have demonstrated the ability of dietary fatty acids to regulate the production of this adipokine. Thus, EPA has been shown to stimulate visfatin gene expression both in vitro (Lorente-Cebrián et al. 2009) and in vivo (Pérez-Echarri et al. 2009b). However, palmitate and oleate have been shown to down-regulate visfatin gene expression in 3T3-L1 adipocytes, which was mentioned as a potential mechanism to directly induce insulin resistance by oleate and palmitate in vitro (Wen et al. 2006). Concerning apelin, previous studies have shown that it can restore glucose tolerance in obese and insulin-resistant mice (Dray et al. 2008). It has been described that EPA upregulates apelin secretion and gene expression in 3T3-L1 adipocytes (Lorente-Cebrián et al. 2010). Moreover, dietary supplementation with EPA increased apelin gene expression, and a negative relationship between HOMA index with visceral apelin mRNA and serum apelin:total WAT ratio was observed in lean and overweight (cafeteria dietfed) rats (Pérez-Echarri et al. 2009b). Lipoic acid and trans-resveratrol-3-O-sulfate also stimulate apelin secretion or apelin mRNA, respectively in 3T3-L1 adipocytes (Fernández-Galilea et al. 2011; Eseberri et al. 2013).

Serum chemerin levels are elevated in obesity both in rodents and humans, and decreased after weight loss (Sell et al. 2010). Circulating chemerin concentrations are strongly associated with markers of inflammation and components of the metabolic syndrome (Ernst and Sinal 2010). A recent study has demonstrated that both lipoic acid and resveratrol are able to inhibit both basal and TNF-α-stimulated chemerin production in 3T3-L1 adipocytes. This inhibitory effect of lipoic acid on chemerin was also observed in cultured human adipocytes from overweight/obese subjects and after dietary supplementation with lipoic acid to lean and diet-induced obese rats (Prieto-Hontoria et al. 2016).

Low-grade inflammation has been identified as a key factor in the development of MetS features affecting obese subjects. In obesity, the expanding adipose tissue makes a substantial contribution to the development of obesity-linked inflammation via dysregulated secretion of proinflammatory cytokines, chemokines, and adipokines and the reduction of anti-inflammatory adipokines (Moreno-Aliaga et al. 2005a). Several studies have clearly demonstrated that dietary factors modulate the proinflammatory state linked to obesity. Treatment of obese subjects with n-3 PUFA in a clinical setting reduced circulating levels of both proinflammatory cytokines and acute phase proteins (White and Marette 2006). Moreover, n-3 PUFA have been shown to ameliorate inflammation within the adipose tissue of obese both rodents (Pérez-Matute et al. 2007c) and humans (Itariu et al. 2012; Huerta et al. 2016).

The beneficial actions of n-3 PUFA were initially believed to be mediated by a decrease in the production of classic inflammatory mediators such as arachidonic acid-derived eicosanoids and inflammatory cytokines (Martínez-Fernández et al. 2015). However, in recent years, n-3 PUFA have been demonstrated to serve as substrates for the conversion to a novel series of specialized proresolving lipid mediators designated resolvins, protectins and maresins, which have been proposed to mediate the protective and beneficial anti-inflammatory actions underlying the effects of n-3 PUFA (Serhan et al. 2002; González-Périz and Clària 2010; Spite et al. 2014). In this context, it has been identified that obesity is accompanied by impaired, local adipose tissue production of some proresolving lipid mediators, such as 17-HDHA and PD1, which represents one of the earliest alterations in diet-induced inflammation (Claria et al. 2012; Neuhofer et al. 2013). A study in *ob/ob* mice showed that increased intake of n-3 PUFA not only inhibited the formation of eicosanoids derived from the n-6 PUFA arachidonic acid, but also increased the generation of protective n-3 PUFA-derived lipid mediators (protectins and resolvins), which mimicked the insulin-sensitizing and antisteatotic effects exerted by n-3 PUFA (González-Périz et al. 2009). Interestingly, a trial in severely obese-nondiabetic patients has also shown that supplementation with highly purified n-3 PUFA significantly promoted the production of n-3 PUFA-derived specialized pro-resolving lipid mediators, including RvE1, 17-HDHA, PD1, and RvD1 in visceral adipose tissue in parallel with the decrease of adipose tissue and systemic inflammation (Itariu et al. 2012).

In summary, there is strong evidence that both diet-derived nutrients as well as non-nutritional factors can regulate both WAT metabolism and the secretion of key bioactive adipokines involved in the regulation of food intake, body composition, as well as glucose and lipid metabolism (Fig. 10.2).

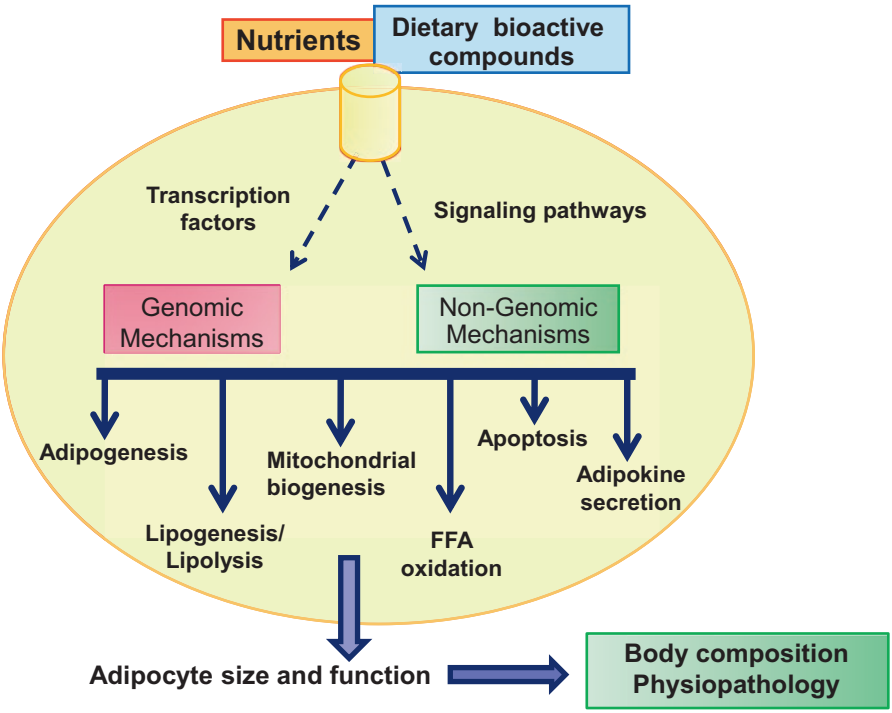

Fig. 10.2 Potential mechanism involved in the regulation of white adipose tissue (WAT) biology and function by nutrients and other dietary bioactive molecules

10.9 Clinical Implications and Future Directions for Research

Body weight and composition determinants are complex with multifactorial origin and interactions, which in many cases appear as a polygenic condition affected by diverse environmental factors. Nutrition is considered to have the most important lifelong environmental modifiable impact on human health. However, it is well known that dietary factors can differently affect depending on individual genetic background. In fact, not all individuals habitually eating a high-fat diet are obese; some have a similar BMI to low-fat consumers despite the consumption of substantially more fat and energy (Mercer 2001; Marrades et al. 2007). Moreover, individual genetic make-up has been shown to determine differential responses to weight loss interventions, and reliable predictors of successful slimming are poorly understood. Therefore, achieving effective weight and fat mass loss must take into account many environmental, behavioral, and genetic influences (Moreno-Aliaga et al. 2005b; Martinez et al. 2014b). During the last years, the development of

Nutritional Genomics, a science studying the relationship between human genome, nutrition, and health, is contributing to understand how diet and genomes interact. Nowadays, Nutritional Genomics has the challenge to answer the following different questions:

1. How an individual's genetic make-up predisposes for dietary susceptibility to obesity or influences the response to weight-loss interventions. In this context, Nutrigenetics has revealed insights into obesity susceptibility and can help differentiate responders from nonresponders in dietary interventions, but the predictive power of single-nucleotide polymorphisms in disease susceptibility genes has so far been limited in terms of helping to foresee a health trajectory (Kussmann et al. 2010; Marti et al. 2010; Goni et al. 2014; Ferguson et al. 2016).
2. How nutrition influences the expression of the genes, proteins, and metabolites. Thus, Nutrigenomics focuses in the study of the effect of nutrients on health through altering genome, proteome, metabolome, and the resulting changes in physiology. Therefore, Nutrigenomics builds on the developments of three omics disciplines transcriptomics, proteomics, and metabolomics (Corthésy-Theulaz et al. 2005; Bondia-Pons et al. 2015; Ferguson et al. 2016).
3. How epigenetic factors influences inter-individual differences in obesity susceptibility. Epigenetics studies the heritable changes in gene expression that do not involve changes to the underlying DNA sequence. These processes include DNA methylation, covalent histone modifications, chromatin folding, and, more recently described, the regulatory action of miRNAs and polycomb group complexes (Campión et al. 2009). Epigenetic mechanisms are established during prenatal and early postnatal development and function throughout life to maintain the diverse gene expression patterns of different cell types within complex organisms. Several studies have provided strong evidences that dietary factors during development can induce permanent alterations in epigenetic gene regulation, and epigenetic dysregulation can contribute to increased fat mass. At this level, the major tasks are: development of robust epigenetic biomarkers of weight loss and description of those more susceptible to be modified by dietary exposures, identification of the active/doses ingredients that alter the epigenome, interplay of other obesity-related factors on epigenetic regulation, determination of the period of life in which best results are obtained, and understanding the importance of the inheritance of these epigenetic marks (Milagro et al. 2013).
4. The integration of Nutrigenetics/Nutrigenomics and Epigenetics is a prerequisite for developing nutritional systems biology, which will constitute a powerful approach to unravel the complex interaction between food components and diet with cells, organs, and the whole body (Daniel et al. 2008; Martínez 2014). Finally, the major challenge will be in such translating research into dietary guidelines, leading to healthier foods and personalized nutrition (Fig. 10.3). Indeed, personalized nutrition will facilitate the prescription of customized dietary patterns to manage adipose tissue biology as well as to reduce excessive adiposity in obese subjects and maintain fat stores in lean individuals (van Ommen 2007; Abete et al. 2012). The role of the macronutrient content and dis-

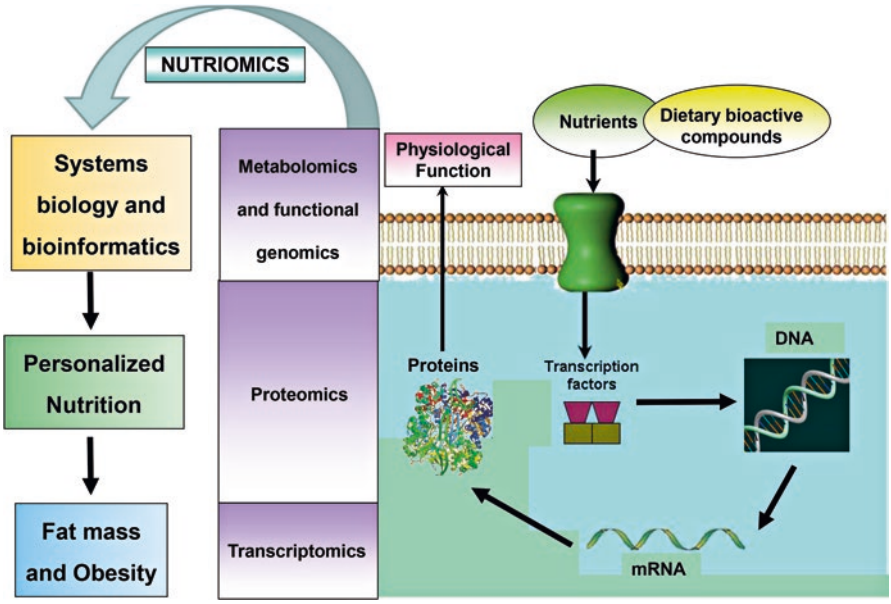

Fig. 10.3 Evolution of "Nutriomics" including epigenomics, transcriptomics, proteomics, and metabolomics will permit better understanding of how dietary factors affect both energy metabolism and fat mass, leading to healthier foods and personalized nutrition

tribution as well as some specific nutrients such as amino acids, fatty acids, fiber, and bioactive compounds should be further investigated in relation not only to fuel supply but also to an energy efficiency perspective, with emphasis on fat mass deposition, and body composition. Precise nutrition, taking into account not only the genetic make-up but also individual aspects such as dietary habits, lifestyles, dietary preferences and dislikes, epigenetic and perinatal nutrition, chronobiology and socioeconomic, is paving the way for a healthier advice for well-being life quality and longevity (San-Cristobal et al. 2013; Goni et al. 2015; Bahcall 2015; Ferguson et al. 2016).

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Chapter 11
The Genetic Determinants of Common Obesity-Susceptibility

Ruth J.F. Loos

Abstract Despite a relatively high heritability, the search for obesity-susceptibility genes has been challenging. While candidate gene studies and genome-wide linkage studies identified only a handful of genetic variants convincingly associated with obesity-related traits, the genome-wide association approach has truly revolutionised gene discovery for many common diseases and traits, including obesity. Over the past decade, large-scale genome-wide association studies for a range of adiposity traits, including BMI, WHR, extreme obesity, and body fat percentage, have identified at least 250 obesity-susceptibility loci, most of which had not previously been linked to body weight regulation. Although the combined contribution of these genetic loci to variation in obesity risk is small and their predictive value is low, the identified loci have shed new light on the complex physiology that governs the regulation of energy balance and fat distribution. Pathway and tissue enrichment analyses applied to associated loci have provided strong support for a role of the central neuronal system in overall obesity and of peripheral physiology in body fat distribution. The identification of new obesity genes could eventually lead to the discovery of drug targets for more effective preventive and therapeutic interventions. While the rapid progress in gene discovery has raised hopes towards the development of genetic risk profiles to guide individual weight management, the current evidence suggests that the available genetic data is not sufficient for such personalized implementations.

Keywords Obesity • Genetic epidemiology • Heritability • Candidate gene study • Genome-wide linkage study • Genome-wide association study • Translation • Genetic prediction

R.J.F. Loos (✉)
The Icahn School of Medicine at Mount Sinai, The Charles Bronfman Institute for Personalized Medicine, The Mindich Child Health and Development Institute, One Gustave L. Levy Place, Box 1003, New York, NY 10029, USA
e-mail: ruth.loos@mssm.edu

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11.1 Introduction

The prevalence of obesity and overweight continues to increase steadily worldwide, causing not only serious personal health problems but also imposing a substantial economic burden on societies (NCD Risk Factor Collaboration 2016). Between 1960 and 1980, 30% of adults in the U.S. were overweight and 10% were obese. Current estimates, however, show that the prevalence has more than doubled over the past three decades; i.e. almost 70% of adults in the U.S. were found to be overweight of who nearly half are obese (Flegal et al. 1998, 2012). Other Western countries have witnessed similar sharp increases following closely behind those reported for the U.S. population (World Obesity Federation 2016). Of concern is that obesity is no longer confined to Western societies and a substantial increase in its prevalence has been observed worldwide (NCD Risk Factor Collaboration 2016).

It has been well established that rapid globalization of the westernized lifestyle is fuelling this growing obesity epidemic. Yet, not everyone in the present-day obesogenic environment becomes obese and intensive efforts of overweight and obese people to loose weight have typically variable success. These observations suggest that lifestyle factors are not the only culprit in the recent obesity epidemic and highlight the multifactorial nature of the condition. Indeed, obesity arises through the joint actions of multiple genetic and environmental factors. More specifically, the obesogenic environment increases the risk of obesity, but more so in those who are genetically susceptible.

After providing evidence for a genetic contribution to obesity-susceptibility, this chapter reviews recent advances made in the field of common obesity genetics with a focus on the genetic loci that were established by large-scale candidate gene and genome-wide studies. The substantial progress made by genome-wide association studies (GWAS) in particular warrants specific attention. Therefore, we report on the latest discoveries, their impact for public health and clinical practice, their potential to unravel the underlying pathophysiology, and the ways ahead to find more obesity-susceptibility loci is being discussed.

11.2 Evidence for a Genetic Contribution to Obesity-Susceptibility

11.2.1 Evidence from Descriptive Epidemiological Studies

Descriptive epidemiological studies based on families and migrants provided the first evidence of a genetic contribution to obesity-susceptibility. Such studies rely on the relatedness between family members or between members of the same ethnic group to estimate the role of genes to a disease or trait. However, as members of the same family or of the same ethnic group not only share a genetic background but also a similar environment, inferences on the genetic contribution are only

suggestive; i.e. the influence of a genetic component can often not be distinguished from that of the shared environmental component.

Family studies calculate the familial risk, represented by the lambda coefficient (λ_R) or the standardized relative risk ratio, which compares the recurrence of a disease between family members (with various degrees of relatedness), with the risk of the disease in the general population. Estimates of λ_R based on BMI data from twin and family studies suggest that the risk of obesity is 1.5 to 5 times higher for an individual with a family history of obesity compared to the risk in the population at large (Allison et al. 1996; Lee et al. 1997; Ziegler et al. 1997; Katzmarzyk et al. 1999). This familial risk is higher when the degree of relatedness with the obese relative is greater; i.e. λ_R varies between 1.4 and 2.5 when an individual has an obese sibling, whereas λ_R ranges from 2.12 to 5.24 when the obese sibling was a monozygotic twin (Ziegler et al. 1997). Familial risk of obesity doubles if the related individual is extremely obese (BMI $\geq$ 45 kg/m^2) (Lee et al. 1997). Data from the Canada Fitness Survey showed that the increased familial risk of obesity was not only due to a shared genetic background, but also due to shared non-genetic factors as the risk of obesity was also increased, yet to a lesser extent, between (unrelated) spouses (Katzmarzyk et al. 1999).

In migrant studies, the disease risk of migrants is compared to that of the native-born population of the country to which they migrated and also to that of the population in their countries of origin. If the migrants' disease risk remains similar to that of the population in their country of origin, it suggests that a shared genetic background predominates potential environmental influences, while the opposite is inferred when the migrants' disease risk becomes similar to that of the native-born population of the country to which they migrated. In a paper that reviewed the health of migrants in the U.S., foreign-born individuals had a lower body mass and were less likely to be overweight or obese upon arrival than U.S.-born individuals (Cunningham et al. 2008). However, the foreign-born individuals tended to catch-up with U.S.-born individuals the more time they spent in the U.S. and after spending a decade in the U.S. the average BMI of foreign-born and U.S.-born individuals was the same (Cunningham et al. 2008). Furthermore, second-generation migrants were more often overweight and obese than their parents, who had move to the U.S. from Asia a generation earlier (Singh and Lin 2013). However, this difference between parents and adult offspring varied depending on their ancestral background (Singh and Lin 2013). Together, these studies suggest that (the American) lifestyle increases the risk of obesity, but that the effect of lifestyle depends on the genetic background. A classic example of how ethnic origin determines obesity-susceptibility is that of the Pima Indians. Pima Indians are American Indians living in central and southern Arizona (U.S.) and in Sonora (Mexico). The Pima Indians in Arizona live in the same 'obesogenic' environment as the white Americans of European descent, yet their prevalence of obesity is twice as high (69%) than that of white Americans (33%), suggesting that Pima Indians are more genetically susceptible to obesity (Knowler et al. 1991). Of interest is that Pima Indians living in the 'restrictive' environment of the remote Mexican Sierra Madre Mountains in Sonora have a much lower prevalence of obesity (13%) despite sharing the same genetic background as the Pima Indians in Arizona (Ravussin et al. 1994).

This observation suggests interaction between genetic susceptibility and lifestyle; i.e. Pima Indians have an increased susceptibility to obesity, but only when they live in an 'obesogenic' environment.

While descriptive epidemiological studies have been useful in providing suggestive evidence for a genetic contribution to obesity-susceptibility, they do not allow quantifying how much genes and environment explain of the variation in obesity risk, which is what heritability studies aim to do.

11.2.2 Evidence from Heritability Studies

Heritability studies have shown that genetic factors contribute typically between 40 and 70% to the inter-individual variation in common obesity (Maes et al. 1997; Elks et al. 2012a). But estimates as low as 5% and as high as 90% have been reported. This wide range in heritability estimates is in part due to study design, with twin studies (heritability = h^2 = 40%–90%) often reporting higher estimates than family (h^2 = 20–50%) or adoption (h^2 = 20–60%) studies (Maes et al. 1997; Elks et al. 2012a). Also the statistical 'modelling' of hypotheses is believed to contribute to the variation in heritability estimates; e.g. whether or not a 'shared environmental' contribution is presumed to be present, or whether interactions between genes and between genes and environments are assumed.

Furthermore, heritability estimates are population-specific, which could explain another part of the wide range in estimates reported. For example, the heritability of obesity estimated in a population with little variation in environmental factors (e.g. convent, prison, and during war-times) will likely be higher than for a population that has a large variety in lifestyles (e.g. present-day westernized countries). Longitudinal twin studies have suggested that the heritability of obesity-susceptibility increases throughout childhood and adolescence until the onset of adulthood, after which the genetic contribution decreases again (Korkeila et al. 1991; Haworth et al. 2008; Lajunen et al. 2009; Silventoinen et al. 2009; Elks et al. 2012a).

Taken together, the wide range suggests that heritability estimates for obesity-susceptibility should be interpreted with caution, accounting for the population for which the estimation was made and for the study design that was used. Nevertheless, as most reported estimates tend to lie within the 40–70% range, a search for obesity-susceptibility genes seems warranted.

11.3 Approaches to Identify Obesity-Susceptibility Genes

Scientists have been searching for obesity-susceptibility genes since the mid-1990s. Early success in the field was largely confined to monogenic obesity, which is typically severe and has often an early-onset. Several mutations that segregate in families or occur de novo have been found to cause major disruptions in the function of genes in which they are located. These genes often encode ligands and receptors

implicated in the leptin-melanocortin pathways that are critical in the regulation of body weight through controlling energy sensing, food intake and appetite (O'Rahilly 2009; van der Klaauw and Farooqi 2015).

While the study of monogenic obesity has already led to valuable insights into biological pathways that lead to weight gain, the mutations are rare, affecting only a fraction of the population. The search for (common) genetic variation that contributes to common forms of obesity, ubiquitous in the general population, has proven to be more challenging. The fact that common obesity is a multifactorial condition with no simple pattern of (Mendelian) inheritance, caused by many genetics variants that each have only a small effect, that interact with each other and with environmental factors, will no doubt have contributed to the limited success of many gene discovery efforts.

In their search for common obesity-susceptibility loci, genetic epidemiologists have applied two main approaches; i.e. the hypothesis-driven approach by using candidate gene studies, and the hypothesis-generating approach by using genome-wide screening studies (Box 11.1). The developments in the field have been largely technology-driven; i.e. progress in genotyping technology has not only facilitated the development of catalogues with detailed insights in human genetic variation (such as the Human Genome Project (International Human Genome Sequencing Consortium 2004), The International HapMap (The International HapMap Consortium 2007), The 1000 Genomes (Genomes Project Consortium et al. 2015; Sudmant et al. 2015) and the Haplotype Reference Consortium (The Haplotype Reference Consortium 2016)), but they have also increased the speed, amount and resolution with which samples can be genotyped. These technological developments have increased the pace of discoveries over time, particularly since the advent of genome-wide association studies, which has led to the identification of many loci robustly associated with common diseases and traits, including obesity (Hindorff et al. 2014). Here, the contribution of the main gene-discovery approaches to the field of common obesity is being reviewed.

Box 11.1: Genetic Epidemiological Approaches to Identify Genes

Genetic epidemiologists have relied mainly on candidate gene and genome-wide screening approaches to identify genetic variants associated with (common) diseases or traits in the general population.

The Candidate Gene Approach

The candidate gene approach is a *hypothesis-driven* approach and relies on the current understanding of the biology and pathophysiology that underlies the susceptibility to obesity. Genes for which there is evidence for a role in the regulation of the energy balance in animal models or in extreme/monogenic forms of obesity are tested for association with obesity-related traits at the population level. Candidate gene studies have been performed since the early 1990s; i.e. as soon as technology allowed genotyping at a population level.

The Genome-Wide Screening Approach

The genome-wide screening approach is a *hypothesis-generating* method that, through screening genetic variation across the whole genome, aims to identify new, unanticipated genetic variants associated with a disease or trait of interest. As this approach is not constraint by the boundaries of an a priori hypothesis, it is expected that the newly identified genetic loci were not previously presumed to be implicated in the disease or trait, and therefore, will provide insights into new pathways and biology that underlie obesity-susceptibility. The genome-wide screening approach has been implemented in linkage and association studies.

Genome-wide linkage studies—Genome-wide linkage studies rely on the relatedness of study participants and test whether certain chromosomal regions co-segregate with a disease or trait across generations. A genome-wide linkage scan requires 400–600 highly polymorphic markers, genotyped at 10-cM intervals. The linkage method relies on the recombination between parental chromosomes during meiosis and the subsequent transmission of these 'recombined' chromosomes to the offspring. As there is a 'natural' limitation to the number of chromosomal crossovers that occur between parental genomes during meiosis, the resolution of genome-wide linkage scans is typically low and increasing the number of markers to more than 600 will not improve the resolution. Because of the rather low resolution, genome-wide linkage studies will identify broad intervals that harbour many genes. Therefore, a linkage 'peak' will often require follow-up genotyping to fine-map the region and to pinpoint the gene(s) that underlie(s) the linkage signal. The genome-wide linkage approach has been available since the mid-1990s, thanks to progress in genotyping technology and publicly available databases that catalogue the highly polymorphic markers. While this approach has been effective in identifying genetic loci for rare diseases, with a simple (Mendelian) pattern of inheritance and a strong (mono-)genetic influence, it has been less successful in identifying genetic loci for common multifactorial diseases and traits.

Genome-wide association studies—Genome-wide association studies screen the whole genome at much higher resolution than genome-wide linkage studies and are thus able to better narrow-down the associated locus. Genome-wide association does not rely on familial relatedness and can therefore achieve larger sample sizes than typical family-based studies. A key feature of genome-wide association studies is the robust study design; i.e. they consist of a discovery stage, which is the actual genome-wide association, and a follow-up stage. SNPs that show significant association in the discovery stage are taken forward to the follow-up stage to confirm (or refute) the association

observed in the discovery stage. Associations are considered significant if *P*-values reach a significance threshold of $<5 \times 10^{-8}$. Genome-wide association studies typically examine the association of a trait or disease with ~2.5 million SNPs across the genome. Although its resolution is much higher than that of genome-wide linkage studies, the identification of the 'causal' gene or variant often remains a major challenge. A catalogue of loci identified at the genome-wide significance level can be found at www.genome.gov/GWAS studies. Substantial advances in high-throughput genotyping technology and a detailed knowledge of the human genetic architecture have enabled genome-wide association studies that have been available since 2005.

11.4 Candidate Gene Studies

Candidate gene studies are hypothesis-driven and rely on the current understanding of the biology that underlies obesity-susceptibility (Box 11.1). In the past two decades, hundreds of genes have been proposed to be candidate genes for obesity and obesity-related traits (Rankinen et al. 2006). Their candidacy is based on their role in the regulation of energy homeostasis observed in animal studies or because mutations in the respective genes lead to extreme and early-onset obesity in humans. At the start of candidate gene studies, in the mid-1990s, genotyping was expensive and laborious and information on the genetic architecture of the human genome was rather scarce, such that only one or a few genetic variants in a particular candidate gene were tested. Over time, candidate genes studies became more comprehensive as decreasing genotyping costs and availability of catalogues of genetic variation allowed a more systematic examination all common variation in the genes of interest.

The most commonly examined genetic variants tested for association in candidate gene studies are single nucleotide polymorphisms (SNPs). SNPs are the most basic and abundant type of genetic variation and are evenly spread throughout the human genome. Recent results of the 1000 Genomes projects estimated that there are at least 85 million SNPs across populations (Genomes Project Consortium et al. 2015). SNPs are bi-allelic—one copy is inherited from each parent—such that an individual can be homozygous for the major allele (e.g. A/A), heterozygous (A/a) or homozygous for the minor allele (a/a). A candidate gene study examines whether either of the two alleles is associated with an increased risk of obesity (dichotomous) or with higher levels of an obesity-related trait (continuous; e.g. BMI) (Fig. 11.1).

Despite the large number of candidate gene studies, most of which had valid hypotheses for a given gene to be implicated in obesity-susceptibility, few candidate genes have been shown to be consistently associated with common obesity or related traits in the general population. The main reasons for the limited success of

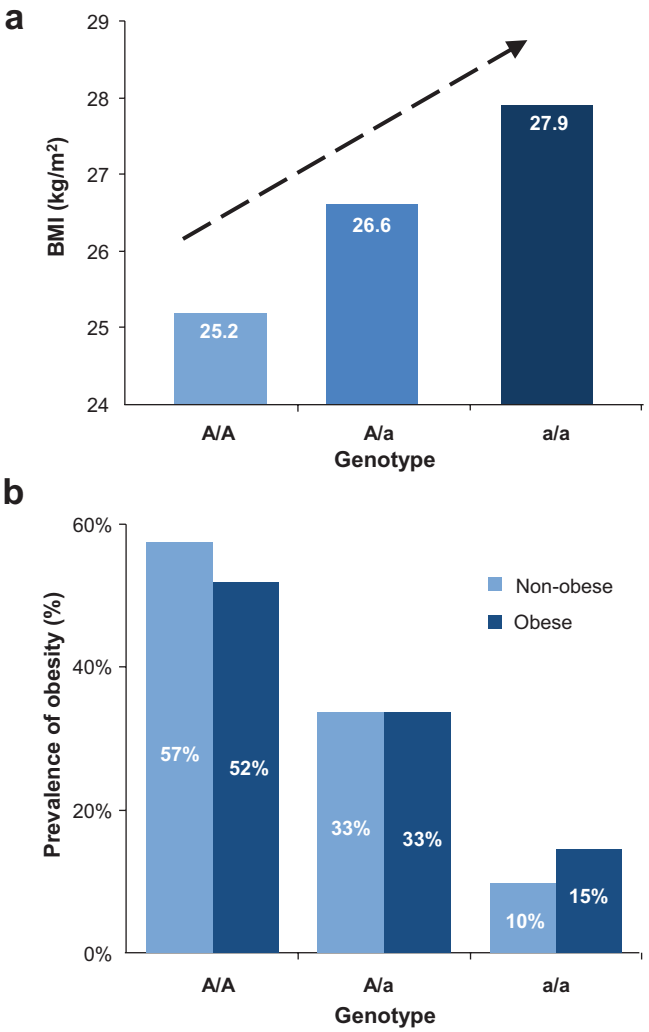

Fig. 11.1 Example of association between a bi-allelic SNP (A/a) and BMI (panel **a**) or obesity risk (panel **b**), assuming an additive effect of the a-allele. This example shows that each additional a-allele increases BMI and risk of obesity

the candidate gene approach are that (1) sample sizes studied are often too small (n<1000) and thus insufficiently powered to identify the small to modest effects that are expected for common obesity, (2) the genetic variation of the gene of interest was not surveyed comprehensively, and (3) the candidacy was based on limited biological insights.

In more recent years, however, an increasing number of candidate gene studies have tested for associations in larger populations (n>5000) and increasingly more

meta-analyses of all available published (and unpublished) data have been performed. Such large-scale studies have greater statistical power, needed to confirm or refute associations with confidence.

Here, we summarize results of large-scale (n>5000) association studies and meta-analyses that have shown robust results for genetic variants in candidate genes, often later confirmed in even large genome-wide association studies.

The melanocortin 4 receptor (MC4R)—*MC4R* has a strong biological candidacy. MC4R is predominantly expressed in the brain and plays a key role in the regulation of food intake and energy homeostasis (Fan et al. 1997; Huszar et al. 1997). Up to 6% of individuals with severe, early-onset obesity are believed to carry pathogenic mutations in *MC4R*, making *MC4R* deficiency the commonest form of monogenic obesity (Vaisse et al. 2000; Farooqi et al. 2003). Patients with *MC4R* deficiency exhibit hyperphagia, increased fat and lean mass, greater bone mineral density, and accelerated linear growth (Farooqi et al. 2003).

While the role of *MC4R* mutations in the development of extreme and early-onset obesity has been well-established for several years, convincing evidence that also common genetic variation in *MC4R* contributes to common obesity-susceptibility has only recently started to emerge (Loos 2011). The two most common *MC4R* variants, V103I (rs2229616) and I251L (rs5282087), each result in a non-synonymous change with potential functional implications (Xiang et al. 2006). Numerous, typically small, studies examined these two *MC4R* variants, but none found significant association with obesity-related traits, apart from one sizeable population-based study that observed a significant protective effect of the 103I–allele (frequency: 2–3% of the population) on obesity risk (Heid et al. 2005). Since 2004, four consecutive meta-analyses, each including a growing number of association studies, confirmed that 103I–allele carriers have a 20% lower risk of obesity than V103 V homozygotes (Geller et al. 2004; Stutzmann et al. 2007; Young et al. 2007; Wang et al. 2010). In addition, a meta-analysis of data on the I251L *MC4R* variant provided strong evidence for a protective effect with a nearly 50% reduced risk of obesity for carriers of the 251 L-allele (frequency: 1–2%) (Stutzmann et al. 2007). It should be noted that both meta-analyses are based mainly (for V103I) or exclusively (for I251L) on data from case-control studies for (extreme) obesity, which may result in effect sizes that are somewhat inflated than if data had been obtained from population-based cohorts. Large-scale GWAS later confirmed the association between the V103I variants and BMI (Speliotes et al. 2010; Locke et al. 2015).

β-adrenergic receptor 3 (ADRB3)—*ADRB3* is an obvious candidate gene for obesity as it is part of the adrenergic system, which is known to play a key role in energy metabolism. ADRB3 is primarily expressed in adipose tissue where it is involved the regulation of lipolysis and thermogenesis through activation of the sympathetic nervous system (Lafontan and Berlan 1993; Enocksson et al. 1995). So far, no mutations in *ADRB3* have been reported to be associated with monogenic obesity. However, in 1995, a common variant that leads to the replacement of tryptophan by arginine (Trp64Arg, rs4994) in the receptor protein was identified through restriction enzyme and sequence analyses (Walston et al. 1995). This variant was found to

be associated with the onset of type 2 diabetes, insulin resistance and weight gain in Pima Indian (Walston et al. 1995), French (Clement et al. 1995) and Finish populations (Widen et al. 1995). Following these first reports in 1995, more than 100 studies have been published on the association between the Trp64Arg variant and obesity-related traits, but results have been inconsistent. The first three consecutive meta-analyses on the association with BMI (all N < 10,000), were inconclusive. However, the fourth and most recent meta-analysis was more than four times larger than any of the three previous meta-analyses, including data of 44,833 individuals from 97 populations (Kurokawa et al. 2008). Significant association between the Trp64Arg variant and BMI was observed in East Asians only, with Arg64-allele carriers having a 0.31 kg/m^2 higher BMI compared to the Trp64Trp homozygotes, whereas no associations were observed in Caucasians (Kurokawa et al. 2008). Of interest is that the Arg64-allele is also more frequent in East Asians (frequency: ~18%) than in Europeans (~7.5%). The functional effect of the Trp64Arg polymorphism on the expression and activity of *ADRB3* remains unclear. In vitro experiments in rodent and human cell lines found that the Arg64 variant reduces the ability to stimulate adenyl cyclase activity compared with the Trp64 variant (Pietri-Rouxel et al. 1997; Kimura et al. 2000). Furthermore, lipolysis in human adipocytes was lower in cells with the Arg64-variant compared with cells with the Trp64 variant (Umekawa et al. 1999). However, others did not observe in vitro functional effects of the Arg64Trp variant (Urhammer et al. 2000).

Prohormone convertase 1/3 (PCSK1)—The *PCSK1* gene is another strong candidate for obesity as it encodes an enzyme, expressed in neuroendocrine cells, that converts pro-hormones into functional key hormones that are involved in the regulation of central and peripheral energy metabolism. Mutations in *PCSK1* lead to a PC1/3 deficiency, resulting in a syndrome characterised by extreme childhood obesity (Jackson et al. 1997, 2003; Farooqi et al. 2007; Bandsma et al. 2013; Frank et al. 2013; Martin et al. 2013; Pickett et al. 2013; Philippe et al. 2015). A large-scale study provided evidence that also common variants in *PCSK1* might be associated with risk of obesity (Benzinou et al. 2008). After sequencing *PCSK1* coding regions in a small sample of obese individuals, nine variants that captured the common genetic variation in *PCSK1* were genotyped in 13,659 individuals of European ancestry. Two non-synonymous variants, Asn221Asp (rs6232) and the Gln665Glu-Ser690Thr pair (tagged by rs6235), were consistently associated with obesity in adults and children. Each additional minor allele (frequency: 4–7%) of the Asn221Asp variant increased the risk of obesity by 1.34-fold, while each additional minor allele (frequency: 25–30%) of the Gln665Glu-Ser690Thr pair increased the risk by 1.22-fold. Two recent large-scale meta-analyses (N>200,000) convincingly confirmed association for both variants with BMI and obesity risk (all P < 10^{-5}) (Stijnen et al. 2014; Nead et al. 2015). However, effect sizes tended to be smaller than in the original study, which may be due to study design (case-control and family-based cohorts) and/or the so-called winner's curse. Specifically, the Asn221Asp variant was associated with an 1.15 to 1.19-fold increased risk of obesity, and for the Gln665Glu-Ser690Thr pair obesity risk was increased by 1.09 to 1.08-fold (Stijnen et al. 2014; Nead et al. 2015). Functional characterization of these

two variants suggested a modest deleterious effect of the Asn221Asp variant, but no functional role for the Gln665Glu-Ser690Thr was observed (Benzinou et al. 2008).

Brain-derived neurotrophic factor (BDNF)—BDNF, a nerve growth factor that signals via the tyrosine kinase receptor tropomycin-related kinase B (TrkB), is believed to act primarily in the hypothalamus, downstream of the leptin–proopiomelanocortin signaling pathway (Xu et al. 2003; Unger et al. 2007; Wang et al. 2007). *BDNF* has been mainly studied for its presumed role in the regulation of development, stress response, survival, and mood disorders. However, evidence for a role of *BDNF* in the regulation of energy homeostasis comes from animal studies as well as from a case reports. *BDNF* mutant mice show a reduced expression of the gene in the hypothalamus, they are hyperphagic, obese and hyperactive (Kernie et al. 2000; Rios et al. 2001; Fox and Byerly 2004). While no mutations in humans have been described, a de novo chromosomal inversion at chr11p, a region encompassing *BDNF*, was detected in an 8-y-old girl who was hyperphagic, severely obese and hyperactive (Gray et al. 2006). Furthermore, in patients with the WAGR syndrome, those with *BDNF* haploinsufficiency had a higher BMI and all had developed obesity in childhood (Han et al. 2008). The *BDNF* polymorphism most commonly studied in association studies is the non-synonymous Val66Met (rs6265) variant (Met66-allele frequency: ~20%). The replacement of valine by methionine at codon 66 appears to result in an impaired intracellular trafficking and reduced activity-dependent secretion of BDNF in hippocampal neurons (Chen et al. 2004), poorer episodic memory and abnormal hippocampal activation using functional magnetic resonance imaging (Egan et al. 2003). While several small studies found no evidence of association, a large-scale study, including 10,109 women, reported that Met66Met homozygotes had a significantly lower BMI (-0.76 kg/m^2) than Val66-allele carriers (Shugart et al. 2009). Large-scale GWAS have reported highly significant associations between variants in the locus that harbours the Val66Met and BMI (Speliotes et al. 2010; Locke et al. 2015).

While such large-scale candidate gene studies have sufficient power to identify small effects, they are also powered to refute associations. For example, none of four studies with each more than 5000 participants found association between the ENPP1 Lys121Gln variant and obesity-related traits (Meyre et al. 2005; Grarup et al. 2006; Lyon et al. 2006; Weedon et al. 2006). Also for other genes, despite their sometimes strong biological candidacy, there was sufficient data to refute association with obesity-related traits; for example, no associations were observed for the Ala54Thr variant in *fatty acid binding protein 2* (*FABP2*) (Zhao et al. 2011), seven variants in the *grehlin receptor* (*GHSR*) (Gjesing et al. 2010), the −174G>C variant near *interleukin 6* (*IL6*) (Qi et al. 2007; Huth et al. 2009), and the −759C/T variant near the *serotonin 5-HT-2C receptor* (*HTR2C*) (Vimaleswaran et al. 2010). It should be noted that the strong evidence for absence of association pertains to the specific variants tested and does not rule out association for variants elsewhere in or near the candidate gene. Furthermore, the fact that many other proposed candidate genes have not been confirmed does not mean that they do not affect obesity risk. Larger studies that interrogate all functional variants in and near these candidate genes will be needed to convincingly confirm or refute their role in body weight regulation.

Taken together, despite 20 years of candidate gene efforts, this approach has had only limited success. Large-scale studies and meta-analyses have identified common variants, mostly non-synonymous and/or functional, in at least four candidate genes (*MC4R*, *ADRB3*, *PCSK1*, *BDNF*) to be robustly associated with obesity-related traits. It is reassuring that also GWAS (see below) show strong evidence of association with BMI for at least three of those candidate genes i.e. for *MC4R*, *PCSK1* and *BDNF* (Locke et al. 2015).

11.5 Genome-Wide Linkage Studies

The genome-wide linkage approach is a hypothesis-generating method that aims to identify new, unanticipated genetic loci that co-segregate with a disease or trait of interest across generations (Box 11.1). Genome-wide linkage studies screen the genome with a rather low resolution and typically identify broad intervals that require follow-up genotyping to pinpoint the causal genes that underlie the linkage signal.

Since the first genome-wide linkage study on body fat percentage in Pima Indians in 1997 (Norman et al. 1997), the number of chromosomal loci linked to obesity-related traits has grown exponentially. The last Human Obesity Gene map update summarised the literature up to 2005 and reported 253 loci from 61 genome-wide linkage scans, of which 15 loci have been replicated in at least three studies (Rankinen et al. 2006). Most of these replicated loci cover a large genomic region that harbours many genes and, so far, none of these loci have been narrowed down sufficiently to pinpoint the genes or variants that underlie the linkage signal. Furthermore, a meta-analysis of 37 genome-wide linkage studies with data on more than 31,000 individuals from 10,000 families of European origin could not locate a single obesity or BMI locus with convincing evidence, despite sufficient power to identify loci with even small effects (Saunders et al. 2007). This meta-analysis suggests that genome-wide linkage is not an effective approach for identifying genetic variants for common obesity.

11.6 Genome-Wide Association Studies

Similar to genome-wide linkage, genome-wide association is a hypothesis-generating approach that aims to identify new unanticipated loci for a disease or trait of interest (Box 11.1).

The genome-wide association approach has been enabled through major advances in high-throughput genotyping technology. These technological developments first facilitated the rapid expansion of our understanding of the human genetic architecture, which is being catalogued in publicly available ‘maps’ (such as the Human Genome Project, the International HapMap, and the 1000 Genomes Project).

Subsequently, these maps, in concert with the high-throughput genotyping technology, have provided the methodological basis for the production of smartly designed arrays that allow the genotyping of more than one million genetic variants in a single experiment. This change in genotyping capacity has dramatically increased the pace of discoveries for many common diseases and traits. Currently, GWAS have identified more than 14,000 SNPs for over 5000 diseases and traits (Welter et al. 2014), including at least 300 loci for obesity-related traits. The main reasons for its success are threefold; (1) the whole genome is being surveyed at a high resolution, (2) association can be tested using unrelated individuals, who are easier to recruit than related individuals, such that sample sizes can be large and statistical power high, and (3) the study design of GWAS is robust.

11.6.1 The Genome-Wide Association Study Design

GWAS typically consist of two stages; a discovery stage and a replication stage.

The discovery stage—The discovery stage comprises the actual genome-wide association analysis. Hundreds of thousands of genetic variants, typically SNPs, are being genotyped across the genome using high-density genotyping arrays. Genotyping can be done in cohorts to test for association with a continuous trait (e.g. BMI, waist-to-hip ratio) or in cases and controls to test for association with risk of a disease (e.g. obesity). Each SNP is subsequently tested for association with the trait or disease of interest in a similar way as association is tested in candidate gene studies (Fig. 11.1).

Studies with large sample sizes at the discovery stage tend to be more successful, in particular for common traits with moderate heritability, as they have greater statistical power to detect associations of SNPs with small effect sizes. The need for large sample sizes has led to the formation of international consortia that involve a growing number of studies of whom scientists have agreed to pool data to guarantee continued gene discovery. Some prominent consortia in the context of obesity related traits include the GIANT (Genetic Investigation for Anthropometric Traits, (The GIANT Consortium 2016)) Consortium, the AAAGC (African Ancestry Anthropometric Genetics Consortium), the AGEN (Asian Genetic Epidemiology Network, (AGEN 2016)), and the EGG (Early Growth Genetics, (The EGG Consortium 2013)) Consortium. These consortia use genome-wide meta-analyses to combine summary statistics of a series of individual GWAS into one analysis. Because individual studies may have used different genotyping arrays, meta-analyses using genotyped data only would be limited to the subset of SNPs that is common to all arrays. Therefore, to make more efficient use of the available data, a statistical method called *imputation* is being applied to the genotyped data. In brief, based on the observed haplotype structure of the genotyped SNPs (i.e. correlation between SNPs) in a study and based on that of a reference panel (e.g. HapMap,

1000 Genomes, Haplotype Reference Consortium), genotypes of >35 million of untyped SNPs are inferred for each of the individuals of the separate studies. Imputation of genotypes is now routinely done using one of the publicly available imputation software programs, such as IMPUTE (Howie et al. 2011), or MACH (Li et al. 2010b). As such, all studies in a consortium will have genotype data available on the same SNPs across the whole genome. Each study subsequently performs a genome-wide association analysis using the genotyped and imputed SNPs. Next, the summary statistics for the association of each SNP, of each of the participating genome-wide association studies, are meta-analysed to calculate the overall significance of the associations. Results of such a genome-wide association (meta-)analysis are presented in a Manhattan plot, which shows the *P*-values of the associations for the millions of SNPs according to their position in the genome (Fig. 11.2).

Given that millions of tests are performed in a genome-wide association study, the chance of false positive findings is very high. To account for multiple testing, the nominal *P*-value to consider an association as significant is very stringent. A *P*-value of $<5\text{x}10^{-8}$, which corresponds to a 5% genome-wide type I error rate, has been recommended as the minimum significance threshold to be reached after validation of the association in the replication stage (Bakker et al. 2008). Therefore, SNPs for which association *P*-values reach $<10^{-7}$ or $<10^{-6}$ at the discovery stage are taken forward to the next stage with the expectation that, if the SNP is a true 'hit', the association will reach a *P*-value of $<5 \times 10^{-8}$ at the replication stage. It should be

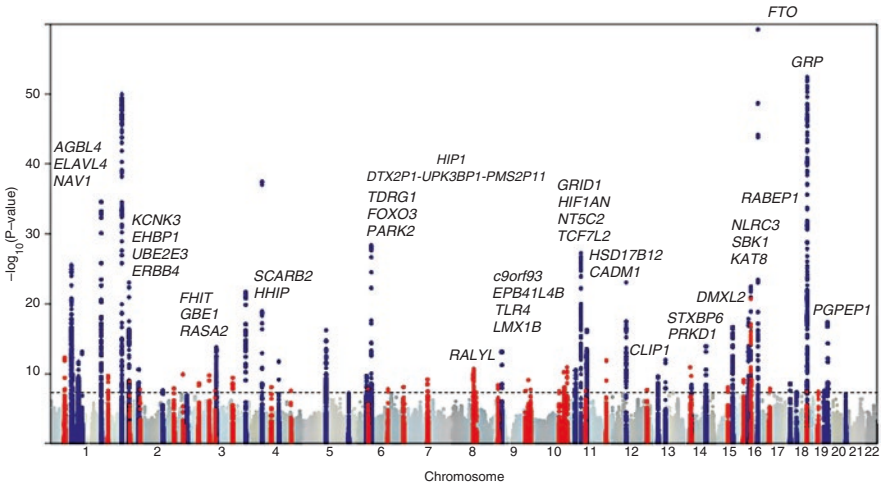

Fig. 11.2 Manhattan plot of the association between genome-wide data and BMI in the meta-analysis of the GIANT consortium. The –log10 P-values for the association of each single nucleotide polymorphism with BMI are shown on the y-axis, which was truncated at $P<10^{-60}$. The SNPs are plotted on the x-axis according to their chromosomal location. The SNPs that had previously been shown to associate with BMI are shown in *blue*, the newly identified SNPs are shown in *red*. Adapted from Locke et al. (2015), with permission from Nature Publishing Group

noted that associations for SNPs in the same locus often show similar significance levels because they are in high linkage disequilibrium (i.e. highly correlated). All SNPs that are part of such a cluster represent the same association signal, such that typically only one of the SNPs is taken forward for replication.

The replication stage—The SNPs that were taken forward from the discovery stage are tested for association in replication samples, which is a new series of samples that have the same study design as that used in the discovery stage. Ideally the replication sample size is at least as large as the sample used at the discovery stage to provide the replication sample with sufficient statistical power to identify the effects observed in the discovery stage. Eventually, the results of the discovery and replication stage are meta-analysed. SNPs for which the *P*-values reach the critical threshold of $<5 \times 10^{-8}$ are considered 'confirmed loci' ('hits'), whereas the other SNPs, for which the association becomes less significant after replication, were likely false positive findings at the discovery stage.

While it has been recommended to use a nominal P-value threshold of $<5.0 \times 10^{-8}$, which corresponds to a 5% genome-wide type I error rate, more liberal thresholds have been used by early genome-wide association studies.

Loci for which association is confirmed at the replication stage are then followed up for more in-depth analyses; e.g. to assess their effects on related traits, to fine-map the locus to identify the causal variant or gene, to examine their functional implications and identify the biological mechanisms that underlie the phenotype or disease of interest, e.g. body weight regulation, and fat distribution.

As the size of the discovery stage has increased to extreme scales ($N > 250{,}000$), finding appropriately sized replication samples has become challenging. As such, the latest GWAS do not have a replication stage, but instead more effort is put into follow-up analyses to validate the observed association.

11.6.2 The Discovery of More That 300 Obesity-Susceptibility Loci

Since the introduction of the genome-wide association approach in 2005, more than 300 genetic loci have been identified that are unequivocally associated with obesity-related traits (Table 11.1, Fig. 11.3). Large-scale high-density GWAS and meta-analyses have been performed for common outcomes, such as BMI, waist-to-hip ratio (WHR), extreme and early-onset obesity, childhood obesity, but also less commonly studies outcomes such as body fat percentage, visceral and subcutaneous adipose tissue, circulating leptin levels, and even weight loss after bariatric surgery (Table 11.1). These have been performed predominantly in adults of white European ancestry. However, the past few years has seen a growing number of GWAS in populations of Asian and African origin, as well as in children and adolescents. Here, the latest discoveries are reviewed by phenotype outcomes.

Table 11.1 Overview of all large-scale genome-wide association studies performed so far, sorted by obesity outcomes and year of publication

Study	PMID	Pub year	Outcome	Ancestry	Sample size discovery (N_D) and replication (N_R)
Frayling et al. (2007)	17,434,869	2007	BMI	European	N_D = 4862; N_R = 38,759
Scuteri et al. (2007)	17,658,951	2007	BMI	European	N_D = 4741; N_R = 2893
Chambers et al. (2008)	18,454,146	2008	BMI	European	N_D = 2684; N_R = 11,955
Loos et al. (2008)	18,454,148	2008	BMI	European	N_D = 16,876; N_R = 60,352
Thorleifsson et al. (2009)	19,079,260	2009	BMI	European	N_D = 31,392; N_R = 5586
Willer et al. (2009)	19,079,261	2009	BMI	European	N_D = 32,387; N_R = 45,018
Speliotes et al. (2010)	20,935,630	2010	BMI	European	N_D = 123,865; N_R = 125,931
Wen et al. (2012)	22,344,219	2012	BMI	East Asian	N_D = 27,715; N_R = 55,333
Okada et al. (2012)	22,344,221	2012	BMI	East Asian	N_D = 26,620; N_R = 35,625
Berndt et al. (2013)	23,563,607	2013	BMI tails, obesity classes	European	N_D = 158,864; N_R = 109,703
Monda et al. (2013)	23,583,978	2013	BMI	African	N_D = 39,144; N_R = 32,268
Wen et al. (2014)	24,861,553	2014	BMI	East Asian	N_D = 86,757; N_R = 47,352
Locke et al. (2015)	25,673,413	2015	BMI	All ancestry	N_T = 339,224
Winkler et al. (2015)	26,426,971	2015	BMI (interaction with age and sex)	European (predominantly)	N_T = 320,485
Bradfield et al. (2012)	22,484,627	2012	Childhood obesity	European	N_D = 13,848; N_R = 6901
Felix et al. (2016)	26,604,143	2016	Childhood BMI	European	N_D = 35,668; N_R = 11,873
Hinney et al. (2007)	18,159,244	2007	Extreme and early onset obesity	European	N_D = 931; N_R = 644 families
Meyre et al. (2009)	19,151,714	2009	Extreme and early onset obesity	European	N_D = 2696; N_R = 14,186
Scherag et al. (2010)	20,421,936	2010	Extreme and early onset obesity	European	N_D = 2258; N_R = 3141
Wheeler et al. (2013)	23,563,609	2013	Extreme and early onset obesity	European	N_D = 6889; N_R = 2961
Lindgren et al. (2009)	19,557,161	2009	Waist circumference	European	N_D = 38,580; N_R = 70,689
Heard-Costa et al. (2009)	19,557,197	2009	Waist circumference	European	N_D = 31,373; N_R = 38,641
Heid et al. (2010)	20,935,629	2010	WHR_{adjBMI}	European	N_D = 77,167; N_R = 113,636

Berndt et al. (2013)	23,563,607	2013	WHR tails	European	N_D = 100,605; N_R = 75,220
Liu et al. (2013)	23,966,867	2015	WHR_{adjBMI}	African	N_D = 23,564; N_R = 10,174
Shungin et al. (2015)	25,673,412	2015	WHR_{adjBMI}	All ancestry	N_T = 224,459
Winkler et al. (2015)	26,426,971	2015	WHR_{adjBMI}	European (predominantly)	N_T = 210,028
Wen et al. (2016)	26,785,701	2016	WHR_{adjBMI}	East Asian	N_D = 53,052; N_R = 17,110
Kilpelainen et al. (2011c)	21,706,003	2011	Body fat percentage	European	N_D = 32,161; N_R = 19,979
Lu et al. (2016)	26,833,246	2016	Body fat percentage	European (predominantly)	N_T = 100,716
Fox et al. (2012)	22,589,738	2012	Subcutaneous and visceral adipose tissue	European	N_T = 10,557
Sung et al. (2016)	26,480,920	2015	Subcutaneous and visceral adipose tissue	European and African	N_D = 2513; N_R = 2943
Kilpelainen et al. (2016)	26,833,098	2016	Circulating leptin levels	European (predominantly)	N_D = 36,626; N_R = 39,576
Hatoum et al. (2013)	23,643,386	2013	Weight loss after bariatric surgery	European	N_D = 693; N_R = 693

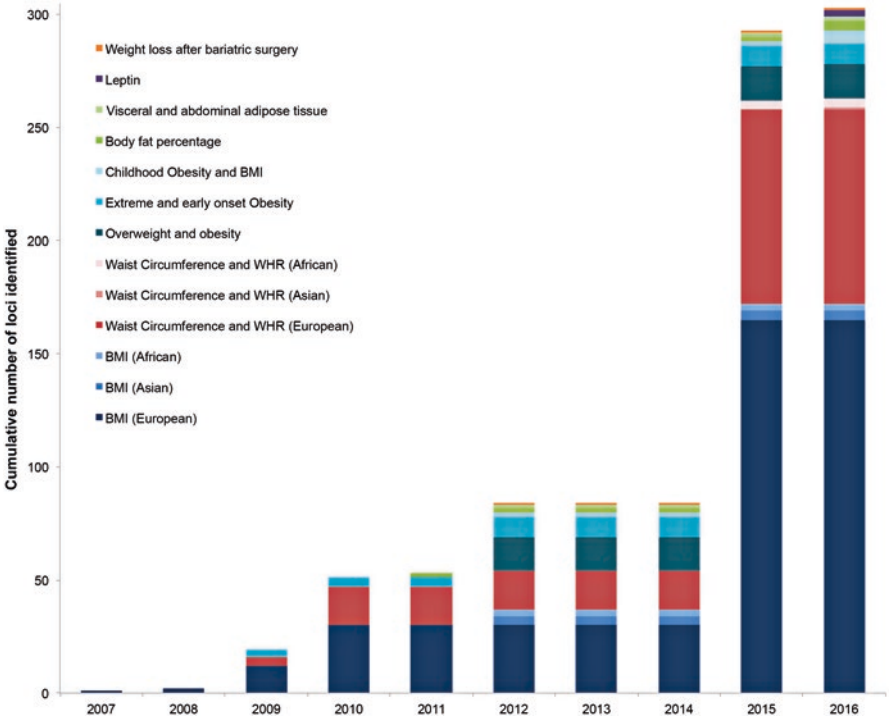

Fig. 11.3 Cumulative number of obesity-susceptibility loci discovered since 2007, colors correspond to outcome and ancestry

11.6.2.1 Genome-Wide Association Studies for Body Mass Index

Most GWAS for obesity-related traits have been performed for BMI, which is an inexpensive and non-invasive measure of adiposity in adults and available in many studies. Over the past decade, at least 13 large-scale genome-wide association (meta-)analyses have been performed, each characterised by a larger sample size and a growing number of discoveries. The latest studies exceed 300,000 individuals and the number of loci identified through GWAS for BMI stands at 175 (Table 11.1, Fig. 11.3).

In 2007, two independent GWAS each identified *FTO* (fat mass and obesity associated gene) as the first gene of which genetic variation in the first intron was incontrovertibly associated with BMI and related adiposity traits (Frayling et al. 2007; Scuteri et al. 2007). The association between *FTO* variants and obesity-related phenotypes has since been consistently replicated across all age- and ancestry groups, yet, it remains unclear what the causal gene(s) and variant(s) in this locus are (see more below) (Loos and Yeo 2014).

Soon after the discovery of the FTO locus, a GWAS meta-analysis of 16,876 individuals confirmed the *FTO* locus and identified one new locus near *MC4R* (Loos

et al. 2008). At the same time, a GWAS in 2684 Indian Asians reported highly significant associations for genetic variants in the same locus (Chambers et al. 2008). The frequency of the BMI-increasing allele is significantly higher in Indian Asians (36%) than in white-Europeans (27%), which might in part explain why this locus could be identified with a relatively small sample of Indian Asians in the discovery stage.

While the discovery sample sizes of the first GWAS were relatively small, they were sufficiently large to harvest the *FTO* and *MC4R* loci as the 'low-hanging-fruits'. However, scientists realised that for more discoveries, collaborative efforts were needed to increase the sample size and thus power of the study. As such, research groups from around the world, combined forces to form consortia. The GIANT Consortium was among the first genetic consortia, and studies the genetic architecture of anthropometric traits in adults, predominantly but not only, from European ancestry. Other consortia, that formed later, have focused more on populations of African (AAGC) and East Asian (AGEN) ancestry.

The GIANT consortium's first GWAS meta-analysis included data from 32,387 adults of European ancestry (Willer et al. 2009). At the same time, the Icelandic company, deCODE Genetics, performed a meta-analysis of four GWAS for BMI, including 30,232 individuals of European descent and 1160 African Americans (Thorleifsson et al. 2009). Together, these two GWAS identified ten novel BMI-associated loci, while also confirming the *FTO* and near *MC4R* loci. In 2010, the GIANT consortium quadrupled its discovery stage to 123,865 individuals (Speliotes et al. 2010). All previously established BMI loci were confirmed, and 18 additional loci were identified. In the following years, the GIANT consortium continued to grow and results from new analyses were published. In 2013, GIANT reported on a GWAS in which BMI was analysed categorically (normal weight vs. overweight or obesity (class I, II, III); highest 5th vs. lowest 5th percentiles of BMI distribution), identifying seven novel loci. In 2015, they reported on a two GWAS, one that included nearly 340,000 individuals from 125 studies, resulting in 56 additional BMI-associated loci (Locke et al. 2015). The second GWAS examined whether age and sex influenced genome-wide genetic associations with BMI (Winkler et al. 2015). Besides four age-specific loci, this GWAS also identified 73 additional loci associated with BMI in the overall sample.

In the mean time, three large-scale GWAS in individuals of East Asian ancestry identified eight loci (Okada et al. 2012; Wen et al. 2012, 2014) and a GWAS in populations of African ancestry identified three additional BMI-associated loci (Monda et al. 2013) that had not been identified before in GWAS of European ancestry populations.

While most studies focused on BMI in adults, two GWAS meta-analyses by the EGG Consortium included children and adolescents only. The first GWAS identified two novel loci associated with childhood obesity (BMI $\geq$ 95th percentile) (Bradfield et al. 2012), and the second study identified four new loci for BMI in childhood (Felix et al. 2016).

11.6.2.2 Genome-Wide Association Studies for Extreme and Early-Onset Obesity

It has been speculated that individuals with early-onset and/or morbid obesity may be enriched for variants that predispose to obesity in the general population. Therefore, GWAS for extreme obesity may have more statistical power to identify obesity-susceptibility loci. So far, there have been four such studies (Table 11.1, Fig. 11.3).

The first GWAS for risk of early-onset extreme obesity was small and only confirmed the *FTO* locus (Hinney et al. 2007). The second GWAS for early-onset (before age 6 years) and morbid adult obesity (BMI $\geq$ 40 kg/m^2) was almost three times larger than the first and identified three new loci (Meyre et al. 2009). In addition to *FTO* and near-*MC4R,* loci in the Niemann-Pick disease type C-1 gene (*NPC1*), near the v-maf musculoaponeurotic fibrosarcoma oncogene homologue gene (*MAF*) and near the phosphotriesterase related gene (*PTER*) showed robust association (Meyre et al. 2009). The third GWAS focussed on early-onset obesity only and combined the data from the first two studies identifying one additional locus (near-*MSRA*) (Scherag et al. 2010) that had 1 year earlier been identified in a GWAS for waist circumference by the GIANT consortium (Lindgren et al. 2009). The most recent GWAS for early onset extreme obesity (BMI > 3SD) was twice as large as the previous ones, but still much smaller than the GWAS for BMI (Wheeler et al. 2013). Yet, this GWAS identified four new loci that had not been identified before by much the larger GWAS.

11.6.2.3 Genome-Wide Association Studies for Waist Circumference and WHR

While BMI is a valid measure of overall adiposity in adults, it does not allow distinguishing between specific fat depots, some of which confer greater metabolic risk than others. More specifically, central adiposity has been proposed to be more strongly associated with metabolic and cardiovascular disease than BMI (Pischon et al. 2008). To better understand the pathogenesis of fat distribution, GWAS have been performed to identify genetic loci for waist circumference and WHR.

The first two GWAS meta-analyses that focussed on central obesity both examined waist circumference and WHR as the main outcomes (Heard-Costa et al. 2009; Lindgren et al. 2009) (Table 11.1, Fig. 11.3). One of the studies, by the GIANT Consortium, identified two new loci (in *TFAP2B*, and near the methionine sulfoxide reductase A gene (*MSRA*)) and a third new locus (near the lysophospholipase-like 1 gene (*LYPLAL1*)) for WHR in women only. The second GWAS, undertaken by the CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium, identified one new locus in the neurexin 3 gene (NRXN3) for waist circumference (Table 11.1, Fig. 11.3).

Because of the high correlation between BMI and waist circumference, the three loci found for waist circumference (*TFAP2B*, near-*MSRA*, *NRXN3*) were also asso-

ciated with BMI, suggesting that these loci are likely more involved in overall adiposity and not specific to central fat deposition. The *LYPLAL1* locus, however, was not associated with BMI, suggesting that this locus more specifically affects fat distribution, at least in women.

Subsequently, the GIANT and CHARGE consortia combined forces and further expanded their discovery stage to include 77,167 individuals (Table 11.1) (Heid et al. 2010). In this study, the main outcome was WHR adjusted for BMI (WHR_{adjBMI}). The adjustment of WHR for BMI allows focussing more specifically on body fat *distribution*, rather than on *overall* obesity. The *LYPLAL1* locus was confirmed. Furthermore, 13 new loci were found to show robust association with WHR_{adjBMI} (Fig. 11.3). Most recently, two large-scale GWAS by the GIANT Consortium, one focussing on main effects (Shungin et al. 2015) and the other on differences between men and women (Winkler et al. 2015), each included data of more than 200,000 individuals of mainly European ancestry and identified 70 additional variants associated with WHR_{adjBMI}. Despite much smaller sample sizes, a GWAS in populations of African ancestry identified four novel loci (Liu et al. 2013) and another GWAS in East Asians identified one additional locus for WHR_{adjBMI} (Wen et al. 2016) that had not been identified in previous GWAS of individuals of European descent that were 3 to 4 times larger. This observation emphasises the importance of studying population of diverse ancestries, as allele frequencies and effect sizes may differ. Of interest is that for nearly half of the WHR_{adjBMI} loci the association was significantly stronger in women than in men. The reason for this sexual dimorphism, which was observed across European, Asian and African ancestry populations, remains unclear. Sex differences in body fat distribution become first apparent during puberty and are attributed to the influence of sex hormones. However, there is no evidence that the identified loci represent genes involved in these hormonal pathways.

11.6.2.4 Genome-Wide Association Studies for More Refined Obesity-Related Phenotypes

Since height, weight, waist and hip circumference are inexpensive and non-invasive measurements, the most studied obesity traits are BMI, as a proxy for overall adiposity, and WHR_{adjBMI} as a proxy for body fat distribution. However, these traits tend to be very heterogeneous; e.g. two individuals may have the same BMI, but their body fat percentage can be very different. Such phenotypic heterogeneity will reduce the statistical power to new discoveries.

Body fat percentage—Body fat percentage, as a more accurate estimate of body composition, was studied in two large-scale GWAS (Table 11.1, Fig. 11.3) (Kilpelainen et al. 2011b; Lu et al. 2016). While many of the previously identified BMI-associated loci were confirmed, the GWAS for body fat percentage identified four novel loci (in/near *IRS1*, *SPRY2*, *IGF2BP1*, *PLA2G6*) that had not been identified before in the much larger GWAS for BMI, obesity or WHR.

Visceral and adipose tissue—Two GWAS have been performed for visceral (VAT) and adipose tissue (SAT), which was quantified using computed tomography (CT).

The advantage of using imaging techniques (such as CT) over anthropometric measures (such as WHR) lies in the ability to partition fat depots in SAT and VAT. However, an important limitation in the context of GWAS is that imaging techniques are expensive and therefore the availability of these refined phenotypes is relatively scarce. Nevertheless, these GWAS identified two new loci, one (near *THNSL2*) for VAT in women only and a second one (*MLLT10*) (Table 11.1, Fig. 11.3) (Fox et al. 2012; Sung et al. 2016) highlighting how more refined and homogenous phenotypes can increase statistical power even when sample sizes are smaller.

Circulating leptin levels—Leptin is secreted by adipocyte proportional to overall adiposity. GWAS on circulating leptin levels, one with and another one without adjusting for BMI, identified three loci that had not been identified before using other adiposity phenotypes, one of which is near *LEP* (i.e. the gene that encodes leptin), and two other loci (*GCKR*, *SLC32A1*) (Table 11.1, Fig. 11.3) (Kilpelainen et al. 2016). Of interest is that association between these loci and circulating leptin levels persisted after adjusting for BMI, suggesting that the association was not mediated by adiposity and that the genes underlying the association influence leptin levels do not affect adiposity.

Weight loss after bariatric surgery—A small GWAS on weight loss after Roux-en-Y gastric bypass (RYGB) surgery identified a locus near *ST8SIA2* and *SLCO3A1*, underscoring the biological nature to the response to RYGB (Table 11.1, Fig. 11.3) (Hatoum et al. 2013). Of interest is that none of the BMI-associated loci identified at the time were associated with weight loss after RYGB, and that the weight loss loci were not associated with BMI in the GIANT Consortium, suggesting that the mechanisms that underlie *weight loss* differ from those that determine general adiposity levels.

Taken together, large-scale GWAS have identified at least 300 loci in which genetic variants show convincing association with obesity traits (Table 11.1, Fig. 11.3). It should be noted that even though these loci were first identified for one outcome (e.g. BMI), many of the loci also associate with other obesity traits (e.g. obesity risk, or body fat percentage).

11.6.3 Translation of New Discoveries

There is no doubt that the genome-wide association approach has been extremely successful in identifying new obesity-susceptibility loci. However, their clinical relevance for the general population and their contribution to understanding the functional mechanisms that underlie body weight regulation has been questioned. A major challenge is translation of this new knowledge into public health and clinical practice.

11.6.3.1 Clinical Relevance of New Discoveries

The flurry of discoveries has raised hopes towards a more personalised approach in obesity prevention and treatment. Some believe that the established obesity-susceptibility variants will contribute to the development of genetic risk profiles that predict early in life who is at risk to become obesity in later life. However, the estimated effect sizes of the established obesity-susceptibility loci, their explained variance and their predictive ability towards obesity, suggest that there is currently not sufficient evidence for such personalised implementations.

Effect Sizes, Risk and Prediction of Established Loci

As discovery of new loci is the primary aim, the significance of association has been the main focus for most GWAS so far. Despite highly significant associations and consistent and repeated replication, however, the effects of the established obesity-susceptibility loci on the obesity outcomes are small.

The BMI loci—Of the more than 170 established loci for BMI, the firstly identified locus, in *FTO*, has the largest, yet small, effect on obesity-susceptibility. According to the large-scales GWAS discussed above, each risk-allele increases BMI by 0.26 to 0.66 kg/m^2, which is equivalent to 750–1900 g in body weight for a person of 1.70 m tall. The risk of obesity increases by 1.20 to 1.32 odds for each additional risk allele. The *FTO* locus was easily identified by GWAS with modest sample sizes because of its relatively large effect size and high prevalence of the BMI-increasing allele (46%) in individuals of white European descent (Frayling et al. 2007; Hinney et al. 2007; Scuteri et al. 2007). Despite being common and having the largest effect of all loci, variants in *FTO* explain barely 0.32% of the phenotypic variation in BMI (Locke et al. 2015).

As sample sizes of subsequent GWAS increased (Table 11.1), statistical power to identify variants with smaller effects and/or lower allele frequency increased. This is illustrated in Fig. 11.4, which shows effect sizes (in gram/allele, for an individual of 1.7 m tall) of the 97 BMI-associated variants that reached significance in the latest and largest GWAS for BMI by the GIANT Consortium (Locke et al. 2015). Effect sizes of the 96 BMI-associated loci, besides *FTO*, ranged from as low as 0.066 to up to 0.3 kg/m^2 per risk-allele for BMI (or 190 to 855 g for a 1.70 m–tall person), and the explained variance ranged from 0.01 to 0.11%. Of these 97 loci, 41 loci (in blue) had been identified in earlier, smaller, GWAS (Speliotes et al. 2010), whereas 56 loci (in red) were newly identified with the larger GWAS (Locke et al. 2015). Effect sizes of these 56 loci tend to be smaller for a given allele frequency (Fig. 11.4). Together, these 97 BMI-associated loci explain only 2.7% of the phenotypic variance in BMI (Locke et al. 2015). A GWAS that focused on interactions with age and sex identified more than 73 additional BMI-associated loci that had similar effect sizes as those reported before (Winkler et al. 2015). While currently no data is available on the explained variance for all >170 BMI-associated loci, it is

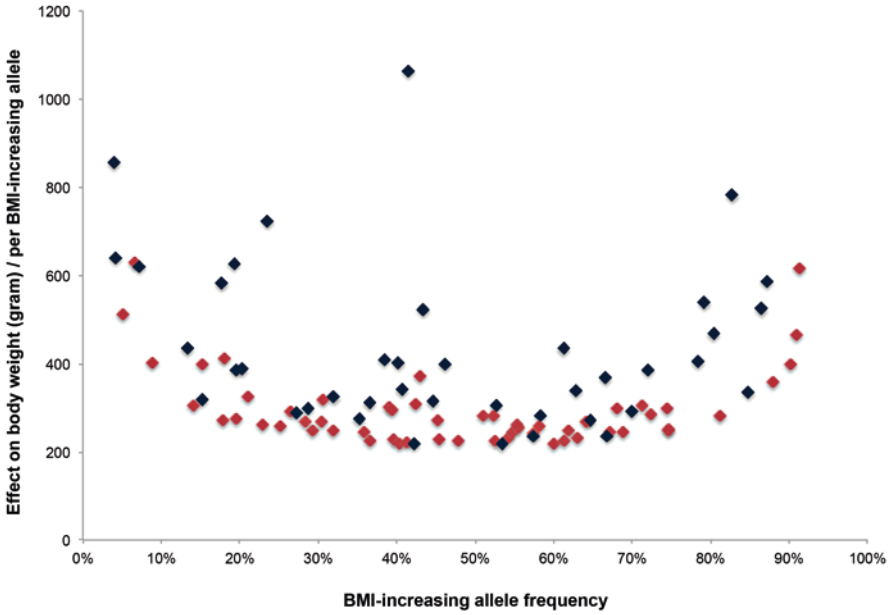

Fig. 11.4 The per-allele effect size (y-axis, in gram per BMI-increasing allele, assuming a 1.7 m tall person) for each of the 97 genetic loci for BMI loci, by the BMI-increasing allele frequency (x-axis), from the most recent GIANT analyses (Locke et al. 2015)

expected that these additional loci will not explain much more of the BMI variation (Locke et al. 2015).

In an analysis of 8164 individuals from the Health and Retirement Study, the combined effect of the 97 SNPs representing all BMI loci was estimated, by constructing a genetic risk score, which sums the number of BMI-increasing alleles an individual inherited (Locke et al. 2015). Each additional BMI-increasing allele in the genetic-susceptibility score was found to increase BMI by 0.1 kg/m^2 (or 290 g increase in body weight for a 1.70 m–tall person). The difference in average BMI between individuals with a high genetic-susceptibility score (having $\geq$104 BMI-increasing alleles, 1.8% of the population) and those with a low genetic-susceptibility (having $\leq$78 BMI-increasing alleles, 1.2% of the population) score amounted to 1.8 kg/m^2 (or 5.2 kg in body weight for a 1.70 m–tall person). In view of using genetic profiles to predict whether a newborn is at risk of becoming an obese adult, this study also examined whether the genetic susceptibility score could be used as a genetic test and found that it has a low predictive value (i.e. the area under the ROC curve was 0.60) (Locke et al. 2015). In fact, the family history of obesity has a better predictive value than the genetic susceptibility score (Whitaker et al. 1997).

Thus, despite overwhelming significances and repeated replications, the explained variance and predictive value of the currently identified loci is too low to be used for genetic profiling and personalised management of obesity.

The WHR loci—The average standardised effect sizes of the WHR_{adjBMI} loci, as reported in the latest large-scale GWAS including more than 210,000 individuals, are of a similar magnitude as those of the BMI loci (0.018 to 0.125 SD/allele) (Shungin et al. 2015; Winkler et al. 2015). Of interest is that effects on $WHR_{(adjBMI)}$ tend to be larger in women than in men; ranging from 0.017 to 0.176 SD/allele in women and from 0.002 to 0.082 SD/allele in men. Consistent with the larger effect sizes in women, WHR loci combined explained more of variation in $WHR_{(adjBMI)}$ among women (1.6–2.4%) than among men (0.7–0.8%) (Shungin et al. 2015; Winkler et al. 2015).

Impact of Obesity Loci Across Ancestries

Of the 33 large-scale GWAS reported so far, only six GWAS were performed in non-European ancestry populations only (Table 11.1). Despite the much smaller sample sizes compared to the European ancestry GWAS, these six non-European ancestry GWAS have identified 16 of the more than 300 obesity loci so far (Fig. 11.3). Even though the majority of loci were first identified in European ancestry populations, associations often "transfer" to populations of other ancestries and vice versa, loci identified in non-European ancestry population often replicate in European ancestry populations (Lu and Loos 2013; Locke et al. 2015). For example, of the 97 variants that showed significant association with BMI in GIANT's most recent GWAS, effect estimates for 79% in African-descent samples and 91% in East Asian samples showed directional consistency with those observed in European-only analyses (Locke et al. 2015). Similarly, of the 14 WHR_{adjBMI} loci identified in European ancestry (Heid et al. 2010), 85% showed directionally consistent association in African ancestry population (Liu et al. 2013). While the transferability of loci across populations from different ancestry seems generally high, the contribution of each of the loci to the obesity outcomes may differ as allele frequencies and effect sizes may differ across population. It should be noted that detailed cross-ancestry analyses tends to be challenging as replication of associations observed in large-scale GWAS (in e.g. European ancestry populations) would require similar sizes samples for the GWAS in other ancestries, which is often not realistic.

Impact of Established Loci in Childhood and Adolescence

So far, most obesity-susceptibility loci have been identified through GWAS of adults and first evidence suggests that loci associated with BMI in adulthood often associate also with BMI in childhood (Speliotes et al. 2010; Hoed et al. 2013; Felix et al. 2016). However, as recent loci have been identified in enormously large GWAS in adults, confirming associations with obesity traits in childhood is challenging, as sample sizes are often not large enough.

Among the 33 large-scale GWAS, two studies have focussed on BMI and obesity risk during childhood, and four studies included adults and children who developed extreme obesity early in life (Table 11.1). Together, these six studies identified 14 new obesity associated variants (Fig. 11.3). Of the nine loci identified in GWAS for early onset and extreme obesity, five loci have so far not been identified for common obesity or for BMI in adults in the general population, suggesting that they may be specific for these extreme forms of obesity. Three of five loci identified for childhood BMI and obesity risk had not been reported before for adult obesity risk, which might indicate that the loci have a larger effect early in life.

Of interest is that BMI-associated loci, at least those identified in the early GWAS on adult BMI, do not associate with birth weight (Andersson et al. 2010; Kilpelainen et al. 2011a), yet, they are associated with greater childhood weight gain soon after birth (Elks et al. 2012b).

Interaction Between Genetic Loci and Lifestyle Factors

It is well-recognised that our westernised lifestyle, which promotes excessive calorie intake and which discourages physical activity, is the major culprit of the obesity epidemic. Yet, not every individual that is exposed to this obesogenic environment becomes obese. It seems that individuals' response to this environment depends on their genetic susceptibility to become obese. Indeed, environmental and genetic factors do not act strictly independently or just additively, but they interact with each other in their causeway to disease. This intricate interplay between genes and environment is also often metaphorically described as "Genes load the gun, but the environment pulls the trigger". The first evidence of gene-lifestyle interaction on risk of obesity and weight gain was provided by the observations in migrants (e.g. Pima Indians described above) and by controlled overfeeding and energy restriction intervention studies with monozygotic twins (Bouchard et al. 1990, 1994; Hainer et al. 2000). The discovery of loci robustly associated with obesity-susceptibility has increased the interest in examining gene-lifestyle interaction at a 'genetic' level; i.e. whether lifestyle can attenuate or exacerbate the strength of association between a genetic locus and obesity-susceptibility.

Most gene-lifestyle interaction studies have focussed on how physical activity affects the association between the *FTO*-locus and BMI. Early on, a large number of studies reported significant interaction between the *FTO* locus and physical activity on BMI, showing that physical activity attenuates the BMI-increasing effect of *FTO*. However, some other studies could not confirm the effect of physical activity on the *FTO*-BMI association. To firmly confirm (or refute) this interaction, a meta-analysis of data from 45 studies in adults (N = 218,166), and nine studies in children and adolescents (N = 19,268) was performed (Kilpelainen et al. 2011c). Consistent with early observations, this meta-analysis confirmed that in adults a physically active lifestyle significantly attenuates the effect of FTO variants on the risk of obesity. Specifically, each FTO risk allele increases the odds of obesity by 30% in inactive individuals but only by 22% in the active individuals. Consistently, the

BMI-increasing effect of FTO was reduced by 30% in the physically active individuals compared with the inactive individuals. In contrast to the adult data, physical activity had no effect on the association between FTO variants and obesity-related traits in children and adolescents.

It remains unclear what biological mechanisms are behind the observed interaction. Variants in the first intron of FTO have been shown to affect the methylation capability, such that this region might be sensitive to epigenetic effects (Bell et al. 2010; Almen et al. 2012). Of interest is that a similar large-scale meta-analysis studying the interaction with dietary factors, did not find evidence that a healthy diet attenuates the association between *FTO* and BMI (Qi et al. 2014b), suggesting that interaction might be specific to physical activity.

Interactions with lifestyle factors for other obesity-susceptibility loci have been examined much less and results are generally inconsistent, as studies tend to be small. Instead of studying individual loci, genetic risk score, which assess the overall genetic susceptibility, have been used in interaction studies. In a large-scale population-based study, including more than 20,000 white British men and women, the interaction with daily physical activity was examined for each of the 12 obesity-susceptibility loci that had been identified for BMI before 2010 (Li et al. 2010a). The genetic risk score, which summed the BMI-increasing alleles of across the 12 loci, was examined in interaction with physical activity. Overall, each additional BMI-increasing allele was associated with 0.154 kg/m^2 (or 445 g for a 1.70 m tall person) increase in BMI. Most importantly, the increase in BMI was significantly more pronounced in sedentary individuals (0.205 kg/m^2 or 592 g per allele) than in physically active individuals (0.131 kg/m^2 or 379 g per allele) (Fig. 11.5) (Li et al. 2010a). Similar interaction effects were observed between the genetic susceptibility score and physical activity for the risk of obesity. Taken together, this study shows that the genetic predisposition to obesity can be reduced by ~40% by having a physically active lifestyle (Li et al. 2010a).

In 2013, a meta-analysis of 11 studies including 111,421 individuals of European descent aimed to replicate the previously observed interaction between genetic susceptibility to obesity and physical activity and to further explore how study-specific characteristics influenced their observations (Ahmad et al. 2013). Using the same GRS based on 12 BMI-associated loci, the meta-analysis showed that each additional risk allele increased BMI by 0.16 kg/m^2 (equivalent to 465 g per allele for a 1.70-m-tall person) (1), which is similar to the overall effect in the previous study (Li et al. 2010a). Importantly, they also replicated the interaction between the GRS and physical activity. More specifically, in physically inactive individuals, each additional risk allele in the GRS increased the BMI by 0.186 kg/m^2 (or 538 g for a 1.70-m-tall person), whereas in physically active individuals, the increase in BMI was 0.150 kg/m^2 per risk allele (or 434 g for a 1.70-m-tall person). Even though the difference between active and inactive individuals was smaller, it was directionally consistent with that of the previous study.

While the evidence that physical activity attenuates the genetic susceptibility to obesity is growing, it remains unclear whether the attenuation is due to specific properties of physical activity as such, or whether a healthy lifestyle in general

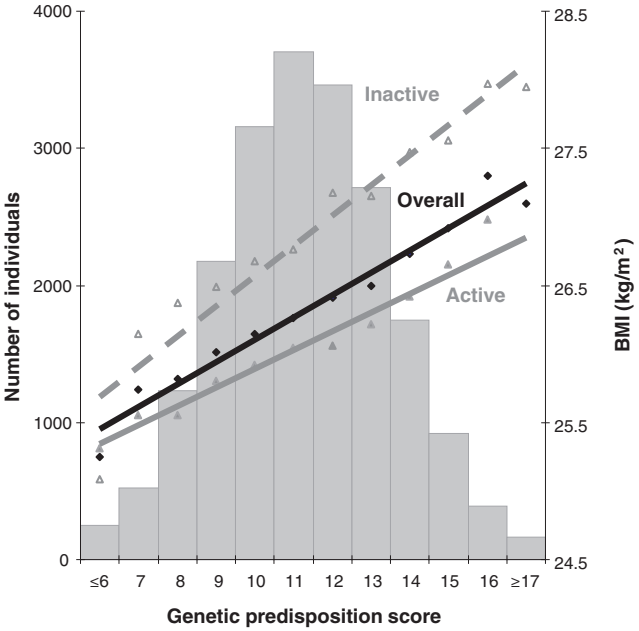

Fig. 11.5 Association between the genetic predisposition score (sum of BMI-increasing alleles from 12 BMI loci) with BMI in all individuals (*solid black line*), in sedentary individuals (*dashed grey line*) and in physically active individuals (*solid grey line*). Adapted from Li et al. (2010a, b)

would induce similar attenuating effects. Recent large-scale studies examining the effect of dietary factors, as another proxy of a healthy lifestyle, on the association between genetic variants and obesity traits have been inconclusive. Some studies found that the association between a GRS and BMI was significantly weaker in individuals who consumed a healthier diet (less fried food and sugar-sweetened beverages) compared to those who consumed more of the unhealthy foods (Qi et al. 2012, 2014a), one study found the opposite (Nettleton et al. 2013), whereas others found no evidence for interactions with dietary intake (Rukh et al. 2013; Qi et al. 2014b).

These findings emphasize the importance of a healthy (physically active) lifestyle in body weight regulation in adults, in particular in those who are genetically predisposed to obesity, and they oppose the often-held fatalist view that a genetic susceptibility is not modifiable.

11.6.3.2 Implications Towards the Etiology of Obesity

It is anticipated that the newly identified genetic loci will shed light on the complex physiology governing the regulation of energy balance and fat distribution. The expectation is that the genetic loci will point towards novel causal pathways and,

subsequently, to the identification of therapeutic targets within these pathways. These could eventually lead to the development of agents for more effective preventive and therapeutic interventions. It should be noted that even loci with small effects can offer important new translational opportunities through the identification of novel modifiable pathways.

Some loci map near genes (at *BBS4*, *BDNF*, *MC4R*, *NPC1*, *PCSK1*, *POMC*, *PPARG, SH2B1*) already known to be involved in body weight regulation and fat distribution, based on insights from monogenic and syndromic forms of obesity as well as from animal models (Chung 2012; Farooqi 2014; van der Klaauw and Farooqi 2015). However, for the vast majority of loci, the physiological mechanisms that link the established loci to weight gain and increased obesity risk are not yet understood.

Loci associated with BMI and overall obesity—So far, the firstly discovered locus, *FTO*, has been studied most extensively in human and animal studies. Yet, it remains unclear how these intronic variants affect body weight regulation (Loos and Yeo 2014). There is good evidence that *FTO* might indeed be the causal gene, as it may function as an amino acid sensor (Gulati et al. 2013), a regulator of grehlin, a mediator of food intake (Karra et al. 2013), a regulator of dopaminergic signaling that governs behaviors (Hess et al. 2013), affecting adipogenesis (Merkestein et al. 2015). However, other research suggests that *FTO*'s intronic variants influence genes downstream (*RPGRIP1L*) that affect energy intake in mice (Stratigopoulos et al. 2008, 2011, 2016), and/or upstream (*IRX3, IRX5*) that, in turn, influence energy expenditure through adipocyte thermogenesis (Smemo et al. 2014; Claussnitzer et al. 2015). Epidemiological studies have so far provided limited evidence for a role of FTO variants in resting or physical activity energy expenditure, whereas there is more support for a central role in the regulation of food intake and appetite (Speakman 2015).

For most other loci, no in-depth analyses such as those performed for the *FTO* locus have been performed. However, pathway and tissue enrichment analyses have provided insight into the *broad* biology involved in body weight regulation (Raychaudhuri et al. 2009; Segre et al. 2010; Pers et al. 2015). For example, the GIANT Consortium applied these approaches to the ~100 BMI-associated loci identified in the largest GWAS so far, emphasising a role of the central nervous system (CNS) in body weight regulation (Locke et al. 2015). In total, 27 out of 31 significantly enriched tissues were in the CNS, including hypothalamus, pituitary gland and hippocampus (Fig. 11.6a), each key sites of central regulation of appetite, hunger, satiety and reward (Locke et al. 2015). Also the most enriched gene sets emphasize CNS pathways, including those related to synaptic function and neurotransmitter signalling, of which some overlap with proposed mechanisms of action of an FDA-approved weight-loss drug (Gibbs et al. 2000; Poulsen et al. 2004). The prominent role for the CNS is consistent with insights from monogenic obesity cases in whom mutations predominantly occur in genes implicated in neuronal circuits that control eating behaviour and energy homeostasis (Farooqi 2014; van der Klaauw and Farooqi 2015).

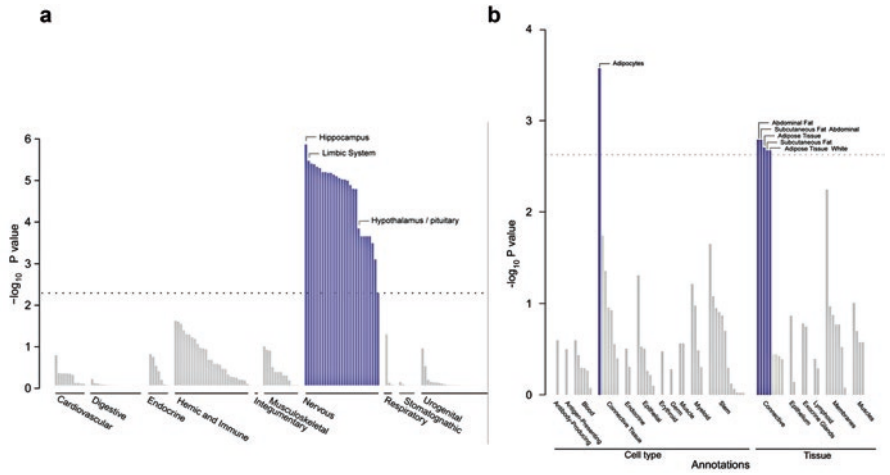

Fig. 11.6 Tissues significantly enriched for genes within BMI-associated loci (panel **a**) and WHR-associated loci (panel **b**). Adapted from Locke et al. (2015) and Shungin et al. (2015), with permission from Nature Publishing Group

Loci identified in GWAS of body fat distribution—The GIANT Consortium applied the same pathway and tissue enrichment analyses to the WHR_{adjBMI} loci, we revealed very different results (Shungin et al. 2015). Specifically, identified loci were enriched for genes expressed in adipose tissue and for putative regulatory elements in adipocytes. Pathway analyses implicated adipogenesis, angiogenesis, transcriptional regulation and insulin resistance as processes affecting fat distribution, providing insight into potential pathophysiological mechanisms (Fig. 11.6b) (Shungin et al. 2015).

Loci identified in GWAS for more refined obesity phenotypes—It has been speculated that by studying more refined and homogenous outcomes, the identified loci will be more directly point towards the underlying biology, as compared to when more heterogeneous phenotypes, such as BMI and WHR, are studied.

For example, the four novel loci identified in GWAS for body fat percentage showed a stronger association with body fat percentage than with BMI and, for three of the loci, cross-phenotype association signatures with a range of cardiometabolic traits (in/near IRS1, IGF2BP1, PLA2G6) was very distinct from loci more strongly associated with BMI, revealing new insights in the link between adiposity and disease risk (Kilpelainen et al. 2011b; Lu et al. 2016). The most striking association signature was observed for the near-*IRS1* loci. The fat-percentage increasing allele of the near-*IRS1* locus was found to be associated with a highly significant reduced risk of type 2 diabetes (Rung et al. 2009) and cardiovascular disease (Samani et al. 2007), as well as with a favourable lipid profile (Teslovich et al. 2010). In targeted follow-up analyses, this association signature was explained

by an effect of this locus on where fat was deposited; i.e. the fat percentage-increasing allele increased the subcutaneous fat, but not the metabolically more harmful visceral fat, emphasising the importance of fat deposition and distribution in the context cardio-metabolic health.

While the GWAS for plasma leptin was relatively small and the number of newly identified loci was limited, the insights gained from follow up analyses are substantial (Kilpelainen et al. 2016). For example, using a mouse knockdown explant model, it was shown that the *Adig* gene, in the *SLC32A1* locus, is the most likely candidate gene in that region as knockdown of Adig resulted in significantly reduced *Lep* expression and leptin secretion (Kilpelainen et al. 2016). ADIG encodes a cytoplasmic adipocyte protein adipogenin, that is, similar to leptin, highly and specifically expressed in the adipose tissue (Hong et al. 2005; Kim et al. 2005; Ren et al. 2016).

11.7 Future Directions

Despite the enormous success of genome-wide association studies, the established loci in combination explain only a fraction of the predicted heritability. Therefore, it has been speculated that more loci remain to be discovered and that the established loci may harbour low-frequency variants, not currently captured by the genome-wide genotyping arrays, that have larger effects. Various approaches have been proposed to identify more genetic loci and to pinpoint the causal variants.

11.7.1 Increased Discovery by Studying (Even) Larger Populations and Screening the Genome with Greater Resolution

The statistical power of GWAS to identify new loci depends largely on the sample size at the discovery stage. The most recent GWAS for BMI and WHR include in the discovery stage included nearly 340,000 and 240,000 individuals (Locke et al. 2015; Shungin et al. 2015), respectively, which is three times larger than the preceding GWAS meta-analyses (Heid et al. 2010; Speliotes et al. 2010). These tripled sample sizes resulted in a substantial increase loci identified (Fig. 11.3). On-going GWAS meta-analyses by the GIANT Consortium are set to be even larger (>1 million), combining the world's available GWAS. Besides increasing sample size, the new GWAS will implement the latest reference panels to impute non-genotyped variants have much greater resolution (1000 Genomes Project, Haplotype Reference Consortium) (Genomes Project Consortium et al. 2015; The Haplotype Reference Consortium 2016). As such, these GWAS meta-analyses will screen the genome for

many more variants (>40 million) and in particular low-frequency variants (minor allele frequency (MAF) = 1–5%), and also rare variants (MAF <1%), will be better represented than before.

With greater sample sizes the statistical power increases, allowing for the discovery of more variants that are either common with small effects or less common with larger effects, consistent with previous observations (Fig. 11.4). It should be noted that even though loci with small effects may not contribute much to explaining the missing heritability, their value lies in the fact that they may point towards new genes of unknown biology.

11.7.2 Identification of Low-Frequency Variation

The risk-allele frequency of the vast majority of obesity-susceptibility loci identified so far ranges from 5 to 95%, and effects are small. While the newest reference panels (1000 Genomes or Haplotype Reference Consortium) will increase the opportunity to identify loci that are less common and that have larger effects. However, imputation of such low frequency variants into genome-wide genotype data is not always successful.

Therefore, to examine low-frequency and rare variants with greater accuracy, whole genome (WGS) and whole exome sequencing (WES) is increasingly performed, which allows investigating the role of every single base-pair of the genome and exome, respectively. It has been speculated that WGS and WES studies will identify rare variants that are more likely to have larger effects and to affect the function of genes and their proteins. Exome-sequencing projects have started to identify disease-causing alleles for complex diseases and traits (Cruchaga et al. 2014; De Rubeis et al. 2014; Fromer et al. 2014; Lange et al. 2014; Purcell et al. 2014). However, for obesity, the largest efforts to date, a case-control study with 1000 overweight and 1000 normal-weight Danes (Albrechtsen et al. 2013) and a second one (UK10K Consortium et al. 2015) with 1468 extremely obese cases and 4000 population controls, have not identified any new rare coding SNVs. These exploratory exome-sequencing studies indicate that to identify rare variants, samples sizes will have to be extremely large (i.e. comparable to those for GWAS). Given that the cost of sequencing is still high, current studies have not been large enough to identify many of such rare variants. Therefore, exome genotyping arrays provide inexpensive array-based alternatives to exome sequencing. These exome chips were designed based on exome sequencing data from 12,000 individuals and comprise ~250,000 nonsynonymous SNVs (Abecasis et al. 2012; Grove et al. 2013). While these arrays have limitations (e.g. incomplete coverage), because of their low-cost (~$70/array), they have already been genotyped in hundreds of thousands of individuals. GIANT's exome-chip meta-analyses for BMI and WHR_{adjBMI} are ongoing that include >500,000 individuals. While sequencing is currently still rather expensive for large-scale projects, exome-arrays provide a good alternative for now.

11.7.3 Studies of Intermediate Traits of Obesity-Susceptibility

Weight gain is the result of a chronic imbalance between energy expenditure and energy intake. Therefore, GWAS of physical activity (i.e. energy expenditure) and dietary factors (i.e. energy intake) could reveal new obesity-predisposing loci. So far, only one genome-wide association study aimed to identify loci for leisure-time exercise behaviour. The study included 1644 Dutch and 978 American individuals that were interchangeably used as a both discovery and a replication cohort (De Moor et al. 2009). Although a few loci were proposed to be associated with exercise behaviour, none of the loci reached genome-wide significance.

Two GWAS for macronutrient intake identified a locus near the fibroblast growth factor 21 (FGF21), a gene known to be involved in glucose and lipid metabolism, to be associated with higher carbohydrate intake, but this locus had no effect on BMI (Chu et al. 2013; Tanaka et al. 2013). The FTO locus was associated with higher protein, independent of its association with BMI (Tanaka et al. 2013).

The main challenge when studying intermediate traits, such as physical activity and food intake, is the accuracy and precision with which these traits are measured, which lowers statistical power, and hampers pooling of data for meta-analyses. Most often questionnaires are used that differ across studies. Therefore, harmonization of measures of lifestyle factors across studies will be essential to achieve a uniform phenotype that can be meta-analysed across cohorts.

11.7.4 Genome-Wide Gene-Lifestyle Interaction Analyses

Thus far, gene-lifestyle interaction studies have focussed on the *FTO* locus or a select set of established obesity-susceptibility loci. However, it is hypothesised that a genome-wide screen of gene-lifestyle interactions may reveal new obesity-susceptibility that are environment-sensitive, e.g. the effect of these loci may be more pronounced in individuals who live an unhealthy lifestyle, while they may have no influence in individuals who live a healthy lifestyle. Similar to GWAS of intermediate traits, described above, gene-environment interaction studies will require the harmonization of lifestyle measures before data can be combined in meta-analyses large enough to detect interaction effects.

11.7.5 Genome-Wide Association of Copy Number Variants (CNVs)

Copy number variants are genomic sequences of roughly 1 kb to 3 Mb in size that are deleted or duplicated in varying numbers and occur commonly in the human genome, but not as frequent as single nucleotide polymorphisms. Several lines of

evidence suggest that they have a role in obesity-susceptibility. Three SNPs of the 97 established BMI loci each represent a CNV (Locke et al. 2015). Furthermore, several systematic genome-wide CNV association studies have provided further evidence for the involvement of small deletions and duplications in obesity risk. The first two genome-wide CNV association studies found convincing evidence for a rare, highly penetrant 593-kb deletion at chromosome 16p11.2 to be associated with morbid obesity (BMI≥40 kg/m^2) (Bochukova et al. 2010; Walters et al. 2010). It was subsequently shown that the corresponding reciprocal duplication is associated with being underweight (Jacquemont et al. 2011). This deletion encompasses *SH2B1* that was previously found to be associated with diet-induced obesity in sh2b-knockout mice and common *SH2B1* variants have also been shown to be convincingly associated with BMI (Locke et al. 2015).

Using an integrated genomic approach, another study found a multi-allelic CNV, encompassing the salivary amylase gene (AMY1), to be associated with BMI and obesity (Falchi et al. 2014). An increased AMY1 copy number was associated with increased gene expression and serum enzyme levels, whereas a reduced AMY1 copy number was associated with increased BMI and obesity risk suggestive of a genetic link between carbohydrate metabolism and BMI. These findings highlight the value of using a variety of strategies to increase our insights into the genetic architecture of human obesity.

11.7.6 Follow-Up of Existing Loci

Besides aiming to identify more susceptibility loci, follow-up of the established loci in molecular and physiological studies will be key to determine the mechanisms through which the loci confer obesity. A major challenge scientists are currently facing, before they can pass on loci to physiologists, is the pinpointing of the causal gene.

The approaches described above to identify new loci can often also be deployed to narrow down the number of potential causal variants. These include the use of 1000 Genomes data or sequencing of the region of interest in extreme cases and controls or in individuals of different ethnicity. It is only when the causal gene is identified and its modes of action are fully understood that this knowledge can be translated into mainstream health care and clinical practice.

11.8 Conclusion

GWAS have revolutionised the search for genetic loci of common obesity. While over the past 15 years, candidate gene studies identified a handful of genetic variants convincingly associated with obesity-related traits, the success of GWAS in terms of gene discovery has been enormous. In less than 4 years time at least 300 obesity-susceptibility loci have been identified, most of which have not previously been linked to body weight regulation.

Follow-up studies of each of these new loci are needed to identify the causal genes and variants and to subsequently elucidate the biological pathways through which these genes confer obesity risk. Physiological experiments have started to shed light on the firstly identified obesity-susceptibility locus, *FTO*, and it is expected that other loci will be examined in similar or greater detail. As translation of basic biomedical discoveries is demanding and takes a lot of effort and time, even after 10 years, it is still too early to evaluate the success of GWAS in terms of their contribution to mainstream health care.

The use of the obesity-susceptibility loci to develop personalised approaches to prevent or treat obesity seems to lie in a future further ahead of us. While, on average, individuals who inherited many obesity-susceptibility loci are more at risk to become obese than those who inherited fewer loci, the identified loci do not have the ability yet to classify 'at risk' individuals with any confidence. It remains questionable whether we will ever have sufficient genetic data to support such personalised approaches to disease management. The major limitation is that the variants identified so far only explain only a fraction of the heritability, and that the Westernised lifestyle puts even those with a low genetic susceptibility at risk of obesity. If genetic profiling is to become applied in clinical practice, we will need to increase the predictive value of the genetic loci and assess their contribution in combination with well-known obesogenic lifestyle factors.

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Chapter 12
Early Origins of Obesity and Developmental Regulation of Adiposity

Shalini Ojha and Helen Budge

Abstract Whilst overweight and obesity result in significant health problems in childhood and adulthood, their origins may lie in earlier life experiences from the nutritional environment of the periconceptional, in utero and postnatal periods. Epidemiological data from human populations show that changes in maternal nutrition during different phases of pregnancy affects the long term health of offspring. Importantly in the context of contemporary populations, maternal overnutrition and obesity also influence offspring health and may induce long term changes which predispose offspring to insulin resistance, obesity and metabolic syndrome in later life. Although changes in maternal nutrition can alter fetal adiposity without overall changes in birthweight, obese mothers are more likely to have large for gestational age babies and these offspring are more prone to becoming overweight and obese in later life. In addition to the effects of the maternal nutritional environment, accelerated growth in the early postnatal period, particularly when proceeded by fetal growth restriction, can be detrimental to long term health and increase the risks of obesity and Type 2 diabetes, consequences similar to those following rapid and early increases in body mass index in childhood. Key pathways of fetal programming include those mediated through glucocorticoids, with their vital role in developmental regulation of adipose tissue, appetite regulation and energy homeostasis regulated by the hypothalamus, and the neurohormones insulin and leptin influencing the actions of neuropeptides in the hypothalamic nuclei. A better understanding of these processes may provide opportunities for the prevention of obesity and improved public health.

Keywords Obesity • Adipose tissue • Nutritional programming • Pregnancy nutrition • BMI • Growth • Metabolic syndrome

S. Ojha
Division of Medical Sciences and Graduate Entry Medicine, University of Nottingham, Nottingham, UK
e-mail: shalini.ojha@nottingham.ac.uk

H. Budge (✉)
Division of Child Health, Obstetrics & Gynaecology, University of Nottingham, Nottingham, UK
e-mail: helen.budge@nottingham.ac.uk

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Overweight and obesity are defined by abnormal or excessive fat accumulation which may impair health and obesity and have significant repercussions on health, being related to various cardiovascular causes of mortality, cancer, Type 2 diabetes, musculoskeletal disorders, work disability and sleep apnoea (Visscher and Seidell 2001).

Obesity, once established, is infamously difficult to reverse and, therefore, the solution to obesity related health problems may lie in its prevention. Traditionally, obesity has been thought to result from an imbalance of energy intake and expenditure, resulting if the intake of energy exceeds its expenditure over a significant period of time. It is intriguing to consider why energy balance occurs in some individuals despite the same obesogenic environmental conditions prevalent in the developed world which in others leads to obesity (Ojha et al. 2015).

It can be hypothesized that the control of body weight and composition depends on an axis with interrelated, and possibly self-controlled, components of food intake, metabolic rate, body fat stores and physical activity. Whilst it is assumed that body weight is ultimately determined by the interaction of genetic, environmental and psychosocial factors acting through several physiological mediators of food intake and energy expenditure (Martinez 2000), the debate over whether obesity is caused by over-eating, lack of physical activity or genetic predisposition remains.

Although the energy balance equation between food intake and energy expenditure may appear deceptively simple, it seems that these variables have a much more complex relationship (Budge et al. 2005). Moreover, recently there is increasing evidence that factors in the periconceptional period, in utero and in early neonatal life may determine later obesity. This may be mediated by their influence on food intake via appetite regulation, nutrient turnover and thermogenesis or by modulation of fat deposition and adiposity. In this chapter, we will discuss the early determinants of adiposity and the current insights into preconceptional, in utero and early life developmental factors which influence later obesity (Ojha et al. 2013). We will discuss how the nutritional environment during the development of the organism impacts upon the physiology of appetite regulation, energy homeostasis, adipose tissue biology and the development of obesity.

12.1 The Theories of the Developmental Origins of Adult Diseases and the Link Between Development and Later Adiposity

Longstanding epidemiological evidence suggests that early life experiences have important implications for long term health. In a Norwegian population, Forsdahl showed that significant poverty in childhood and adolescence, followed by prosperity, is a risk factor for arteriosclerotic heart disease (Forsdahl 1977). Later, in England and Wales, Barker and colleagues demonstrated that ischemic heart disease was strongly correlated with both neonatal and post-neonatal mortality and

suggested that poor nutrition in early life increases susceptibility to the effects of an affluent diet (Barker and Osmond 1986). They further postulated that coronary heart disease is associated with specific patterns of disproportionate fetal growth which result from fetal undernutrition between middle to late gestation (Barker et al. 1993). It is recognised that there are critical windows in fetal development when the process is "plastic" i.e. during periods in which the fetus is undergoing rapid cell proliferation and is very susceptible to environmental influences (McCance and Widdowson 1974). This plasticity provides organisms with the ability to change structure and function in response to environmental cues. Data from the Dutch "Hunger Winter" (the Famine of 1944–45) exemplifies this, documenting the various long term outcomes from significant maternal undernutrition during different periods of gestation (Roseboom et al. 2001). In those exposed to famine in early gestation, even though there was no effect on birth weight, there was an increased risk of later obesity (Ravelli et al. 1999) and metabolic diseases including a three-fold increase in incidence of cardiovascular diseases (Roseboom et al. 2000a).

Hales and Barker have proposed the "thrifty phenotype hypothesis" (Hales and Barker 2001) which postulates that poor fetal nutrition sets in a chain of responses which alters growth and permanently changes the structure and function of the offspring. They proposed that the poorly nourished mother essentially forecasts a poor nutritional environment into which the fetus will be born. Fetal adaptations enable it to survive in the adversity of poor nutrition. However, this becomes detrimental when the postnatal environment changes, with increased abundance of nutrients leading to obesity. Furthermore, the concept of "programming", introduced by Lucas, describes a more general process, whereby a stimulus or insult at a critical period of development has lasting or lifelong significance (Lucas 1991). Gluckman and colleagues (Gluckman et al. 2005) defined predictive adaptive responses (PARs) as a form of developmental plasticity which evolved as adaptive responses to environmental cues acting early in the life cycle. The advantages gained from these adaptations help the offspring survive if the environment remains similar. In these ways, contemporary concepts of the developmental origins of disease have been reached, namely that fetal growth is determined by interaction between fetal environment and fetal genome which, in turn, determines the risk of postnatal disease as well as the individual's capacity to cope with the postnatal environment (Gluckman and Hanson 2004).

The risk of obesity in later life may be determined by both extremes of early nutrition, the risk increasing with early life nutritional deprivation as well as with early life excess due to overnutrition. The Nurses' Health Study in the United States showed an increase in body mass index in midlife in those who weighed more than 10 lb at birth as well as in those who were born with low birth weight (Curhan et al. 1996). Futhermore, increased maternal weight and decreased insulin sensitivity are correlated with fetal growth and, particularly, with increased fat mass at birth (Catalano et al. 1995). In pregnancy, obese women, particularly when they also have Type 2 or gestational diabetes mellitus, make excess nutrients available to the fetus, leading to fetal macrosomia which, in turn, is linked to adolescent and adult obesity. The U.K. Centre for Maternal and Child Enquires reported in 2010 that 5% of U.K.

women who gave birth at ≥24 weeks of gestation had a body mass index (BMI) ≥35 (CMACE 2010). The report also found that the perinatal mortality rate for singleton infants born to mothers with BMI ≥35 was almost double that of the general population and that their babies are at greater risk of being born large for gestational age and/or preterm. Not only were infants of obese mothers more likely to be born large for gestational age, this was amplified when maternal obesity was accompanied by diabetes (CMACE 2010).

Whilst being born large for gestational age presents an obstetric risk to infant and mother, the effects of maternal obesity on the infant persist beyond the newborn period. Maternal obesity prior to pregnancy predisposes offspring to insulin resistance and inflammation (Retnakaran et al. 2003) and increases the risk of overweight in adolescence. The associations between maternal obesity and overnutrition and between obesity and metabolic syndrome in the offspring has been described as the "developmental overnutrition hypothesis" (Armitage et al. 2008) which states that high maternal glucose, free fatty acid and amino acid concentrations result in permanent changes in appetite control, neuroendocrine functioning and/or energy metabolism in the developing fetus which cause obesity and other manifestations of metabolic syndrome in later life. In the face of the obesity epidemic, with increasing prevalence of adolescent obesity and increasing incidence of Type 2 diabetes among young women, there is a vicious cycle of propagation of obesity by the effects of early overnutrition on the fetus and onwards through successive generations (Catalano 2003).

12.2 Evidence from Animal Models

Data from epidemiological studies in human populations such as the British cohorts (Law et al. 1992; Sayer et al. 2004) and the "Dutch Hunger Winter" have provided invaluable evidence suggesting links between early life experiences and later obesity. Although prospective investigations in human cohorts would be of enormous value, these are complex, expensive and confounded by the influences of uncontrollable variables of genetic and environmental origin (Taylor and Poston 2007). Randomised trials to elucidate the relative contributions of different factors and interventions such as sedentary behaviour and maternal nutrition and their modulation by postnatal diet are not practically possible in human populations while in observational studies the effects of the behaviours and other factors of interest are complicated by too many confounding variables. Well-defined experimental studies with the necessary controls can examine precise hypotheses in humans but are usually limited by small numbers and are often ethically impossible (Symonds et al. 2000). The alternative is large observational studies without appropriate controls. In such situations, there are too many confounders and reliance on food diaries or food frequency questionnaires which are not adequately validated. Furthermore, individuals who are under- or over-eating make imprecise records, increasing the likelihood of Type II errors (Symonds et al. 2000; Edington 1999).

The use of animal models is, therefore, essential if the relative contributions of maternal nutrition during fetal development, post-weaning nutrition and sedentary behaviour are to be explored. Animal studies also permit more detailed elucidation of the cellular changes which occur during the evolution of obesity and the changes induced by altered environments (Budge et al. 2005). Several animal models have been used for this purpose, the most common being rodent and sheep models. Like the human, the sheep is a precocial species, carrying one or two fetuses born, at term, after a long gestation (Symonds et al. 2007). However, they have a different pattern of placentation—sheep placentae are cotyledonary synepitheliochoria whilst humans have a discoid haemochorial placenta. Rats, on the other hand are litter bearing with immature offspring born after a short gestation. The rat placenta is more similar to human placenta, although placental differences have not been shown to have substantive modulating effects on nutritional programming. Responses to changes in maternal nutrition at different periods of fetal and early neonatal development can also be better elucidated in the sheep as its diet can be manipulated to coincide with precise periods of fetal organogenesis which are comparable with those during human fetal development (Festing 2006). Sheep are also comparable to humans in a variety of metabolic functions, including brown adipose tissue (BAT) physiology. Both sheep and humans are precocial thermoregulators. BAT is most abundant at the time of birth (Clarke et al. 1997a) which triggers non-shivering thermogenesis (Symonds et al. 2003). On the contrary, rats are altricial species where there is postnatal maturation of uncoupling protein (UCP)1 abundance and the hypothalamo-pituitary axis.

Important long-term impacts also result from changes in organ growth rates, fetal metabolic rate and protein turnover which are similar in sheep and humans, but different in rodents. The hypothalamic–pituitary–adrenal axis, a major player in endocrine control of feeding and adipose tissue metabolism, has a similar maturity pattern in sheep and humans (Fowden et al. 1998) as does the central neural network for the regulation of appetite (Muhlhausler et al. 2004). In rats, these developments occur in the early postnatal period and are dependent on the influence of a neonatal surge in leptin (Bouret et al. 2004). These differences highlight important discrepancies in the pattern of development in various animals. The neuroendocrine mechanisms which modulate appetite and energy homeostasis are largely developed in late gestation in both sheep and humans whilst substantial maturation occurs in the early postnatal period in rodent species. Therefore, sheep models may be a closer estimate of the "programming" effects of nutritional variations and possible interventions in human fetus and neonate.

12.3 The Programming of Adipose Tissue

Adipose tissue is present from very early in fetal development but, for larger animals such as humans and sheep, the majority of adipose tissue deposition occurs in the last one-third of gestation (Clarke et al. 1997a). Fetal adipose tissue exhibits characteristics

of both brown and white adipose tissue, demonstrating an ontogenic rise in the BAT specific uncoupling protein 1 (UCP1) as well as leptin secretion, characteristic of white adipose tissue (Budge et al. 2003; Symonds et al. 2004). It consists of a combination of multilocular and unilocular adipocytes (Yuen et al. 2003). Birth results in a surge of UCP1 synthesis in precocial species such as sheep (Budge et al. 2003), followed by a gradual loss of UCP1 to undetectable levels by 1 month of age (Clarke et al. 1997b). Therefore, in precocial thermoregulators such as humans and sheep, brown fat is most abundant at birth and then in sheep disappears to undetectable levels in the postnatal period. In altricial species such as rodents, maximal UCP1 concentrations occur in the postnatal period and functional brown fat is retained throughout life (Budge et al. 2003), as is now know to be the case in humans (Sacks and Symonds 2013).

In large animals, BAT is present mainly around the core organs such as in perirenal fat depots and constitutes only 2% of birth weight (Symonds and Lomax 1992), as well as in the neck region (Symonds et al. 2012). Although BAT is primarily utilised for thermoregulation following the exposure to the extra-uterine environment, it also plays an important role in energy homeostasis (Symonds et al. 2003). When stimulated, BAT produces up to 300 W/kg tissue of heat compared with 1–2 W/kg tissue by most other tissues (Power 1989). In utero, adipose tissue growth is under marked nutritional constraints, unsurprisingly given that the metabolic demand for fat deposition is higher than that for protein deposition. Therefore, in the persistently hypoxic and hypoglycaemic fetal milieu, adipose tissue is kept firmly regulated (Symonds et al. 2003). However, despite this, fetal adipose tissue is significantly altered by changes in maternal nutrition during fetal development and these changes have the potential to substantially increase the risk of offspring becoming obese in later life (Budge et al. 2005).

At the beginning of the third trimester, only a small amount of adipose tissue is present and, at this stage, leptin and UCP1 appear in the fetus (Yuen et al. 1999; Budge et al. 2004; Casteilla et al. 1987). Leptin synthetic capacity of fetal tissue then increases in late gestation (Yuen et al. 1999). After appearing around mid-gestation, UCP1 becomes more abundant in perirenal fat, gradually increasing to peak soon after birth (Budge et al. 2004). This development of fetal adipose tissue in late gestation appears to be stimulated by an increase in sympathetic innervation, β-adrenergic receptor density and plasma catecholamine concentrations which are likely to be the primary stimuli for appearance of UCP1 (Symonds et al. 2003). Endocrine adaptations also take part in this process of adipose tissue development. Increases in the abundance of prolactin receptors and in plasma prolactin and are seen along with rise in the metabolically active forms of thyroid hormones in the fetal adipose tissue (Symonds et al. 2003). All these are implicated in upregulation of UCP1 gene expression.

Changes in maternal nutrition during various phases of fetal development can alter fetal adiposity as summarised in Fig. 12.1. These responses may not always manifest as differences in fetal body or adipose tissue weight (Budge et al. 2003). The timing of maternal nutritional manipulation is also critical. Maternal nutrient restriction during the time of placental growth does not affect adipose tissue growth initially but the fetus subsequently deposits more adipose tissue with increased

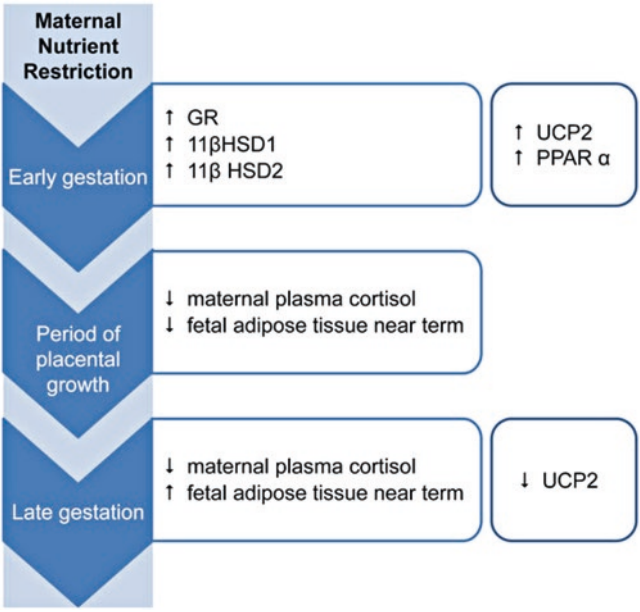

Fig. 12.1 Effects of maternal nutrient restriction on the development of adipose tissue. Nutrient restriction at different phases of development alters the abundances of glucocorticoid receptors (GR), 11β-hydroxy steroid dehydrogenases, (11βHSD), uncoupling protein (UCP) 2 and peroxisome proliferator-activated receptor (PPAR) α in fetal adipose tissue deposition

expression for insulin like growth factor (IGF) I and II receptors (Gardner et al. 2005). In comparison, although offspring of sheep which are nutrient restricted in late gestation may be of similar body weight to those whose mothers were adequately nourished during this period, they develop glucose intolerance, insulin resistance and more fat in young adulthood (Bispham et al. 2005). Similarly, maternal overnutrition also affects adipose tissue deposition and UCP1 expression. Increased maternal nutrition in the latter half of gestation results in heavier offspring with less BAT per kilogram of body weight. However, the BAT in these offspring is richer in UCP1 and has greater thermogenic activity (Budge et al. 2000). Increased maternal nutrition is also associated with the emergence of a strong reciprocal relationship between UCP1 and leptin expression in fetal adipose tissue in late gestation (Muhlhausler et al. 2003).

12.3.1 Role of Glucocorticoids in Programming Obesity

Adipose tissue is the only adult organ which is capable of almost unlimited growth. Glucocorticoids appear to play a vital role in regulation of adipose tissue during fetal development and in later life (Gnanalingham et al. 2005a). They are essential

for the terminal differentiation of adipocytes as seen by the expression of late markers such as glycerol-3-phosphate dehydrogenase (GPDH) activity and triacylglycerol accumulation which are indicative of terminal differentiation in adipocytes (Gaillard et al. 1991). Glucocorticoids also have an action in both the hypertrophic and hyperplastic growth of adipose tissue and influence differentiation, metabolism and gene expression in these cells (Gaillard et al. 1991; Gnanalingham et al. 2005b).

The action of glucocorticoids on adipose tissue is mediated by glucocorticoid receptors (GR) and 11-β-hydroxysteriod dehydrogenase (11βHSD) types 1 and 2. 11βHSD1 behaves predominantly as an 11-oxoreductase, utilising nicotinamide adenine dinucleotide phosphate (NADP) as a cofactor to catalyse the conversion of inactive cortisone to bioactive cortisol and as an intracellular amplifier of glucocorticoid excess to the GR (Bamberger et al. 1996; Budge et al. 2005). The reverse action is catalysed by 11βHSD2 which acts as a nicotinamide adenine dinucleotide (NAD)—dependent dehydrogenase, catalysing the conversion of cortisol to inactive cortisone, a process which maintains the specificity of the mineralocorticoid receptor for aldosterone (Stewart and Krozowski 1999; Budge et al. 2004). Both GR and 11βHSD1 expression increase with fat mass, whilst 11βHSD2 expression decreases (Gnanalingham et al. 2005b; Budge et al. 2005). In sheep offspring, both GR and 11βHSD1 mRNA abundance increase with postnatal age and are maximal at 6 month of age when they demonstrate an inverse relationship with adipose tissue weight (Gnanalingham et al. 2005b). This appears to be exclusive to perirenal adipose tissue, which is the major fat store in the animal, suggesting a differential regulation of glucocorticoid action in adipose tissue and, hence, the possibility that it may be the pathophysiological mediator of later obesity (Gnanalingham et al. 2005b). In addition, 11βHSD1 gene expression increases in adult women with central obesity (Engeli et al. 2004). Further support for its role comes from transgenic mice where those that overexpress 11βHSD1 in adipose tissue have increased corticosterone and develop visceral obesity and glucose intolerance (Masuzaki et al. 2001) whilst those lacking 11βHSD1 are resistant to obesity (Kotelevtsev et al. 1997).

The environment of the fetus, particularly the maternal diet, has a strong influence on glucocorticoid metabolism (Budge et al. 2005) and this may be an important pathway for regulation of fetal and later obesity. Maternal early to mid-gestation nutrient restriction in sheep increases the expression of GR, 11βHSD1 and attenuates the expression of 11βHSD2 in adrenal and kidney in the neonatal offspring even in the absence of changes in birth weight (Whorwood et al. 2001). In perirenal tissue, such changes persist beyond the period of nutrient restriction, despite increased feed intake, suggesting that the gene expression changes have been programmed in the offspring (Whorwood et al. 2001). Furthermore, an increase in glucocorticoid action persists to at least 6 months of age (Gnanalingham et al. 2005b). Maternal nutrient restriction in sheep during the phase of maximal placental growth results in lower maternal plasma cortisol with an increase in fetal adipose tissue deposition near to term (Bispham et al. 2003), whilst undernutrition in late gestation transiently increases maternal cortisol concentrations when combined with fetal surgery (Edwards and McMillen 2001). In the offspring, early- to

mid-gestational nutrient restriction increased glucocorticoid action both near term and at 6 months of age, whilst it was decreased at both 1 and 30 days of postnatal age by late-gestational undernutrition (Gnanalingham et al. 2005b). As this does not correspond with the changes seen in maternal glucocorticoid concentrations, they are likely to reflect alterations in the mitochondria (Gnanalingham et al. 2005b). These modifications in glucocorticoid sensitivity following maternal nutritional variations could be a pivotal adaptation leading to later obesity, fitting with current theories of fetal programming of adult diseases (Budge et al. 2005). These and other studies have illustrated the role of glucocorticoids and 11βHSD in the regulation of adipose tissue, implicating this developmental pathway as a possible mechanism for later obesity.

12.3.2 Uncoupling Protein 2 in the Regulation of Obesity

Whilst UCP1 is specific to brown adipose tissue, UCP2 is expressed more widely in adult human tissue and is upregulated in white fat in response to fat feeding (Fleury et al. 1997). UCP2 has a role in the control of reactive oxygen species production, regulation of ATP synthesis and the regulation of fatty acid oxidation (Boss et al. 2000) and has been linked to hyperinsulinemia and obesity, suggesting a vital role in energy balance and body weight regulation (Fleury et al. 1997). In adipose tissue, UCP2 levels peak at 30 days of postnatal age and decline up to the age of 6 months (Gnanalingham et al. 2005b) and its expression is positively correlated with total and relative adipose tissue weight. This peak at 30 days of age may be a marker of transition from brown to white adipose tissue (Gnanalingham et al. 2005b; Clarke et al. 1997b). The changes in UCP2 expression with maternal nutrient restriction are similar to the effects on glucocorticoid action as its abundance increases with early- to mid-gestational nutrient restriction and decreases with late gestation nutrient restriction (Gnanalingham et al. 2005b). These changes in UCP2 expression are also implicated in the programming effects of maternal nutrition via UCP2 actions in the acquisition of white adipose tissue characteristics and the accumulation of macrophages, which has been implicated in the development of visceral obesity (Gnanalingham et al. 2005b; Weisberg et al. 2003).

12.3.3 Peroxisome Proliferator-Activated Receptors in the Programming of Obesity

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors which have three isotypes present in various tissues including adipose tissue (Grimaldi 2001). Although PPAR-α in the liver has a role in fatty acid oxidation (Reddy and Hashimoto 2001), in BAT it does not appear to participate in

adipogenesis. In contrast, PPAR-γ is a master transcription factor of the adipocyte lineage and is critical for adipogenesis (Grimaldi 2001). PPAR-γ regulates adipose tissue mass through stimulation of lipoprotein lipase (LPL) and glycerol-3-phosphate dehydrogenase (G3PDH) and is involved in the regulation of adipokines such as leptin and adiponectin (Muhlhausler et al. 2007). In response to maternal nutritional restriction between early- to mid-gestation, PPAR-α and UCP2 gene expression increase with adipose tissue mass, particularly when mothers are fed to requirements in the third trimester (Bispham et al. 2005). As both PPAR–α and UCP2 are characteristic of white adipose tissue, this might indicate the potential significance of PPAR–α in regulating early adipose tissue development, particularly in white adipocytes. PPAR-α upregulates fatty acid oxidation and when accompanied by an increase in UCP2 (Bispham et al. 2005) can promote substrate availability to adipose tissue. As IGF- I and II receptors are also upregulated in these circumstances (Bispham et al. 2003), increasing the uptake of glucose, lipid deposition could be promoted.

Maternal overnutrition also impacts on fetal adiposity and its markers. Increased nutrient supply in late gestation results in an increase in the expression of PPAR-γ, lipoprotein lipase, adiponectin and leptin expression in fetal perirenal adipose tissue, suggesting that elevated nutrient supply before birth may result in premature activation of the expression of genes which accelerate the transformation of adipose tissue from a neonatal thermogenic organ to an adult lipid storage organ, laying down the foundations of obesity (Muhlhausler et al. 2007). Periconceptional overnutrition followed by embryo transfer in sheep results in a significant increase in total fat mass in female offspring, with the greatest impact on visceral fat depots, but does not alter the expression of PPAR-γ, G3PDH, LPL or leptin (Rattanatray et al. 2010).

12.3.4 Role of Prolactin

Prolactin has a role in fetal adipose tissue growth and maturation before birth when there is a rise in prolactin receptor (PRLR) expression during the phase of rapid perirenal BAT deposition in sheep (Symonds et al. 1998). In rats, prolactin receptors are widely expressed and increase in PRLR expression is seen in late gestation in a number of fetal tissues (Royster et al. 1995). Administration of prolactin to pregnant rats increases UCP1 abundance in both fetus and newborn offspring, accelerating BAT maturation and enhancing its function, suggesting the role of prolactin in development of BAT (Budge et al. 2002).

Both PRLR 1 and PRLR 2 levels peak between 90 and 125 days of gestation in sheep (Symonds et al. 1998), a time when UCP1 is first detected in BAT (Clarke et al. 1997c). A reduction in fetal nutrition alone does not affect PRLR expression but hypoxia combined with fetal undernutrition (achieved by removal of endometrial caruncles before mating) downregulates PRLR1 gene expression (Symonds

et al. 1998). With an increase in maternal nutrition, fetal plasma prolactin is raised (Stephenson et al. 2001) along with increase in the long isoform of PRLR in BAT (Budge et al. 2000). Interestingly, in the same study, PRLR abundance was not altered in hepatic tissue, a finding which indicates that prolactin has an adipose tissue specific role at this stage of development (Stephenson et al. 2001). This specific relationship between PRLRs and adipose tissue development is also suggested by the effects of experimental placental restriction which significantly reduces fetal plasma prolactin concentrations in late gestation without altering PRLR gene expression in the liver or kidney of the fetus (Phillips et al. 2001).

12.4 Programming of Appetite Regulation and the Hypothalamus

The hypothalamus regulates feeding and energy balance (Bouret 2009) and is a site of action for the central regulatory effects of leptin on energy balance (Elmquist et al. 1999). The arcuate nucleus of the hypothalamus (ARC) receives and integrates signals from peripheral hormones such as leptin and insulin and has a role in peripheral glucose homeostasis.

The central neurohormonal regulation of appetite is also controlled via the action of neuropeptides in hypothalamic nuclei (Fig. 12.2). The major appetite stimulators are neuropeptide Y (NPY) and agouti-related protein (AgRP), whilst the appetite inhibitory factors include pro-opiomelanocortin (POMC), a precursor of α-melanocyte-stimulating hormone (α-MSH), and cocaine-and amphetamine-regulated transcript (CART) (McMillen et al. 2005). NPY neurons are activated by signals from peripheral markers such as glucose, insulin and leptin. These neurons, in turn, project onto other hypothalamic nuclei. Leptin concentrations increase with food intake, decreasing hypothalamic NPY expression, leading to suppression in appetite and hence reduced energy intake (Schwartz 2001). AgRP is co-expressed with NPY and acts as an antagonist for hypothalamic melanocortin receptors. Derived from POMC, α-MSH decreases food intake and its anorexigenic action is increased by leptin which upregulates POMC expression (Schwartz 2001).

Animal studies have suggested that developmental programming of obesity may be due to the influence of the perinatal environment on the developing hypothalamus. This could lead to programming of energy balance "set points". The effects of maternal nutritional modifications (both under- and overnutrition) may be mediated via time-critical influences which alter the expression and actions of specific neuropeptides involved in appetite regulation along with changes in the metabolic regulation of energy homeostasis.

The hypothalami of altricial species, such as rodents, continue to develop until day 20 of postnatal life (Grove et al. 2005). The early neonatal period of precocial species, such as humans, may also be important as although hypothalamic circuits appear to develop in utero in primates (Grayson et al. 2006), maturation may con-

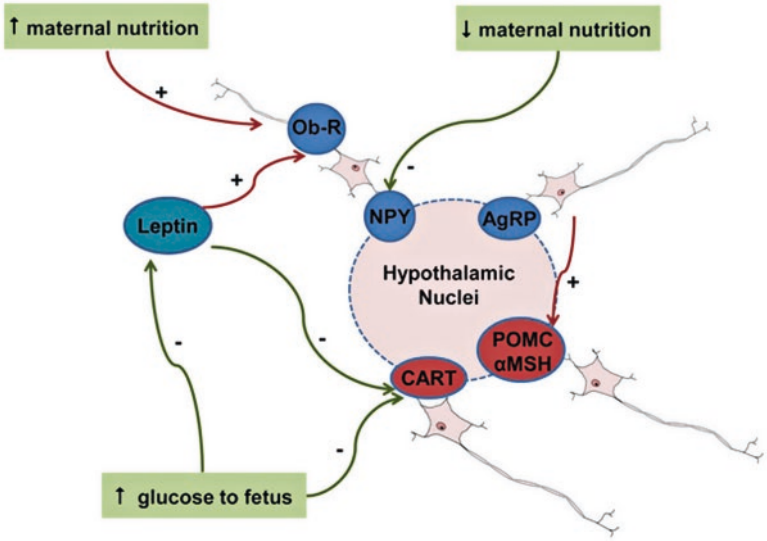

Fig. 12.2 Effects of maternal nutrition on appetite regulation. Decreased maternal nutrition increases the neuropeptide Y (NPY) action on hypothalamic nuclei (HN) whilst leptin acts an appetite suppressant by inhibiting NPY neurons. Increased maternal nutrition reduces leptin receptors in the HN. Increased glucose administration to the fetus (as in diabetic mothers) increases leptin concentrations and stimulates the action of cocaine and amphetamine related transcript (CART). Agouti-related protein (AgRP); pro-opiomelanocortin (POMC); α-melanocyte-stimulating hormone (α-MSH); leptin receptor (Ob-R)

tinue into early postnatal life. Therefore, the perinatal environment, including early neonatal nutrition, can influence hypothalamic programming with implications for later obesity.

Insulin and leptin are the most important peripheral hormone signals of the central nervous system. In early life, leptin acts as a trophic agent and promotes the formation of metabolic pathways. Rodents have gradually increasing leptin concentrations during the first week of life in parallel with the recruitment of non-shivering thermogenesis (Cottrell et al. 2009), even though leptin does not regulate food intake during this period. This has been demonstrated in Lep^{ob}/Lep^{ob} mice (mice lacking leptin), where administration of leptin does not affect food intake, oxygen consumption, body weight or adiposity until weaning (Proulx et al. 2002). Instead of altering metabolism, neonatal leptin appears to be an important signal for the development of hypothalamic circuits controlling food intake and body weight (Bouret and Simerly 2006). This postnatal leptin surge in rodents may originate in adipose tissue (Devaskar et al. 1997), stomach (Oliver et al. 2002), or come from mother's milk (Casabiell et al. 1997). Animal data also indicate that this early critical period for the neurodevelopmental action of leptin seems to be restricted to the first few weeks of life. The existence of a critical period for the developmental effects of leptin suggests changes in leptin concentrations during key periods of

hypothalamic development may induce long-lasting, and potentially irreversible, effects on metabolism (Bouret 2009).

A role for leptin has been shown in the scenario of mismatched in utero and postnatal environments. In a mouse model in which offspring born to mothers with gestational undernutrition were fed a high-fat diet, there was pronounced weight gain and adiposity (Yura et al. 2005). These offspring show a premature onset of the neonatal leptin surge compared to offspring of mothers fed a standard diet. The same authors further demonstrated that exogenous leptin administration to offspring with normal in utero nutrition and a high fat postnatal diet also leads to accelerated weight gain (Yura et al. 2005). Blockage of leptin action during the critical period of early life in rodents has long-term consequences by altering the capacity to respond to leptin during adulthood (Attig et al. 2008), a pattern of long-term leptin insensitivity implicated in adult humans with obesity (Arch et al. 1998). Administration of leptin to offspring of undernourished mothers reverses some of the programming effects of poor nutrition in utero (Vickers et al. 2005). Neonatal rats given leptin during the critical neonatal period show limited neonatal weight gain, and in adulthood caloric intake, locomotor activity, body weight, fat mass, and fasting plasma concentrations of glucose, insulin and leptin are all normalised.

Studies in sheep may be closer to humans as the appetite regulatory network develops before birth in both the species. NPY is present in the sheep hypothalamus prior to birth and fetal undernutrition and glucocorticoids increase NPY gene expression in the fetus (Warnes et al. 1998). Glucose administration to fetal sheep (a surrogate for increased nutrient availability) increases expression of POMC (Muhlhausler et al. 2005), whilst increased maternal nutrition in late pregnancy results in transiently higher relative milk intake, glucose concentration and relative subcutaneous fat mass in early postnatal life (Muhlhausler et al. 2006). The offspring of the well-fed mothers (primarily singletons) have alterations in the expression of the long form of the leptin receptor ORBb in ARC such that there is an inverse relationship between ORBb expression and relative fat mass compared to controls (primarily twins). Increased adiposity is associated with reduced expression of leptin receptors in the ARC. This suggests that exposure to overnutrition in late pregnancy, or fetal number, can cause decreased sensitivity to the actions of leptin (Muhlhausler et al. 2006).

Leptin has also been studied in humans. In pregnancies complicated by maternal diabetes, the fetus is hyperglycaemic and hyperinsulinaemic and cord blood leptin concentrations are increased in parallel with infant adiposity (McMillen et al. 2005). Adults with lower birth or infant weight have higher leptin concentrations than those of higher birth weight with similar degrees of obesity (Phillips et al. 1999). If birth weight is taken as a marker of in utero nutrition, this may be a reflection of the effects of in utero nutrient restriction on adipocyte metabolism and energy homeostasis mediated by serum leptin. Body mass index (BMI) measured at 2 years of age, of infants with intrauterine growth restriction (IUGR), remains significantly lower than those born normal weight (Jaquet et al. 1999). However, although serum leptin was low in IUGR infants at birth, it was raised when measured at 1 year of age compared with those of normal birth weight and there was a loss of the regulatory

effect of BMI and gender. This could be an adaptive leptin resistance to enable so called "catch up" growth. Alternatively, such leptin resistance could be a marker for adipocyte dysfunction.

Leptin concentrations later in life can also be influenced by early neonatal nutrition. In preterm babies, dietary manipulation for an average of only 1 month markedly influences leptin concentrations relative to fat mass up to 16 years later (Singhal et al. 2002). Importantly, the consumption of human milk is associated with a lower leptin to fat mass ratio in comparison to nutrient-enriched preterm formula milk and may represent one possible mechanism of programming by early diet (Singhal et al. 2002).

Animal models and supportive human epidemiological data suggest a fundamental role for leptin in the development and maturation of hypothalamic feeding circuits for long term energy balance. These can be modulated by both in utero and early neonatal nutrition and a premature surge in leptin concentrations can alter weight regulation and energy homeostasis, indicating a time-critical role for leptin. However, although exogenous leptin administered to "programmed" animals can potentially reverse some of the effects, in human studies to date, leptin's potential role as a therapeutic target has not proved to be the much awaited "magic bullet" for preventing obesity (Mantzoros and Flier 2000).

Epidemiological, clinical and experimental results suggest that gestational diabetes or even slightly impaired glucose tolerance during pregnancy are important risk factors for the development of an increased risk of Type 2 and even Type 1 diabetes in the offspring (Dorner and Plagemann 1994) implicating a potential role for insulin in hypothalamic programming. Both perinatal undernutrition and overnutrition can cause hyperinsulinism and lead to permanent dysregulation of the hypothalamus. Malformation of the ventromedial hypothalamic nucleus (Plagemann et al. 1999), suppression of fetal brain NPY concentrations (Singh et al. 1997) and an increase in NPY-positive neurons in the ARC (Plagemann et al. 1998) (a possible marker of acquired hypothalamic insulin resistance) have all been shown in association with alterations in perinatal insulin concentrations.

The effect of untreated maternal diabetes during pregnancy and its consequences for differentiation of hypothalamic nuclei and levels of orexigenic and anorexigenic neurons in the offspring has been demonstrated in an elaborate study on rats (Franke et al. 2005). Exposure to a diabetic intrauterine environment and its prevention by treatment of maternal hyperglycaemia by islet transplantation during gestation has effects on neuronal organisation and expression of orexigenic and anorexigenic neruopeptides in the ARC. There is increased immunopositivity of NPY and AgRP in offspring of mothers with untreated diabetes whilst immunopositivity is decreased for MSH. The change in MSH indicates that exposure to maternal diabetes can alter the processing of POMC to MSH which is an important anorexigenic pathway. Treatment of maternal diabetes by islet cell transplantation (which induces to normoglycaemia) reverses all these effects suggesting that perinatally acquired hypothalamic neuropeptidergic responses are preventable by normalisation of gestational hyperglycaemia (Franke et al. 2005). Animal studies indicate that insulin, particularly fetal or neonatal hyperinsulinism, could induce permanent alterations in hypothalamic organisation affecting energy homeostasis and metabolism throughout life.

In utero nutrition also affects feeding behaviour possibly via the programming of hypothalamic circuits. Offspring hyperphagia in IUGR rats born to nutrient restricted mothers occurs as a result of increased orexigenic hypothalamic signals and reduced anorexigenic physiologic responses (Desai et al. 2007). Programming of central appetite regulation and glucose and lipid metabolism are also affected both by maternal obesity and postnatal overnutrition (Chen et al. 2008). In rats, although maternal obesity does not alter the body or organ weight of newborn offspring, plasma leptin concentrations and hypothalamic NPY, POMC, melanocortin 4 receptor (MC4R), leptin receptor (Ob-Rb), signal transducer and activator of transcription 3 (STAT3), SOCS3 (suppressor of cytokine signaling 3) and mammalian target of rapamycin (mTOR) are all reduced (Morris and Chen 2009). Subsequently, postnatal overnutrition leads to greater weight gain, reduced NPY, increased POMC expression and downregulation of hypothalamic glucose transporter (GLUT) 4 and mTOR expression (factors involved in brain glucose sensing) (Chen et al. 2008). Maternal and postnatal overnutrition also reduces muscle GLUT 4 expression which may explain the resulting glucose intolerance (Chen et al. 2008). This pattern of alterations in glucose handling and in regulators of appetite in response to maternal and postnatal overnutrition could be the foundation of leptin and insulin resistance associated with later obesity and highlights that amplified effects occur when maternal obesity is combined with exposure of the offspring to an obesogenic environment.

12.5 Programming of Level of Physical Activity

Whether reduced physical activity, or increased food intake driven by appetite, is the primary driver for obesity remains an area of continued debate. In evolutionary terms, man was dependent on physical activity for procurement of food and genes evolved to regulate efficient intake and utilisation of fuel stores to ensure survival in an environment of inconsistent food supply (Chakravarthy and Booth 2004). In the current era, the continuous supply of food without any requirement for overt physical activity produces an imbalance in energy intake and expenditure and leads to weight gain. Nevertheless, few studies have analysed the programming effects of physical activity and its effects on later obesity.

When obese individuals lose weight or lean individuals gain weight, their movements associated with routine life (nonexercise activity thermogenesis or NEAT) is unchanged (Levine et al. 2005), suggesting that they may be biologically determined. In rats, an adverse prenatal environment can lead to development of both abnormal eating and exercise behaviour. In this rat model, offspring of undernourished mothers were more sedentary in postnatal life than those born to mothers fed ad libitum and, although present in both genders, males were more inactive than females (Vickers et al. 2003). Therefore, it appears that there may be some effect of the in utero environment of the physical activity of offspring that contributes to obesity in later life.

12.6 Effects of Maternal Undernutrition

Maternal undernutrition can significantly alter the physiology and metabolic course of the offspring. This has been classically demonstrated in humans exposed to the Dutch "Hunger Famine" cohort. Several animal studies have also explored the effects of maternal undernutrition. In a rat model, offspring whose mothers were randomly assigned to received 30% of the ad libitum amount consumed by controls exhibited fetal growth retardation (Vickers et al. 2000). Fetal undernutrition induces inappropriate hyperphagia in adult life and postnatal hypercaloric nutrition further amplifies the abnormalities induced by fetal undernutrition (Vickers et al. 2000). Although offspring of undernourished mothers have markedly increased fasting plasma leptin and insulin concentrations which should decrease appetite, exposure to a postnatal hypercaloric diet, amplifies the hyperphagia, suggesting an inappropriate response due to insulin and leptin resistance induced by early programming. However, it should be noted that these animals were severely nutrient restricted and the model may not be applicable to contemporary human situations. In another rat model where pregnant mothers fed half of the daily intake of controls during the last week of gestation until weaning maternal undernutrition induced both short and long term effects on the hypothalamo-pituitary-adrenal axis (Vieau et al. 2007). There was chronic hyperactivity of the HPA axis leading to high glucocorticoid levels in adulthood. Similarly, large animal studies also indicate that it is only when there is a very severe and prolonged reduction in maternal food intake that birthweight is consistently compromised (Mostyn and Symonds 2009).

Behaviour and lifestyle choices which exacerbate obesity and associated conditions may also have a prenatal origin. Rodent offspring of mothers who were undernourished in pregnancy are significantly more sedentary in postnatal life than those born to ad libitum-fed mothers, independent of postnatal diet (Vickers et al. 2003). Furthermore, this sedentary behaviour is exacerbated by postnatal hypercaloric nutrition. Such findings imply that that "programmed" adults may be more resistant to public health policies and interventions aimed at increasing physical exercise and reducing food intake.

12.6.1 Effect of Undernutrition in Various Stages of Development

The Dutch Famine studies have also demonstrated that there are different consequences of exposure to undernutrition in different trimesters of pregnancy (Roseboom et al. 2001). These differential effects are not surprising in view of the chronological development and growth of fetal organ systems, with cardiovascular growth occurring early in gestation, that of the kidney occurring in mid-gestation and adipose and muscle development occurring late in fetal development. Exposure to the Dutch Famine during early gestation had no effect upon birthweight. However,

as adults, these offspring exhibited a more atherogenic lipid profile (Roseboom et al. 2000b) and increased risks of obesity (Ravelli et al. 1999) and metabolic diseases, including a threefold increased incidence of cardiovascular disease (Roseboom et al. 2000a).

In animal models of maternal undernutrition, peri-implantation undernutrition in sheep (between 0 and 30 days of gestation, where term is around 145 days) does not affect birth weight or offspring growth to 1 year of age although baroreflex sensitivity, which may be precursor of hypertension in later life (Gardner et al. 2004), and the hypothalamo-pituitary-adrenal axis are altered (Gardner et al. 2006). When maternal nutrient restriction is targeted at the period of maximal placental growth (i.e. 28–80 days gestation in sheep), not only is placental growth altered (Dandrea et al. 2001) but maternal plasma cortisol, leptin, thyroxine, and IGF-I are reduced without effects on birth weight, prolactin or glucose concentrations. Interestingly, maternal undernutrition in early-mid gestation increases fetal adipose tissue deposition as measured near to term, a response that is independent of maternal food intake in late gestation (Bispham 2003). These maternal adaptations to undernutrition in pregnancy may act to reduce maternal requirements for nutrients, particularly glucose, therefore partitioning it to the fetus (Symonds et al. 2007). Enhanced fetal fat stores achieved by promoting nutrient supply to the fetus will be beneficial in the short term, promoting metabolic adaptations at birth (especially when in utero nutrient restriction is "predicting" poor nutrition availability after birth), but may set the fetus for excess fat deposition after birth if nutrients are no longer limited (Symonds et al. 2007).

In both sheep and humans, fetal adipose tissue is primarily deposited during the final third of gestation. Over this period, there is an increased abundance of circulating hormones within the fetal circulation which are important in regulating fetal adipose tissue development, and include IGF-I and leptin. The increases in their concentrations are determined by maternal nutrition between early to mid-gestation. Maternal nutrient restriction during this period results in increased expression of both the IGF-I and IGF-II receptors, in conjunction with enhanced adipose tissue deposition, irrespective of the level of maternal nutrition in late gestation (Symonds et al. 2004). As these previously nutrient restricted fetuses have an increased abundance of GLUT 1 (Dandrea et al. 2001), the enhanced responsiveness to IGF may promote the anabolic effects of glucose on fetal adipose tissue growth. Therefore, maternal nutrient restriction in mid-gestation results in enhanced fetal fat deposition in combination with enhanced IGF receptor abundance and glucose supply, which could exacerbate the deposition of fat following the restoration of the maternal diet (Bispham et al. 2003; Symonds et al. 2004).

For sheep, whilst nutrient restriction up to 110d gestation promotes adipose tissue deposition, nutrient restriction in late gestation, decreases it (Gopalakrishnan et al. 2001). Adipose tissue deposition in offspring can also be reduced by manipulating the maternal metabolic and hormonal environment by increasing food intake in late gestation (Symonds et al. 2003). Indeed, late gestation appears to be the period when maternal nutrition restriction has the greatest effect on birth weight (Symonds et al. 2007). These effects are similar to the findings from the Dutch

studies where exposure to famine in late gestation had the greatest effect upon fetal growth, with offspring at birth being lighter, shorter and thinner with small head circumferences (Roseboom et al. 2001).

Sheep studies have demonstrated that although more fat is present at term when mothers are nutrient restricted during the period of maximal placental growth (Bispham et al. 2003), the offspring of mothers who are nutrient restricted in late gestation go on to have greater adiposity as young adults, along with glucose intolerance and insulin resistance (Gardner et al. 2005). This insulin resistance occurs in conjunction with altered glucose uptake in adipose tissue but not in skeletal muscle and there is an increase in adipose tissue insulin receptors in nutrient restricted offspring (Gardner et al. 2005). There is also a reduction in GLUT 4, the major insulin responsive glucose transporter, in adipose tissue suggesting that impaired glucose tolerance is related to the ability of adipose tissue to take up glucose in an insulin responsive manner with a reduction in its abundance is closely associated with insulin resistance (Budge et al. 2005).

In summary, animal studies support evidence from the Dutch "Hunger Winter" that specific periods of famine exposure may impact upon specific physiological control systems in adult life producing differential effects on regulation of adiposity (Budge et al. 2005). These differential effects of maternal nutritional restriction on fetal adiposity suggest that intervention strategies aimed at these critical periods of development have the potential to reduce an individual's predisposition to obesity in adult life (Symonds et al. 2004).

12.7 Effects of Maternal Overnutrition

Starting with the Dutch "Hunger Winter", many studies have focussed on studying the effects of maternal undernutrition on long term outcomes for the fetus. However, the Western World and possibly, in very near future, developing nations (Yajnik 2004), are in the midst of an obesity epidemic. This results in more women being obese both at time of conception and throughout pregnancy. The infants of these obese women are nurtured in the same obesogenic environment as their parents, making them susceptible to postnatal excesses and amplifying effects of in utero overnutrition as summarised in Fig. 12.3.

A study of pregnant women in nine US states showed a 69% increase in pre-pregnancy obesity in one decade (Kim et al. 2007). Studies in the UK also show a similar trend where the number of women who are obese at the start of the second trimester have doubled (Yu et al. 2006). In addition to pre-pregnancy obesity, weight gain during pregnancy can also be excessive. A study of pregnancy outcomes in obese women in Missouri found that 46% gained more than 25 lb of weight during pregnancy (Kiel et al. 2007) and that all pregnancy complications studied were reduced when less weight was gained. With significant implications for maternal and fetal outcomes (Catalano and Ehrenberg 2006), maternal obesity is being increasingly recognized as a major public health issue. In addition to the ill-effects

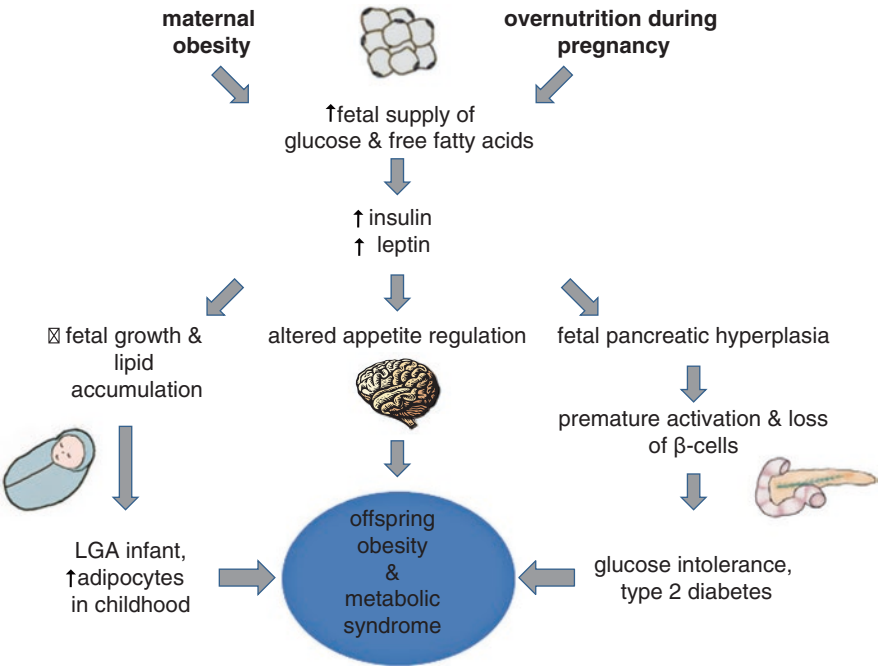

Fig. 12.3 Maternal obesity and overnutrition can programme the fetus to adult obesity and metabolic syndrome

of obesity itself, high maternal weight is associated with a substantially higher risk of gestational diabetes mellitus (Chu et al. 2007), exposing the fetus to further risks due to hyperglycemia and hyperinsulinemia during development.

Maternal obesity has been reported to have varying influences on birth weight in animals. Whilst several studies have not established a link (Chen et al. 2008; Gorski et al. 2006; Caluwaerts et al. 2007; Shankar et al. 2008), some have reported a decrease (Howie et al. 2009). Studies in sheep showed no effect on birth weight when mothers were fed 160% of metabolisable energy requirements during pregnancy (Muhlhausler et al. 2006).

Mechanisms linking maternal and offspring obesity include high maternal glucose, free fatty acid and amino acid concentrations causing permanent programming of energy homeostasis in the fetus (Armitage et al. 2008). A maternal diet rich in energy, fat, sugar and salt during gestation and lactation in rats, induces a preference for similar diet in offspring and increases their body weight (Bayol et al. 2007). Offspring of obese mothers who are cross-fostered to lean mothers fed on a normal diet gain greater body weight and higher percentage of body fat when fed a high-fat diet (Shankar et al. 2008). Effects of maternal obesity are also seen on body composition (Bayol et al. 2009), inflammatory markers (Yan et al. 2010), insulin signalling and mitochondrial activity in muscles (Shelley et al. 2009).

Some influence may be due the composition of the diet rather than the absolute calorie content. In rats, female offspring of mothers who are fed high fat diets have raised blood pressure at 6 and 12 months of age (Khan et al. 2003). This increase is seen with a saturated fat supplemented diet but not with increased maternal polyunsaturated fatty acid intake (Armitage et al. 2004). A high fat diet in rats also affects glucose homeostasis with increased insulin: glucose ratio, higher glucose and triglyceride levels and higher adiposity in the offspring (Guo and Jen 1995). Rats fed a diet rich in omega-6 fatty acids produce offspring with increased proportion of total body and abdominal fat with increase in hepatic triglyceride concentrations and hepatic insulin resistance (Buckley et al. 2005). In contrast, other studies emphasize the effects of essential fatty acid deficiency in the maternal diet on altered leptin expression and adiposity in the offspring (Korotkova et al. 2001). Furthermore, prenatal and suckling exposure to a diet rich in animal fat results in insulin resistance and pancreatic beta cell dysfunction, preceded by altered mitochondrial gene expression (Taylor et al. 2005). Maternal high carbohydrate diets may have a different influence to high fat diets—offspring of rats fed high fat diet have greater appetite stimulation in response to intraventricular-NPY injection (Kozak et al. 2000). These studies suggest that the proportion and quality of fat and other macronutrients in maternal diet, rather than merely the total calorie intake, may be important for metabolic programming.

These animal studies support human observational data that maternal obesity and overnutrition can program the offspring for later obesity and glucose intolerance. Furthermore, children of obese women are more likely to become overweight and develop insulin resistance later in life, if their mothers had diabetes during pregnancy (Taylor and Poston 2007). Therefore, obesity and its related consequences may be a self-perpetuating problem passed through generations and progressively worsened by the facilitative obesogenic environment.

In humans, the increasing prevalence of maternal obesity and overweight (both pregravid weight and weight gain during pregnancy) have been implicated in the causation of the excess of large for gestation age (LGA) and macrosomic babies (Catalano and Ehrenberg 2006; CMACE 2010). Each kilogram of maternal weight gain during pregnancy significantly increases birth weight except in mothers whose prepregnancy weight is more than 135% of ideal for height (Abrams and Laros 1986). As the relationship between birthweight and adult BMI is U- or J shaped (Curhan et al. 1996; Fall et al. 1995), LGA infants are more likely to become obese as adults. The programmed individual may become obese by increasing the number of adipocytes and by producing pancreatic beta-cell hyperplasia which results in hyperinsulinaemia, insulin-resistance and increased deposition of lipids in adipose tissue stores (Levin 2006). High insulin levels seen in overnourished mothers (Taylor et al. 2005) along with alterations in leptin concentrations may impact on neuronal differentiation, synapse formation and maturation in the hypothalamus which may increase the body weight "set point" with increased appetite, reduced basal metabolic rate and altered energy balance resulting in the metabolic syndrome phenotype (Armitage et al. 2004). Among low-income families in Ohio, maternal obesity in early pregnancy doubled the risk of obesity at 2–4 years of age (Whitaker

2004). Maternal pregravid weight and diabetes also increases the risk of obesity in adolescence (Catalano and Ehrenberg 2006). With the substantially increased morbidity associated with maternal obesity and the possible trans-generational cycle it perpetuates, there is an imperative need to understand the mechanisms behind this programming effect and aim to establish successful obesity prevention strategies.

12.8 Early Postnatal Growth and Adiposity Rebound

In the Avon longitudinal study in the UK, children who have intrauterine restraint of fetal growth have more so called "catch-up" growth and go on to be fatter with more central fat distribution at 5 years of age compared with controls (Ong et al. 2000). Such accelerated postnatal growth is also associated with raised blood pressure (Huxley et al. 2000; Adair et al. 2009) and death from coronary heart disease (Eriksson et al. 1999). In a Swedish cohort, the highest death rates from coronary heart disease occurred in boys who were thin at birth but who gained weight centiles in childhood such that they had an average or above average body mass from the age of 7 years (Eriksson et al. 1999).

The programming effects of overfeeding immediately after fetal growth retardation have been studied in animal models. In rats, growth retarded offspring of undernourished mothers recoup their weight when fed adequately (by reducing the litter size) during lactation (Bieswal et al. 2006). After weaning, they continue to gain weight and become significantly heavier than control animals. This weight difference is exaggerated if a high calorie diet is provided to the previously growth restricted animal, an effect more prominent if the gestational undernutrition is achieved with a low calorie diet rather than with an isocaloric protein restricted diet.

Male offspring of mice, who are undernourished during pregnancy, live longer if they are growth restricted during the suckling period. This slowing of postnatal growth also appears to protect against an obesity-inducing diet later on (Ozanne and Hales 2004). Conversely, male mice which are poorly nourished in utero but cross-fostered to normally fed dams exhibit rapid "catch-up" growth and die at a younger age. Life expectancy further reduces with subsequent consumption of a high calorie and high fat diet (Ozanne and Hales 2004).

There is a link between in utero nutrient and growth restriction followed by accelerated postnatal growth and later emergence of insulin resistance, glucose intolerance and visceral obesity. Insulin receptors in the skeletal muscle of sheep are more abundant in response to growth restriction, an effect that persists in postnatal life (Muhlhausler et al. 2009). When nutrition availability improves in postnatal life, this abundance of insulin receptors, along with upregulation of insulin signalling molecules (Muhlhausler et al. 2009), results in accelerated growth of the previously growth restricted animal (Morrison et al. 2010). However, the increased insulin sensitivity changes into insulin resistance, a pattern recognizable as early as 1 year of age (Soto et al. 2003).

Adiponectin, an adipokine, is paradoxically reduced in obese subjects (Arita et al. 1999) and appears to play a central role in development of Type 2 diabetes. A high concentration of adiponectin is associated with reduced relative risk of Type 2 diabetes (Spranger et al. 2003). Children who are born small for gestational age (SGA) have lower adiponectin concentrations compared with those who are short but of appropriate weight for gestational age and with those who are obese (Cianfarani et al. 2004). Additionally, adiponectin is significantly lower in SGA children whose height is appropriate for age, sex and genetic potential (as indicated by mean parental height) when compared to those who are short (Cianfarani et al. 2004), possibly signifying that accelerated postnatal growth increases the risk of obesity and Type 2 diabetes in later life.

Children who are born SGA continue to gain body fat and abdominal fat mass between 2 and 4 years of age despite having largely achieved height and weight similar to children born appropriate for gestation age by 2 years of age (Ibanez et al. 2006). This is accompanied by increases in insulin resistance and IGF1 (Ibanez et al. 2006). Total and abdominal fat mass is further increased between 4 and 6 years of age and visceral fat is already present at 6 years of age (Ibanez et al. 2008), even in non-obese children.

In the process of growth during childhood, BMI increases rapidly during the first year of life followed by a decline. It reaches a minimum in early childhood and then starts to increase up to the end of growth. Adiposity rebound has been defined as the point of least BMI at which the sustained increase begins (Rolland-Cachera et al. 1984). The difference in body composition during "adiposity rebound" has been shown to be due to alterations in body fat rather than changes in lean body mass, children who have early adiposity rebound gaining fat faster (Taylor et al. 2004). The mean age of adiposity rebound was 5.5 years in a US retrospective cohort study (Whitaker et al. 1998) whilst a New Zealand cohort reported 6 years for boys and 5.6 years for girls (Williams et al. 1999). However, the timing of adiposity rebound may be an important factor for the development of obesity, reflecting the changing BMI pattern of the individual. In obese subjects, adiposity rebound occurs around 3 years of age (Rolland-Cachera et al. 1987). An early adiposity rebound has been associated with Type 2 diabetes (Eriksson et al. 2003), higher BMI in adolescence (Rolland-Cachera et al. 1984; Siervogel et al. 1991), early adulthood (Prokopec and Bellisle 1993) and in later adult life (Whitaker et al. 1998) and suggests determinants are established in early life (Rolland-Cachera et al. 2006).

12.9 Conclusion

The high prevalence and health consequences of obesity require urgent preventative strategies. There is ample evidence to show that the origins of adiposity lie in early development, from the periconceptional period through to early childhood. An increasing understanding of these processes and their contribution to later obesity and its accompanying diseases may provide opportunities for long term prevention and prove vital to improving public health.

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