Michael E. Symonds *Editor*

Adipose Tissue Biology

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Contents

Chapter 1
Adipocyte Precursors: Developmental Origins, Self-Renewal, and Plasticity

Christian Dani and Nathalie Billon

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 mesenchymal stem cells down the adipocyte lineage remain largely unknown. Similarly, the study of the developmental origin of adipocytes during embryogenesis has been largely disregarded until now. This review summarizes the surprising findings that have recently emerged from in vivo lineage tracing studies, unraveling unsuspected developmental origins for white adipocytes. We will propose that the differential origin of adipocytes could also reflect functional differences and site-specific regulations of adipose tissue. This chapter also reports recent work that has led to the identification of discrete immature cell populations from which white adipocytes are derived in mice.

A pool of adipocyte progenitors remains present in adipose tissue during adult life. This pool is responsible for the renewal of adipocytes and the potential of this tissue to expand in response to chronic energy overload. However, factors controlling proliferation and differentiation of human adipocyte progenitors are largely unknown. We will present stem cells derived from human adipose tissue (human Multipotent Adipose tissue Derived Stem (hMADS) cells) for studying proliferation and differentiation of adipocyte progenitors and will show that fibroblast growth factor 2 and activin A are key regulators of human adipocyte precursor self-renewal. Finally, we will discuss about the plasticity of hMADS cells.

Keywords Adipocyte precursors • Stem cells • Adipose tissue • Adipocyte development

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1.1 Introduction

Two adipose tissues with different functions coexist in humans, i.e. white and brown adipose tissues. White adipose tissue (WAT) is mainly involved in energy storage and mobilization. WAT is localized in various sites of the body, has an enormous capacity for expansion, and excess of fat accumulation is associated with metabolic disorders. Brown adipose tissue is specialized in energy expenditure. It is a key thermogenic organ, and brown adipocytes burn fat. We will concentrate in this chapter on adipocyte precursors (APs) giving rise to white adipocytes.

The main cellular components of WAT are mature adipocytes and stromal-vascular cells, which include immune cells, endothelial cells, and APs. Expansion of WAT during normal development and in obesity is the result of an increase in size and number of adipocytes. As mature adipocytes do not divide in vivo, regeneration of adipocytes and the increase in adipocyte number depend on self-renewal of a pool of APs that remains present during adult life and that can be recruited to form new fat cells (Hauner et al. 1989; Spalding et al. 2008). Therefore, characterization of the cellular and molecular events involved in the generation of APs and the identification of factors regulating their self-renewal could provide a means for better understanding the mechanisms that lead to hyperplasia and excessive development of adipose tissue.

Adipogenesis is described as a two-step process. The first step consists in the generation of APs, also named preadipocytes, or adipose-derived stem cells depending on their potential to differentiate in adipocyte only or in additional cell types. The second step involves the terminal differentiation of these precursors. Key events controlling terminal differentiation of preadipocytes into adipocytes have been identified. Transcription factors such as CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptors (PPARs) are known to play a critical role in this process, whereas Wnt and Hedgehog signaling pathways are critical regulators of terminal differentiation (Rosen and Spiegelman 2000; Longo et al. 2004; Fontaine et al. 2008). Terminal differentiation has been extensively studied (Rosen and MacDougald 2006) and will not be reviewed in this chapter. We will focus on the earliest steps of adipogenesis, i.e. the generation of APs and regulators of their self-renewal and plasticity (Fig. 1.1).

1.2 Developmental Origins of Adipocyte Precursors

Strikingly, the study of the developmental origin of APs has received very little attention until now. APs are generally described to derive from mesenchymal stem cells (MSCs), which themselves are thought to arise from mesoderm. 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

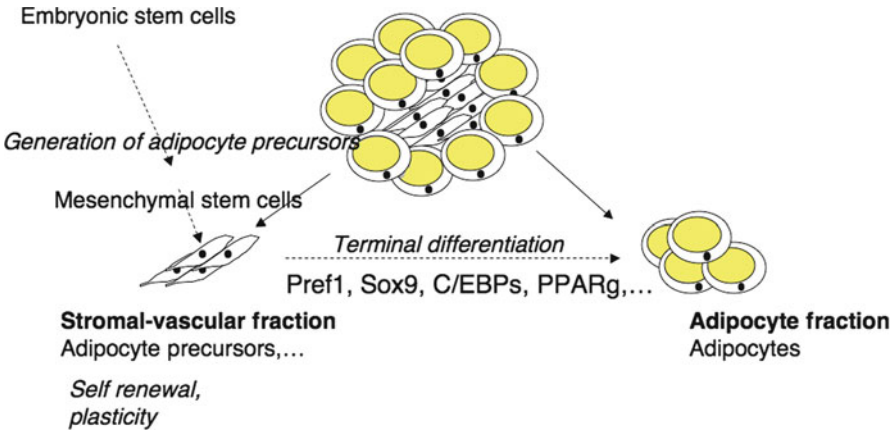

Fig. 1.1 Different steps of the adipocyte development. Adipose tissue is composed of mature adipocytes and stromal-vascular cells including adipocyte precursors (APs). Key events controlling terminal differentiation of APs have been identified. The developmental origins of APs, factors regulating AP self-renewal, as well as the plasticity of APs are discussed in this chapter

population that arises from the neuroectoderm. After neural tube closure, NC cells (NCCs) 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 a powerful model to investigate the earliest steps of adipocyte development and revealed the surprising conclusions regarding the ontogeny of such cells in the NC.

1.2.1 Adipocyte Development in Mouse Embryonic Stem Cells

Mouse embryonic stem cells (mESCs) are proliferating, pluripotent stem cells that have been isolated from the epiblast of blastocyst-stage mouse embryos. They can be propagated indefinitely at the undifferentiated state in vitro. Furthermore, when transplanted into a mouse blastocyst, mESCs integrate into the embryo and contribute to all cell lineages, including germ cells (Smith 1992). When aggregated to form embryoid bodies (EBs) in vitro, they undergo differentiation in ectodermal, mesodermal, and endodermal derivatives (Keller 1995). In addition, ESCs are easily genetically modifiable and can be produced in large numbers, thus offering a unique cell culture model to study the earliest steps of mammalian development. Directed differentiation of mESCs towards the adipocyte lineage was first accomplished in 1997 by Dani et al. (1997), who showed that functional adipocytes could be obtained when mESCs were exposed to appropriate extracellular cues. In this 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 (e.g., insulin, triiodothyronine, and rosiglitazone). Both, lipogenic and lipolytic activities, as well as high levels of expression of adipocyte-specific genes, could be detected in mESC-derived adipocytes. Remarkably, the sequence of expression of key transcription factors known to govern preadipocyte differentiation, such as members of the C/EBP and the PPAR families, was closely conserved during mESC adipogenesis. Thus, this model has provided a powerful system to address the different steps of adipocyte development (Wdziekonski et al. 2003, 2006, 2007; Billon et al. 2010; Schulz et al. 2009; Carnevalli et al. 2010; Tong et al. 2000; Takashima et al. 2007). More recently, adipocytes have been obtained from human ESCs and from human induced pluripotent stem (iPS) cells using a protocol based on mESC studies (e.g., Xiong et al. 2005; Taura et al. 2009; T. Mohsen-kanson and C. Dani, unpublished data).

1.2.1.1 Mesenchymal Stem Cells and Adipocytes Developing from RA-Treated mESCs Derive from the Neuroectoderm, Rather Than from the Mesoderm

In a first attempt to unravel the events underlying the formation of mesenchymal derivatives in RA-treated mES cells, Kawaguchi et al. (2005) 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 of cardiomyocyte formation, suggesting that RA reduces overall mesoderm formation in mESCs (Kawaguchi et al. 2005). Since at high concentrations, RA was shown to promote neural differentiation of mESCs and since some mesenchymal tissues are known to be generated by the NC, which itself derives from neuroectoderm, these authors then analyzed the expression of various NC markers in mES cells. 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 have developed a genetic lineage selection approach in mESCs, which is outlined in Fig. 1.2. We used genetically engineered, selectable *Sox2-βgeo/oct4-tk* mESCs that allow selection for neuroepithelial precursors ($Sox2^+$) and eliminate residual undifferentiated mESCs ($Oct4^+$). After induction of neural differentiation via RA treatment, highly enriched populations of neuroepithelial cells were selected in the presence of G418 and Gancyclovir. We then exposed them to adipogenic signals and showed that indeed, they could give rise to mature adipocytes within 14 days. Interestingly, a significant increase in *sox9*, *sox10*, and *FoxD3* mRNAs was observed prior to adipocyte formation, suggesting that NC-like cells present in the selected population could undergo adipocyte differentiation (Billon et al. 2007, 2008). Together, these data suggest that neuroectoderm/NC is the major source of adipocytes, at least in mESCs exposed to RA (Fig. 1.2a).

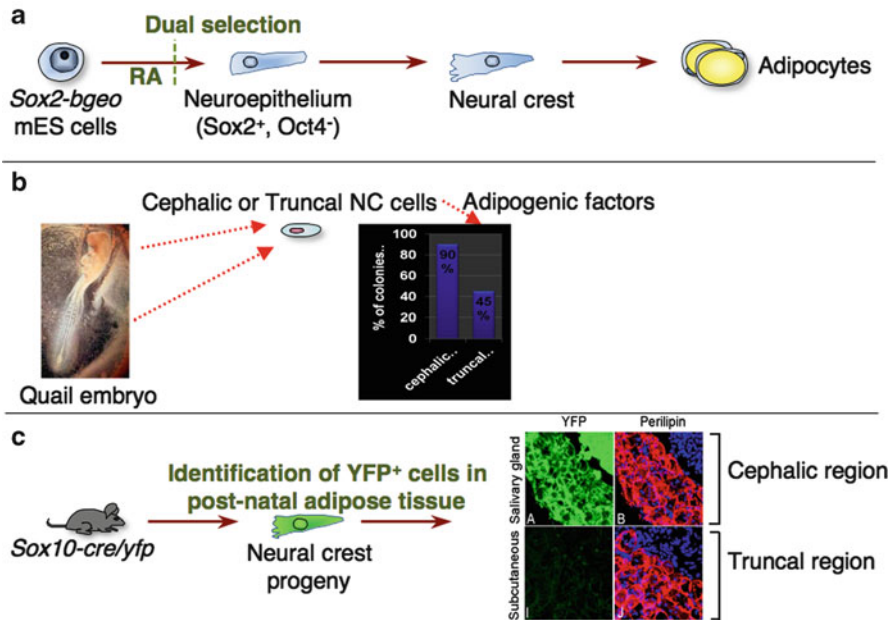

Fig. 1.2 Subset of adipocytes that originated from the neural crest. (**a**) Genetic selection strategy used for the generation of adipocyte of neuroepithelium origin in mESCs. (**b**) Adipogenic potential of cephalic and truncal NCCs isolated from quail embryo. (**c**) Permanent genetic lineage-labeling approaches used in mouse to reveal NC-derived adipocytes in adult cephalic adipose tissues

These findings were later corroborated by Takashima et al. who used an elegant approach to unravel the NCC origin of MSCs in both mESC culture and during mouse development (Takashima et al. 2007). All together, these studies suggest that MSCs, as well as adipocytes generated from RA-exposed mES cells, arise from the neuroectoderm/NC, rather than from the mesoderm.

1.2.2 Study of Adipocyte Precursor Developmental Origins in Quail and Mouse Embryos

To better understand adipocyte lineage specification from the NC, we checked whether adipocytes could be obtained from NCCs isolated from a normal developing embryo. We used primary cultures of quail NCCs, since they have been instrumental in establishing the developmental potentialities of the NC. NCCs were isolated from both the cephalic and thoracic level and maintained in culture media permissive for adipocyte differentiation (Rodriguez et al. 2004). This analysis revealed that typical mature adipocytes could readily be produced from cephalic

NCCs and, to a lesser extent, from truncal NCCs (Billon et al. 2007). Therefore, quail NCCs from both the cephalic and the thoracic level exhibit an adipogenic potential in vitro (Fig. 1.2b). Finally, we have used a lineage tracing approach in mouse to address the origin of the adipocyte lineage in vivo and to provide direct evidence for the contribution of the NC. We have investigated whether subsets of adipocytes originate from the NC using *Sox10-cre*/yfp transgenic mice to map NC derivatives in vivo because to date, Sox10 is considered as the best bona fide NC marker. Indeed, Sox10 is strongly and specifically expressed in the NC from early embryonic development and is not expressed in mesoderm. This study revealed adipocytes derived from NC in cephalic adipose depots, between the salivary gland and the ear area. In contrast, no NC-derived adipocytes could be detected in truncal adipose depots, including subcutaneous, perirenal, periepididymal, and interscapular tissues (Fig. 1.2c). 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 face originates from NC, and not from mesoderm (Billon et al. 2007). 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 reminiscent of the role of RA in mESC adipogenesis. Due to the lack of specific markers of undifferentiated APs in the studies described above, these cells were functionally traced by the appearance of adipocytes, or identified a posteriori, by their potential to differentiate into adipocytes. The AP phenotype allowing their identification in a prospective manner, as well as their tissue localization in adult mice, has recently been addressed (see below).

1.3 Cellular Origins and Tissue Localization of Adipocyte Precursors

Recently, Graff and Friedman laboratories performed critical experiments to identify and localize APs in mouse adipose tissue. Rodeheffer et al. used Fluorescence Activated Cell Sorting (FACS) analysis to isolate various cellular subpopulations from stromal-vascular fraction (SVF) and tested their adipogenic potential both in vitro and in vivo after transplantation in lipoatrophic A-Zip mice. By this approach, the authors identified mouse APs in the SVF of adipose tissue as lin^-/$CD34^+$/$CD29^+$/sca-1^+/CD24+ cells (Rodeheffer et al. 2008). Whether APs originated from NC or from mesoderm display the same immunophenotype remains to be determined. By a different approach, based on the expression of PPARγ in SVF of adipose tissue, (Tang et al. 2008) isolated undifferentiated cells able to undergo adipogenesis in vitro and in vivo in *nude* mice. These cells express markers of preadipocytes but not those of mature adipocytes, indicating that PPARγ can also be used to trace APs. Interestingly, these cells are $CD45^-$/$Ter119^-$/$CD34^+$/$sca1^+$, indicating that they are similar, if not identical, to cells isolated by Friedman laboratory. Thanks to the expression of a reporter gene under the control of PPARγ promoter, APs have

been localized in the mural cell compartment of adipose tissue vasculature in mice (Tang et al. 2008). The immunophenotype of human APs has not yet been fully characterized, although they have been shown to reside in the $CD34^{+}/CD31^{-}$ subpopulation of stromal-vascular cells of adipose tissue (Sengenes et al. 2005).

APs are resident in adipose tissue, but other sources have been recently reported. Bone marrow appeared to be a source of APs in the adipose tissue as it has been reported that a small subpopulation of adipocytes in WAT might arise from bone marrow progenitors (Crossno et al. 2006). More recently, a hematopoietic origin of APs has also been proposed. Indeed, clonal analysis and cell sorting-based studies of hematopoietic progenitors suggested that adipocytes could be derived from hematopoietic stem cells via progenitors for monocytes/macrophages or via myeloid intermediates in mice. These conclusions are supported by previous studies showing that the phenotypes of adipocyte and macrophages are closed. Interestingly, hematopoietic-derived adipocytes seem to accumulate with age in visceral fat depot, where they display higher expression of inflammatory genes than "conventional" adipocytes (Sera et al. 2009; Majka et al. 2010; Cousin et al. 1999). The contribution of these nonresident APs on metabolic diseases remains to be determined.

1.4 Do Adipocyte Precursors Produced from Different Sources Differ in Their Biological Properties?

It is well established that APs isolated from different depots display different features in terms of proliferation, differentiation, and gene expression profiles (Tchkonia et al. 2007; Gesta et al. 2006). In addition, adipocytes derived from these APs have different functional properties and have different contributions to metabolic diseases (Montague et al. 1998). The cellular and molecular mechanisms underlying these fat depot-dependent differences are currently unknown. However, several observations suggest that developmental mechanisms contribute to regional variation in function. Therefore, studies on the origins of APs open at least two questions: are adipocytes derived from different developmental origins or cellular sources functionally different? And what are the developmental origins of APs in humans? As adipocytes can be now generated from human ESCs and from human iPS cells (Xiong et al. 2005; Taura et al. 2009; T. Mohsen-kanson and C. Dani, unpublished data), studies of APs properties related to their cellular and developmental origins can be now addressed in human cellular models.

1.5 Self-Renewal of Human Adipocyte Precursors

A pool of APs remains present in adipose tissue during adult life. This pool is responsible for the renewal of adipocytes and the potential of this tissue to expand in response to chronic energy overload. Therefore, the identification of factors

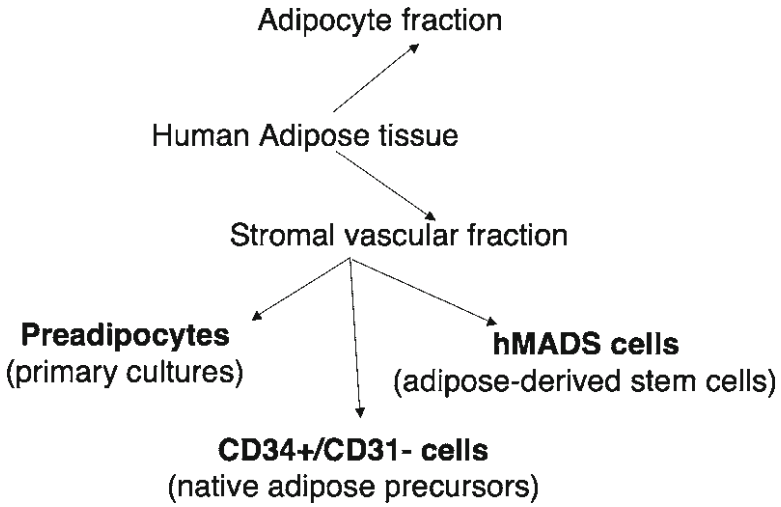

Fig. 1.3 Three types of adipocyte precursors isolated from the stromal-vascular fraction of human adipose progenitors

regulating self-renewal of APs cells could provide a means for better understanding the mechanisms that lead to hyperplasia and excessive development of adipose tissue and could ultimately be translated into clinical interventions. Ex vivo and in vitro cellular models are used to gain insight into cellular and molecular mechanisms of early steps of adipogenesis.

1.5.1 Cellular Models to Investigate Self-Renewal of Human Adipocyte Precursors

Three types of AP populations can be isolated from the SVF of human adipose tissue (Fig. 1.3) to study the regulation of human adipogenesis. Primary cultures of preadipocytes derived from SVF of adipose tissue, although being able to differentiate into adipocytes in vitro, undergo a dramatic decrease in their ability to differentiate, and replicative senescence occurs with serial subculturing, making it difficult to investigate molecular mechanisms in a fully reproducible manner. It has been clearly demonstrated that native APs are contained in the $CD34^{+}/CD31^{-}$ cell population. This population can be easily isolated from the SVF using the immunoselection/depletion protocol as previously described (Bourlier et al. 2008). Finally, human Multipotent Adipose tissue Derived Stem (hMADS) cells are adipocyte progenitors isolated from the SVF of infant adipose tissues (Rodriguez et al. 2005a). As these cells display the characteristics of MSCs, they have been termed hMADS cells.

1.5.1.1 Human Adipose-Derived Stem (hMADS) Cells

hMADS cells exhibit the capacity to self-renew, as cells can be expanded in vitro for more than 160 population doublings (i.e., around 30 passages) while maintaining a normal diploid karyotype. They also differentiate under serum-free adipogenic condition into cells able to exhibit characteristics of human fat cells (Rodriguez et al. 2004). Within 14 days after induction of adipocyte differentiation, more than 90% of cells accumulate intracellular lipids present as multiple droplets. These cells express the major molecular markers, key transcription factors, and nuclear receptors of human white adipocytes. Then, after differentiation, they exhibit the panoply of lipolytic responses, which are characteristic of human adipocytes. Interestingly, hMADs cells respond to the atrial natriuretic peptide, a unique characteristic both in vitro and in vivo of adipocytes from primates (Lafontan et al. 2000). An important feature of differentiated hMADS cells is their ability to secrete leptin and adiponectin within values reported for isolated human adipocytes. More recently, hMADs cells have been described as a faithful model to study human fat cell metabolism (Poitou et al. 2009; Bezaire et al. 2009). Altogether, these data indicate that hMADS cells commit to the adipose lineage at a high rate and differentiate into cells that display a unique combination of properties similar, if not identical, to those of native human adipocytes making them a powerful cellular model to investigate human adipogenesis.

1.5.2 Fibroblast Growth Factor 2 (FGF2) and Activin A, Both Secreted by hMADS Cells, Are Key Regulators of Self-Renewal

Regarding factors regulating proliferation and differentiation of hMADS cells, it has been shown that FGF2 plays a crucial autocrine role (Zaragosi et al. 2006). Analysis of FGF2 secretion revealed that FGF2 is exported to hMADS cell surface without being released into the culture medium, suggesting a strictly autocrine loop. Indeed, treatment of FGF2-expressing hMADS cells with PD173074, a specific FGF receptor inhibitor, decreased dramatically their clonogenicity and differentiation potential. Thus, hMADS cells express a functional autocrine FGF loop that allows maintenance of their self-renewal ability in vitro. Inhibition of Mitogen-Activated Protein Kinase (MEK1) reduced the clonogenic potential of hMADS cells but did not affect their differentiation potential, indicating that the Extracellular Signal Regulated Kinase (ERK)1/2 signaling pathway is partly involved in FGF2-mediated self-renewal. FGF1 is also expressed in human adipose tissue (Widberg et al. 2009). However, the involvement of FGFs in human WAT growth remains to be investigated. Activin A is expressed in the SVF of human adipose tissue and is secreted by undifferentiated hMADS cells and by preadipocytes isolated from different human fat depots. However, its expression is down regulated as soon as cells undergo adipocyte differentiation and is not only a marker of undifferentiated cells

but plays also a functional role in proliferation as observed by activin A supplementation and activin A knockdown expression (Zaragosi et al. 2010). Altogether, data support the hypothesis that activin A represents a novel crucial player controlling self-renewal of human adipose progenitors. We have proposed a model in which activin A is involved in the maintenance of the pool of adipose progenitors in adipose tissue of lean subjects by promoting proliferation and inhibiting differentiation. The molecular mechanisms involved in activin A effects have been identified. Sustained activation or inhibition of the activin A pathway impairs or promotes adipocyte differentiation via C/EBPβ-LAP and Smad2 pathway, respectively, in an autocrine/paracrine manner (Zaragosi et al. 2010). It has been proposed recently that the bone morphogenetic protein pathway, which shares signaling components with the activin pathway, regulates both adipose cell fate determination, differentiation of committed preadipocytes, as well as function of mature adipocytes in mouse models (Schulz and Tseng 2009). Altogether, these data support the hypothesis that the Smad pathway regulates different steps of adipogenesis. Therefore, we propose a model in which FGF2 and activin A, both secreted by undifferentiated cells, are involved in the maintenance of the pool of APs in adipose tissue by promoting proliferation and inhibiting differentiation.

1.5.2.1 Regulation of AP Self-Renewal by Obese Adipose Tissue Microenvironment

Obesity is associated with new macrophages that are recruited into adipose tissue and is accompanied by chronic low-grade inflammation in this tissue (Weisberg et al. 2003; Xu et al. 2003). Interestingly, an increase in the proportion of $CD34^+/CD31^-$ cells exhibiting proliferative potential is observed in obese adipose tissue (Maumus et al. 2008). In addition, it has been recently reported that the differentiation potential of human preadipocytes is inversely correlated with obesity, whereas the pool of precursors cells was positively correlated to BMI (Permana et al. 2004; Isakson et al. 2009), suggesting that the obese microenvironment is capable of inducing proliferation of human preadipocytes while inhibiting their differentiation. Concordantly, human macrophages conditioned medium stimulates proliferation of human preadipocytes in vitro (Lacasa et al. 2007; Keophiphath et al. 2009). Therefore, a model of cross talk between APs and macrophages, in which immunoinflammatory cells that accumulate within adipose tissue with obesity might contribute to fat mass enlargement through paracrine effects on APs, can be proposed. We observed that levels of secreted activin A and of FGF2 are dramatically increased in hMADS cells maintained in the presence of factors secreted by macrophages isolated from obese adipose tissues. Adipose-tissue macrophage secreted factors involved in stimulation of activin A expression remain to be identified. IL-1β and/or TNFα are potent candidates as previous studies have shown in other cell models that activin A secretion is increased upon treatment with these two cytokines (Mohan et al. 2001). Therefore, we propose a model in which FGF2 and activin A, both secreted by undifferentiated cells and induced by signals secreted from

adipose tissue-derived immune cells, are involved in the maintenance of the pool of APs in adipose tissue by promoting proliferation and inhibiting differentiation. The disappearance of macrophages (Cancello et al. 2005), and by consequences, the reduction of activin A levels in adipose tissue, for instance as a consequence of dieting, might be favorable to the formation of additional adipocytes from adipose progenitors upon ending dietary restriction, a situation reminiscent of the "yoyo" phenomenon. Related with the concept of adipose tissue expandability (Sethi and Vidal-Puig 2007), blocking activin A signals that prevent differentiation of APs in adipose tissue of obese patients could represent a new therapeutic avenue to increase the number of new adipocytes and therefore to decrease the accumulation of fat in ectopic tissues not specialized to store large amount of triglycerides. Further studies are required to validate activin A as a candidate biomarker for obesity and associated metabolic complications.

Finally, we would like to point out that APs are also present in skeletal muscles. In 2010, two papers have been published showing a critical role of undifferentiated APs in muscle of mouse models (Uezumi et al. 2010; Joe et al. 2010). Proliferation and differentiation of APs seem to be controlled in healthy skeletal muscle. However, in several pathological situations including obesity, type II diabetes, aging, and muscular dystrophies (Wren et al. 2008; Goodpaster and Wolf 2004), APs undergo adipocyte differentiation, and adipocytes accumulate and replace a large proportion of muscle fibers. As previously described in human adipose tissue, APs are contained in the $CD34^+$ cell population of human skeletal muscle (Pisani et al. 2010). However, it is not know whether skeletal muscle and adipose tissue APs are identical. Nevertheless, clinical knowledge of muscular dystrophy disease may lead to the identification of new regulators of AP biology.

1.6 Plasticity of Human Adipocyte Precursors

Zuk et al. first reported that human adipose tissue contains a population of uncharacterized cells, harvested by liposuction, able in vitro to undergo adipogenic, osteogenic, chondrogenic, and myogenic differentiation (Zuk et al. 2001, 2002), suggesting that APs could be multipotent stem cells. A few years later, isolation and characterization of hMADS cells demonstrated that human adipose tissue is a rich source of multipotent stem cells (Rodriguez et al. 2005a, b). hMADS cells display the potential to undergo differentiation into adipocytes, osteoblasts, and chondrocytes at the single cell level (Rodriguez et al. 2005a; Zaragosi et al. 2006) (Fig. 1.4). The plasticity of hMADS cells led us to investigate their therapeutic potential. Actually, transplantation of hMADS cells into *mdx* mouse, an animal model for Duchenne muscular dystrophy, results in substantial expression of human dystrophin on a long-term basis, and engraftment takes place in non-immunocompromised animals (Rodriguez et al. 2005a). hMADS cells have a weak intrinsic myogenic potential. However, ectopic expression of MyoD1 dramatically increases the ability of hMADS cells to form myotubes in vitro and in vivo (Goudenege et al. 2009).

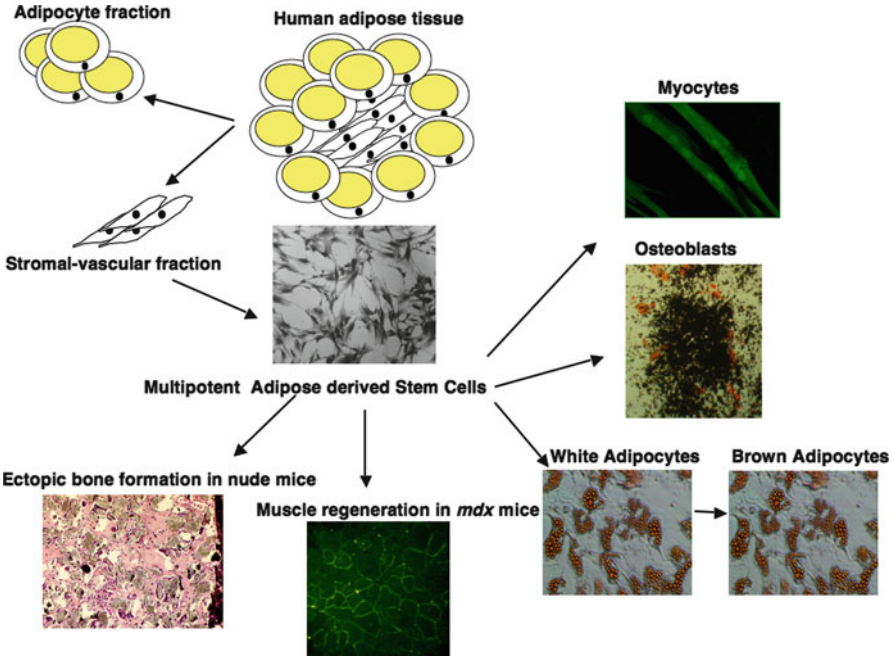

Fig. 1.4 Plasticity of human adipose derived Stem (hMADS) cells. hMADS cells are isolated from the stromal-vascular fraction of young donor adipose tissues. In vitro, they can undergo differentiation into osteoblasts, skeletal myocytes (after ectopic expression of MyoD gene), and white adipocytes, which can turn into brown adipocytes. In vivo, they are able to contribute to muscle regeneration after transplantation into mdx mice or to form ectopic bone after subcutaneous transplantation

When transplanted with a scaffold, hMADS cells are able to form ectopic bone in mouse, suggesting that cells can be used for bone repair (Elabd et al. 2007). More recently, culture conditions to turn hMADSc-white adipocytes into brown adipocytes have been reported (Elabd et al. 2009). Upon chronic exposure to a specific PPARγ agonist, but not to a PPARβ/δ or PPARα agonists, white adipocytes derived from hMADS cells are able to switch to a functional brown phenotype by expressing uncoupling protein 1 (UCP1) protein. This switch is accompanied by an increase in oxygen consumption and uncoupling. The existence of a common precursor for white and brown adipocytes has been a debate for several years. Recently, elegant experiments in mouse have reported the surprising findings of a common precursor between brown adipocytes and skeletal myocytes while white adipocytes derived from a different lineage (Timmons et al. 2007; Seale et al. 2009). These studies also report the existence of a second type of brown adipocytes, localized in WAT, that do not derive from an adipocyte/myocyte precursor. The ability of hMADS cells to differentiate into both white and brown adipocytes strongly suggests that a common precursor for these two types of adipocytes may exist in humans.

Altogether, the plasticity of APs suggests that these cells could be an important tool for cell-mediated therapy. They also represent an invaluable cell model to screen for drugs stimulating the formation and/or the uncoupling capacity of human brown adipocytes that could help to dissipate excess caloric intake of individuals.

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Chapter 2
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

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2.1 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.

2.2 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.

2.2.1 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.

2.2.2 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.

2.2.3 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.

2.2.4 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.

2.3 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 division prior to differentiation, whereas others (such as mouse C3H10T1/2 and

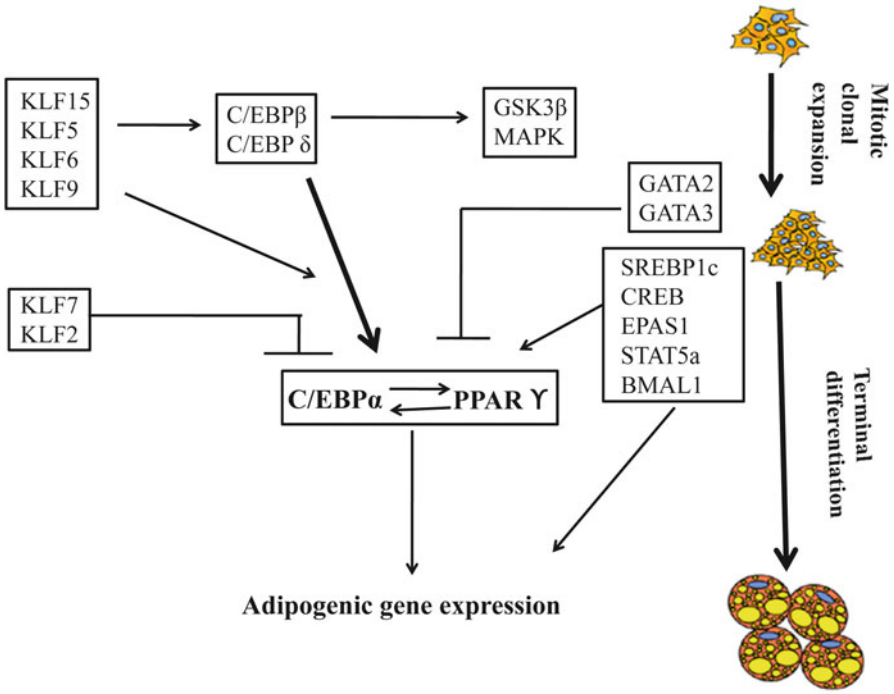

Fig. 2.1 Transcriptional regulation of adipocyte differentiation during 3T3-L1 mitotic clonal expansion and terminal differentiation

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. 2.1).

2.4 Nuclear Regulation of Adipocyte Differentiation

2.4.1 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. 2.2).

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 adipose 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

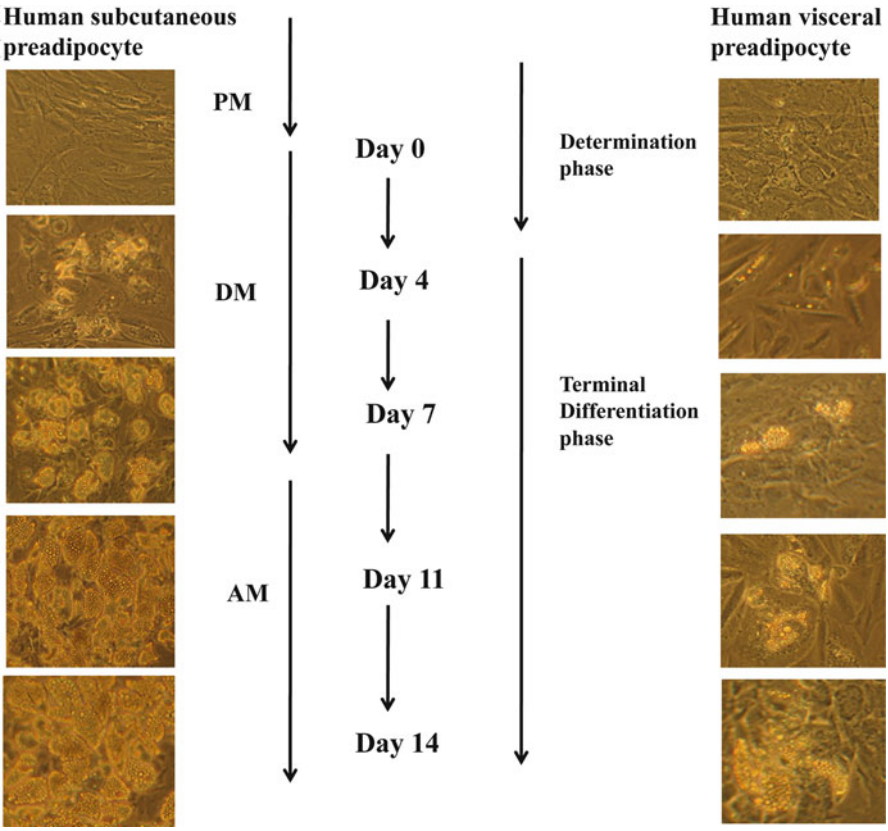

Fig. 2.2 Adipogenesis phases of human subcutaneous and visceral preadipocytes. PM is proliferatium medium and composed of DMEM/Nutrient Mix F-12 medium (1:1, v/v), HEPES, FBS, penicillin and streptomycin. DM is differentiation medium and composed of PM, human insulin, DXM, isobutylmethylxanthine and 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

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, b). 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 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).

2.4.2 *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).

2.5 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.

2.5.1 *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).

2.5.2 *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. 2005a, b; 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).

2.5.3 Other Players in the Regulation of Adipogenesis

2.5.3.1 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.

2.5.3.2 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).

2.5.3.3 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).

2.6 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 3
Brown Adipose Tissue

Martin Klingenspor 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 heater 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 recent 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 • Brown adipose tissue • White adipose tissue • Adipocyte • Progenitor • Mitochondria • ATP synthesis • Non-shivering thermogenesis • Progenitor • Proliferation • Differentiation

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3.1 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 heater 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.

3.2 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 and Neuweiler 2004, p. 130). 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 rodent studies, the subcutaneous interscapular brown adipose tissue depot has been investigated. The interscapular brown adipose tissue is organized into two lobes and displays the prototypical morphology of this heater organ. It is densely

Table 3.1 Mass-specific metabolic rates in 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)	References
Rat	28	0.57	160	Foster and Frydman (1979)
	6	1.44	330	
Mouse	23[a]	1.26	420	Thurlby and Trayhurn (1980)
Djungarian hamster	23[a]	0.48	190	Puchalski et al. (1987)
	–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°C 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

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 tenfold 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 to 190 mW and 330 to 480 mW per gram of brown adipose tissue in warm and cold acclimated rodents, respectively (Table 3.1; 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).

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.

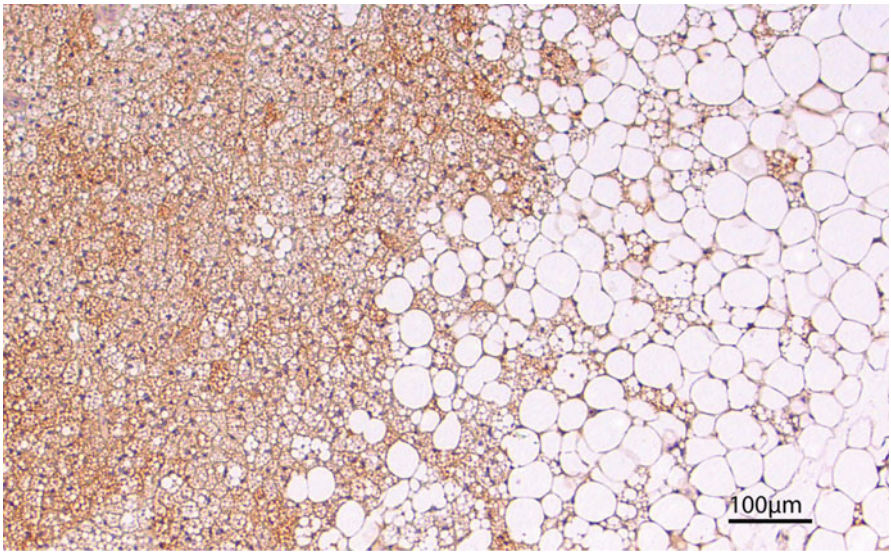

Fig. 3.1 Histology of brown and white adipose tissue. A section of paraffin-embedded interscapular brown adipose tissue was treated with haematoxylin to stain nuclei in *blue*. UCP1 was immunodetected and is indicated by a *brownish* color. Typical multilocular brown adipocytes positive for UCP1 can be seen in the *left half* of the picture, while unilocular white adipocytes devoid of UCP1 dominate the *right half* (image kindly provided by David Lasar)

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. 3.1). 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. 3.6.1), 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, p. 842). More than 400 years after this first anatomical description of brown adipose tissue, the thermogenic function of brown adipose tissue was first 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 defense, 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 Nicholls 2001; Cannon and Nedergaard 2004).

3.3 Molecular Mechanism of Thermogenesis in Brown Adipose Tissue

3.3.1 Mitochondrial Bioenergetics

Early on, 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. This was puzzling 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 transmembraneous 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.

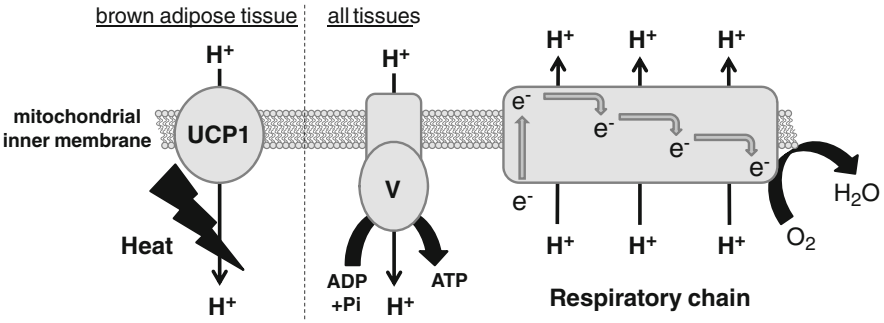

Fig. 3.2 Uncoupling the respiratory chain. The respiratory chain generates a proton gradient across the mitochondrial inner membrane by translocation of protons from the matrix into the intermembrane space. This process is driven by energy-rich electrons from nutrient macromolecules which stepwise release 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 electrons delivered from reduction equivalents out of the citric acid cycle and β-oxidation. After discharging their energy 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. 3.2).

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. 3.2). 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).

3.3.2 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 catalyzed by the uncoupling protein 1 (UCP1), a member of the mitochondrial transporter family. UCP1 was discovered as a purine nucleotide binding protein inserted into the inner mitochondrial membrane with an apparent molecular weight of 32 kD (Heaton et al. 1978; Ricquier and Kader 1976). In reference to the size and the preferential binding of GDP, it was initially termed 32 kD 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 β-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.

3.3.3 Mode of UCP1 Action

In brown adipocytes, in vivo UCP1 activity is under tight control and only dissipates proton motive force in response to appropriate stimuli. 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 triphosphate moiety of purine nucleotides in the cell. A cytosolic concentration of ~1 mM Mg-ATP is required to fully inhibit UCP1 (Nicholls and Locke 1984). It is not finally settled whether UCP1 is completely inactive in the presence of purine nucleotides under

fatty acid-free conditions or may still exhibit some basal leak activity (Parker et al. 2009; Shabalina et al. 2010). 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). This explains the early observation that the oxidation of endogenous fatty acids by addition of ATP, CoA and carnitine conveys coupled respiration in isolated brown adipose tissue mitochondria (Hittelman et al. 1969). 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). In a 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 (=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, but it is not known which of them is correct.

3.4 Activation of Non-Shivering Thermogenesis

A mouse or a rat when acutely transferred from room temperature to the cold (5°C) shows vasoconstriction and a slight drop in body temperature resulting in reduced thermal conductance and an immediate increase in thermoregulatory heat production by shivering and by non-shivering thermogenesis in brown adipose tissue. The latter invokes the activation of UCP1 resulting in uncoupled respiration of brown adipocyte mitochondria. However, 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.

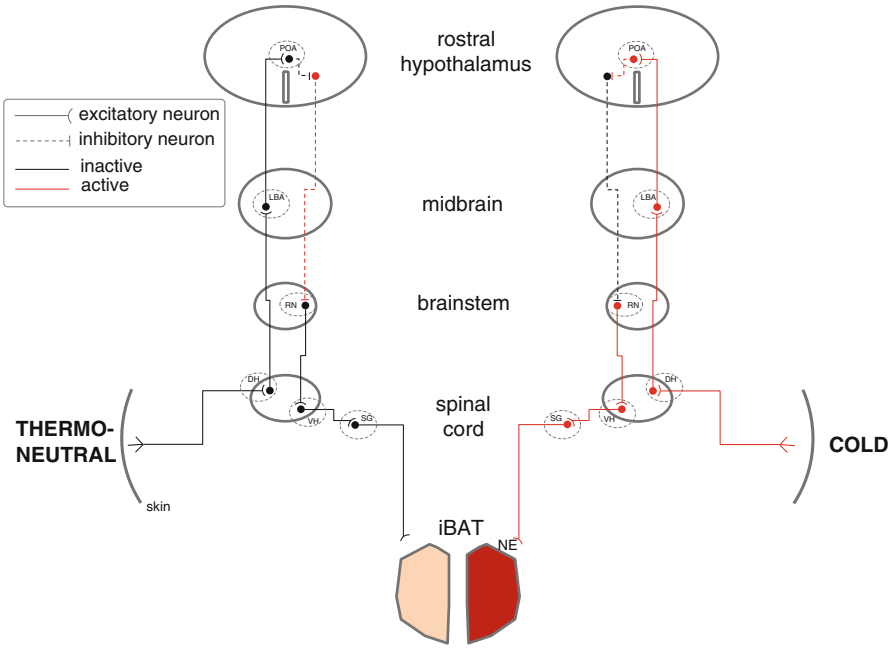

Fig. 3.3 Schematic illustration of the thermal somatosensory reflex. Thermal afferent signals elicited by thermoceptors in the skin are transmitted to the brain and activate inhibitory GABAergic interneurons in the POA of the rostral hypothalamus. In the activated state these interneurons block the activity 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

3.4.1 Neuronal Control of Brown Adipose Tissue Thermogenesis

Upon cold exposure, thermal afferent signals elicited by thermoceptors in the skin are transmitted to the brain and result in efferent stimulation of vasoconstriction in peripheral blood vessels, shivering in skeletal muscle and non-shivering thermogenesis in brown adipose tissue. A current model of this thermal somatosensory reflex suggests that peripheral thermoceptors transmit cold sensation through afferent neuronal projections to a command center in the brain, the preoptic area (POA) in the rostral hypothalamus (Morrison et al. 2008). At thermoneutrality, efferent inhibitory neurons projecting from the POA to the dorsal medial hypothalamus and the brain stem tonically block sympathetic outflow to brown adipose tissue (Nakamura and Morrison 2008).

Transduction of thermal information from the skin via the POA to brown adipose tissue involves multiple steps of neurotransmission and processing (Fig. 3.3).

Primary somatosensory neurons first deliver cold sensation of skin thermoceptors to the dorsal horn of the spinal cord, from where secondary afferent fibers relay this information to the lateral parabrachial nucleus (LBA) in the midbrain. LBA neurons are then activated and stimulate inhibitory GABAergic interneurons in the POA. These interneurons upon stimulation diminish the activity of efferent inhibitory neurons projecting from the POA to caudal brain regions, including the raphe nuclei in the brain stem. Retrograde tracing of the central origins of sympathetic neurons innervating brown adipose tissue identified rostral hypothalamic nuclei, like the nucleus paraventricularis, and several regions in the brain stem including the medullary raphe pallidus and obscurus nuclei (Bamshad et al. 1999). The medullary raphe nuclei are currently regarded as the prime brain stem region involved in the control of brown adipose tissue thermogenesis (but also of peripheral vasoconstriction). Functional studies in interscapular brown adipose tissue support the view that efferent sympathetic premotor neurons emerging from these raphe nuclei descend to preganglionic sympathetic fibers which project from the third and fourth thoracic segments into the stellate ganglion and stimulate the postganglionic sympathetic fibers (Morrison 2004). These postganglionic fibers directly innervate brown adipose tissue and upon preganglionic (cholinergic) stimulation activate non-shivering thermogenesis by the release of their transmitter norepinephrine.

According to this model, skin cooling elicits feedforward activation of inhibitory GABAergic interneurons in the POA resulting in the disinhibition of medullary raphe nuclei and increased firing rates of efferent sympathetic neurons innervating brown adipose tissue. Several studies, however, suggest that independent of this neuronal pathway which implements an essential role of hypothalamic nuclei (POA) in cold responses, the caudal brain stem also appears to receive direct sensoric input from peripheral thermoceptors and can directly elicit increased sympathetic outflow to brown adipose tissue (Bartness et al. 2010; Nautiyal et al. 2008). Moreover, the known thermoregulatory responses controlled by the central nervous system are the result of the integration of thermal information not only from the skin, but also from thermoception in the core of the body, the spine and the brain itself.

3.4.2 Neuroendocrine Stimulation of Brown Adipose Tissue Thermogenesis

Several lines of evidence suggest that brown adipose tissue not only serves in the defense of body temperature but may also dissipate food energy in the defense of energy balance (Himms-Hagen 1979; Nedergaard and Cannon 2010; Rothwell and Stock 1979). 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 peripheral signals and central mechanisms and pathways involved to elicit diet-induced thermogenesis in brown adipose tissue are only partially understood. Many peripheral signals derived from adipose tissue and the gastrointestinal tract are involved in the

regulation of energy intake and expenditure, and it has been suggested that brown adipose tissue is the effector organ for the catabolic action of some of these signals (Spiegelman and Flier 2001). Once these signals reach the brain, they are integrated in the hypothalamus and the brain stem, which are the primary metabolic sensors of the brain. Different neuronal subpopulations within these sensors secrete neuropeptides which either stimulate or inhibit food intake and energy expenditure (Morton et al. 2006).

Soon after the discovery of leptin which is secreted from adipose tissue in direct proportion to body fat mass, it was reported that intraperitoneal leptin injection in mice causes an increase of sympathetic nerve activity in brown adipose tissue without affecting food intake (Collins et al. 1996). Leptin binds to leptin receptors in neurons of the arcuate nucleus in the hypothalamus and stimulates the synthesis and neurosecretion of alpha-melanocyte stimulating hormone (α-MSH). Binding of α-MSH to melanocortin receptors, mainly MC4R, not only inhibits food intake but also increases the sympathetic outflow to brown adipose tissue. Peripheral leptin injection stimulates UCP1 expression of brown adipose tissue in wildtype, but not in $Mc4r^{-/-}$ mice (Ste et al. 2000). A large proportion of neurons in the paraventricular hypothalamus identified by retrograde transsynaptic tracing to be part of the efferent outflow from the brain to the sympathetic innervation of brown adipose tissue also show Mc4r expression (Song et al. 2008; Voss-Andreae et al. 2007). Injection of the melanocortin receptor agonist MTII into the paraventricular hypothalamus caused a dose-dependent rise in interscapular brown adipose tissue temperature (Song et al. 2008).

The ventromedial hypothalamus was identified early on as a satiety center and has been implicated in the regulation of the sympathetic outflow to brown adipose tissue by multiple studies. Neuroanatomical evidence for the latter function is rather weak (Bamshad et al. 1999) and the published functional evidence has been questioned (Morrison et al. 2008). A revival of the role of the VMH in controlling brown adipose tissue thermogenesis has occurred recently by demonstrating that in the rat, both systemic hyperthyroidism and T3 injections into the VMH stimulate sympathetic nerve activity in brown adipose tissue (Lopez et al. 2010). The neuronal pathway by which this effect is mediated remains to be elucidated. In any case, it might explain a significant proportion of the long-known necessity of thyroid hormones for brown adipose tissue thermogenesis (reviewed in Silva 2006).

Taken together, considerable knowledge has already accumulated on the endocrine effectors of sympathetic outflow to brown adipose tissue, but it is not clear at which level thermal and metabolic efferent pathways converge.

3.4.3 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

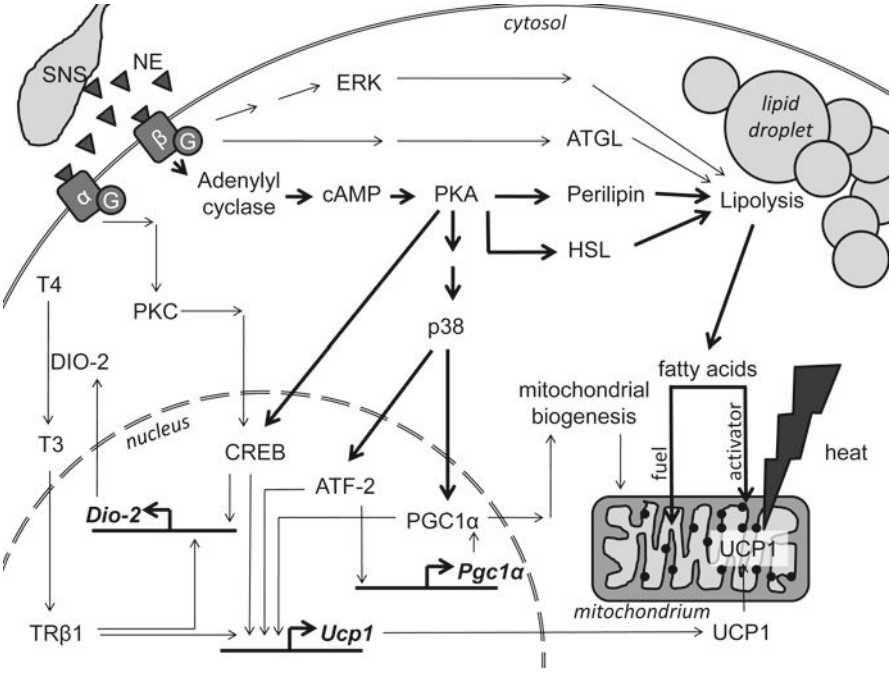

Fig. 3.4 Signaling pathways in brown adipocytes. Release of norepinephrine at the plasma membrane of brown adipocytes leads to activation of G-protein coupled adrenoreceptors. The resulting signaling network prominently relies on PKA which mediates both acute and longer term consequences. The immediate increase in heat generation is affected by fast liberation of fatty acids at the lipid droplets. A network of adaptive gene expression is 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

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 the plasma membrane of brown adipocytes (Fig. 3.4). Adrenoceptors (AR) of all three known β-subtypes (β1-, β1- and β3-AR) are expressed in brown adipocytes of which the β3-adrenoreceptor seems to be most relevant for the acute activation of thermogenesis (Lafontan and Berlan 1993). This G-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. 3.4).

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 are direct PKA targets. Their phosphorylation leads to an increased rate of lipolysis and emergence of cytosolic 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 not entirely known, 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 β-oxidation pathway, while in their unbound non-esterified anion form, they act as potent activators of UCP1 (Sect. 3.3.3). 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.

3.5 Recruitment of Non-Shivering Thermogenesis Capacity

3.5.1 Magnitude of Cold-Induced Increase in Heat Production Capacity

A normal B6 mouse (body 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 to prevent life-threatening hypothermia. When previously housed at thermoneutrality, however, the mouse only has a maximal heat production capacity (HP_{max}) 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. 3.5). Acclimation of the mouse to moderate cold conditions (18°C) for >3 weeks causes an increase of HP_{max} to ~1,000 mW and enables the mouse to pass the acute cold challenge test without problems. A mouse cold acclimated at 5°C will further increase HP_{max} to ~1,200 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

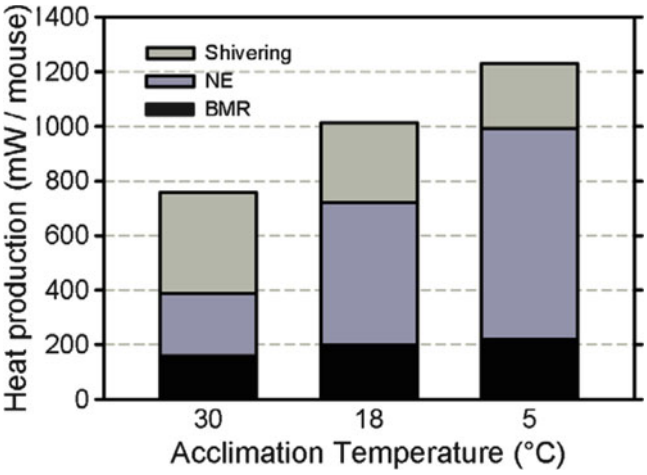

Fig. 3.5 Maximal cold-induced heat production. Contribution of basal metabolic rate and NE-induced thermogenesis to maximal cold-induced heat production. Wildtype B6 mice were acclimated to 30, 18, and 5°C for several weeks. Basal metabolic rate was measured during 3–4 h at 30°C, NE-induced thermogenesis 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)

norepinephrine (Heldmaier 1971). Comparing B6 mice acclimated to 30, 18, and 5°C, a three to fourfold increase in norepinephrine-inducible non-shivering thermogenesis capacity can be observed (Fig. 3.5). 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. 3.5). 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.

3.5.2 *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, among them prominently 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 (see Sect. 3.4.3 and Fig. 3.4). 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. 3.4). 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 peroxisome proliferator activated receptor γ (PPARγ) coactivator 1α (PGC1α) gene. PGC1α is not only positively regulated by p38 via 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 actions beyond the centrally mediated effects on brown adipose tissue already discussed above (see Sect. 3.4.2). 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 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 (Forner et al. 2009; Watanabe et al. 2008). The key organelle implicated in these processes is the mitochondrium. Mitochondrial biogenesis is strongly activated in the cold and results in a more than threefold increase in the amount of mitochondrial protein per brown adipocyte (Rafael et al. 1985; Klingenspor et al. 1996b). 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- (cyclooxygenase-4 [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. 1996b).

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 3–4 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 are all aspects of adaptive hyperplasia of the entire organ brown adipose tissue in response to cold exposure.

3.5.3 *Non-Shivering Thermogenesis Outside of Brown Adipose Tissue*

The recruitment of brown adipose tissue in the cold is clearly the major contributor to increased non-shivering thermogenesis capacity but whether this heater organ is the exclusive site of adaptive thermogenesis in mammals is controversial (Golozoubova et al. 2006; Meyer et al. 2010; Ukropec et al. 2006). The UCP1 knockout mouse has been utilized by several groups to address this question. A B6 UCP1 knockout mouse (body weight = 23 g) when acclimated to thermoneutrality has a HP_{max} of ~710 mW which closely resembles the thermogenic capacity of a wildtype B6 mouse (see Fig. 3.5 and Sect. 3.5.1). In this acclimation state, the presence or absence of UCP1

has no impact on the thermogenic performance in the cold as brown adipose tissue is in a non-recruited (atrophied) state, and both genotypes mainly have to rely on shivering thermogenesis. In contrast, when comparing wildtype and UCP1 knockout mice acclimated to room temperature, the latter become hypothermic when transferred to the cold whereas wildtype mice are cold resistant (Enerback et al. 1997).

Notably, UCP1 knockout mice can survive for months at 5°C when first pre-acclimated to cool conditions (18°C). Pre-acclimation results in an increase of HP_{max} to ~870 mW, and subsequent cold acclimation to 5°C further mounts HP_{max} to ~940 mW (Meyer et al. 2010). A straightforward conclusion from these data is that UCP1 knockout mice do exhibit adaptive thermogenesis, perhaps by increasing the capacity for shivering thermogenesis in skeletal muscle and/or recruitment of non-shivering thermogenesis capacity in other tissues. Pertaining to shivering capacity, the search for structural and functional adaptations of skeletal muscle, however, revealed no striking differential training effects when comparing cold-acclimated wildtype and UCP1 knockout mice (Meyer et al. 2010), despite continuous shivering of UCP1 knockout in the cold (Golozoubova et al. 2001). Therefore, it has been suggested that mice living at thermoneutrality cannot bail out their maximal capacity for shivering thermogenesis capacity in skeletal muscle due to limitations in physical endurance which is improved by pre-acclimation. The metabolic scope, representing the ratio of maximal heat production and basal metabolic rate, is a reliable measure of fitness. The metabolic scope of thermoneutral acclimated wildtype and UCP1 knockout mice at their cold limit is 4.7- and 4.3-fold BMR, corresponding to HP_{max} of 740 and 710 mW, respectively. This closely resembles the sustained metabolic scope of laboratory mice in the cold which ranges between four- and fivefold BMR in subsequent exposures to 8, 0, −10, and −15°C ambient temperature (Konarzewski and Diamond 1994). Based on these data, cold acclimation does not invoke a striking improvement of physical endurance. This is in favor of the view that UCP1 knockout mice in the absence of functional brown adipose tissue can also recruit significant capacity for thermogenesis at other sites of the body. Notably, in cold-acclimated UCP1 knockout mice, a striking remodeling of white adipose tissue occurs with the appearance of multilocular brown adipocyte-like cells, a nearly fourfold increase in mitochondrial respiration capacity (cytochrom-c-oxidase activity) and an increased expression signature of brown adipocytes (Meyer et al. 2010; Ukropec et al. 2006). It remains to be resolved whether this increased respiratory capacity in WAT may contribute to adaptive thermogenesis in UCP1 knockout mice.

3.6 Origin of Brown Adipose Tissue

3.6.1 Brown Adipocytes

For decades, researchers have tried 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 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 a study demonstrated that both brown adipocytes and skeletal muscle myotubes derive from progenitor cells expressing Myf-5, a transcription factor well known for its important role during skeletal muscle cell differentiation (Seale et al. 2008). White adipocytes, in contrast, had never expressed Myf-5. Furthermore, a single transcription factor named PR domain containing 16 (PRDM16) has been identified as a molecular switch deciding the fate of a common progenitor cell ("adipomyoblast") to become either a myotube or a brown adipocyte and vice versa (Seale et al. 2007, 2008). Knocking down PRDM16 in preadipocytes isolated from brown adipose tissue leads to formation of myotubes in primary cell culture while overexpression of PRDM16 in myoblasts leads to differentiation to brown adipocytes.

Despite all differences between white and brown adipocytes, they share several key transcription factors that are involved in adipogenic differentiation (Rosen and MacDougald 2006). It could be argued that brown adipocytes are rather muscle type cells with an adipogenic expression signature than the other way round. These adipogenic factors include the 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). A further transcription factor expressed in both white and brown adipose tissue is the forkhead factor C2 (FOXC2) which upon transgenic, forced expression confers several brown adipocyte characteristics to white adipose tissue (Cederberg et al. 2001). Its specific requirement for brown adipocyte differentiation, however, has not been demonstrated. Thus, the only known factor conferring identity to brown adipocytes during differentiation remains PRDM16.

Apart from endogenous factors, external stimuli have been identified that induce brown adipocyte differentiation. Of paramount importance in this context is certainly norepinephrine which leads to increased proliferation in brown adipose tissue (see Sect. 3.5.2). A novel effector was recently identified in primary cell culture of brown preadipocytes. Treatment with bone morphogenetic protein 7 (BMP7) leads to their differentiation into typical brown fat cells. Conversely, mice devoid of BMP7 show a drastically decreased brown adipose tissue mass and UCP1 expression (Tseng et al. 2008). So far neither the source of this BMP7 signal in vivo nor the information it conveys are known and thus the physiological relevance remains to be assessed.

The fundamental knowledge on the regulation of cellular differentiation of brown adipocytes, however, is not entirely transferable to brown adipocytes found interspersed within white adipose tissue depots. The β-adrenergic recruitment of interspersed brown adipocytes e.g. requires the presence of COX-2, while brown adipocytes in pure brown adipose tissue depots do not (Madsen et al. 2010; Vegiopoulos et al. 2010). A hypothesis recurringly brought forward in this context is that white adipocytes may be able to transdifferentiate into brown adipocytes and thus constitute a second type of brown adipocyte distinct from those found in brown adipose tissue depots. The emergence of fat cells with an intermediate appearance in terms of lipid droplet and mitochondrial number following a β-adrenergic stimulus seems to support this view (Barbatelli et al. 2010). On the other hand, the stromal-vascular (non-adipocyte) fraction of white adipose tissue contains a subpopulation that in primary cell culture forms thermogenically competent, UCP1-containing cells when properly stimulated (Petrovic et al. 2010). It is thus unclear whether the source of brown adipocytes in white adipose tissue depots is mainly transdifferentiation or differentiation of a separate pool of precursor cells within the tissue or both. In view of a possible pharmacological intervention leading to transdifferentiation of fat-storing white into fat-burning brown adipocytes, this particular field needs to be intensely investigated.

3.6.2 Emergence of Brown Adipose Tissue in Mammals

Beyond the ontogenic origin of brown adipocytes, their evolutionary history has been object of investigation. Based on the study of a small subset of investigated species, it is often stated that brown adipose tissue can be found in all Eutherian mammals (>5,000 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*), a species belonging to the Afrotheria, a group of mammals thought to be at the base of the Eutherian radiation (Mzilikazi et al. 2007). On the other hand, the UCP1 gene, encoding the unique molecular marker of brown adipocytes, 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). The actual origin of brown adipose tissue may date back much earlier 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). More physiological studies must be conducted to demonstrate the capacity 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 eight hibernators in a body mass range of <10 g to 5 kg, it was observed that hibernators have about twice the amount of brown adipose tissue (Heldmaier 1971) which most likely represents an 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 (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. 1996a). In fact, the 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 1,600 mW as compared to 1,000 mW in warm-acclimated hamsters in summer (Heldmaier et al. 1990). This is accomplished by a nearly threefold 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. 3.7), such differences between species in the recruitment mechanisms should be kept in mind.

3.6.3 UCP1

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. 3.6) (Jastroch et al. 2005, 2008). Not 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. 3.6) (Jastroch et al. 2008). In evolutionary time, however, marsupials are

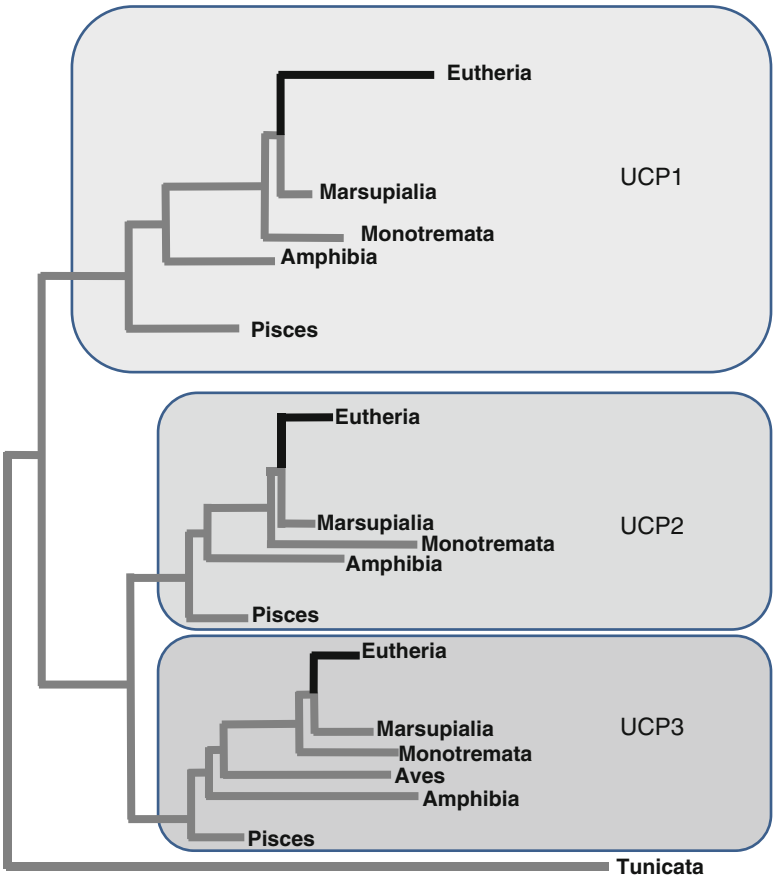

Fig. 3.6 Schematic representation of a UCP species tree. The molecular phylogeny of the UCP family was analyzed using 79 UCP sequences from vertebrates. Branch lengths represent the number of substitutions; the Eutherian branches are highlighted in *black* (for details see Hughes et al. 2009)

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; Rial and Zardoya 2009).

3.7 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. Less successful attempts to reduce the intensity of these adipose FDG PET signals, including the pretreatment 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 AR 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 warm 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. 3.7).

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 analyzed a large number of clinical scans which had been performed on patients with different indications (Cypess et al. 2009; Au-Yong et al. 2009). 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). Notably, the prevalence of brown adipose tissue positive patients was largely altered by season. The percentage of brown adipose tissue positive scans increased fourfold from summer to winter, and also the number of FDG positive depots increased in the winter season (Au-Yong et al. 2009). 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

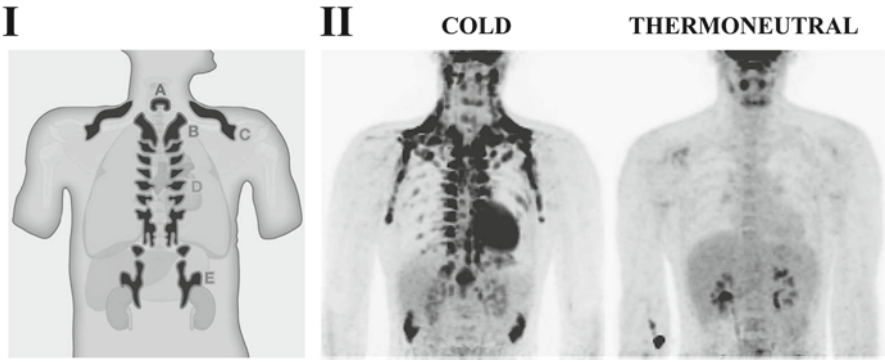

Fig. 3.7 Brown adipose tissue in humans. I: In humans brown adipose tissue depots are found in the tracheal (*A*), mediastinal (*B*), supraclavicular (*C*), paravertebral (*D*) and supra-/perirenal (*E*) areas (depicted from Enerback 2010). II: In a comparison of FDG PET scans taken after cold exposure and in thermoneutral conditions, the localization of active brown adipose tissue is evident (depicted from van Marken Lichtenbelt et al. 2009)

adipose tissue was negligible whereas in the cold condition, a large number of the subjects showed FDG uptake in several brown adipose tissue depots. Cold-induced FDG uptake in brown adipose tissue was detected in 16 out of 31 and 23 out of 24 young 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). 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% (Lee et al. 2010) 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, but notably, the seasonal increase in brown adipose tissue positive scans in winter is more closely associated with the change in photoperiod than ambient temperature (Au-Yong et al. 2009). 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 (Cypess et al. 2009; Saito et al. 2009; van Marken Lichtenbelt et al. 2009; Virtanen 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 urgent 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). Several critical papers have dismissed this possibility in the past (Astrup et al. 1984; Cunningham et al. 1985). In the light of the present FDG PET findings, the 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 was calculated based on glucose (FDG) uptake measurements. Taking into account that only 10% of the oxygen consumption in brown adipose tissue is fueled by glucose, the estimated metabolic rate could allow us to burn ~4 kg of adipose tissue in 1 year (Virtanen et al. 2009), corresponding to ~120 MJ (30 MJ/kg adipose tissue). 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 dissipate heat at a power of ~50 mW/g of tissue which is below the values reported for brown adipose tissue in rodents (Table 3.1). Based on such theoretical assumptions, it seems feasible to expect a significant contribution of brown adipose tissue to the defense of energy balance in humans. 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 dissipate 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 4
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 secrete 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 in WAT distribution and function and the impact of fat distribution on obesity-related diseases.

Keywords White adipose tissue • Anatomy • Metabolism • Adipokines

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4.1 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 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.

4.2 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 cells 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 transcriptional 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 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 capacities. 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 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 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.

4.3 Functions of WAT

4.3.1 Metabolic Function of WAT

One of the main characteristics of the energy metabolism is 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

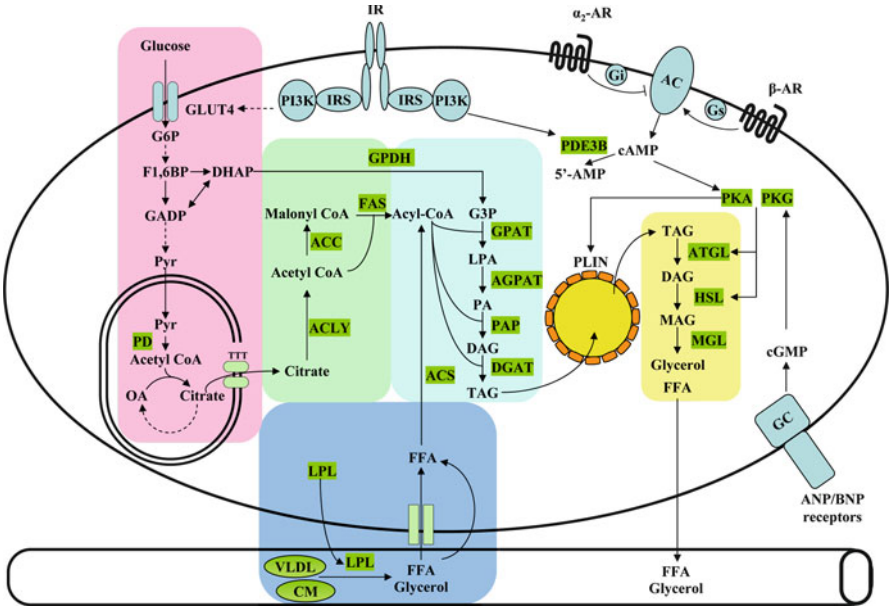

Fig. 4.1 Metabolic functions of WAT. The main metabolic functions of WAT are the storage of energy in the form of triglycerides and the mobilization of this energy when it is required by the body. In WAT, triglycerides can be synthesized (*turquoise box*) following the uptake and metabolism of glucose (*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

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. 4.1).

4.3.1.1 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 in 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.

4.3.1.2 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 white 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. Un-ionized 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. Un-ionized 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 acids analogs in these mice revealed a 60–70% reduction in the uptake of these analogues 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 receptors responsive 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).

4.3.1.3 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 have 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).

4.3.1.4 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).

4.3.2 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 this 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 secreted proteins 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).

4.3.2.1 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 receptor. Thus, *db/db* mice, which only lack the ObRb isoform (Lee et al. 1996), have a phenotype indistinguishable than 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 others 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.

4.3.2.2 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).

4.3.2.3 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 percent of fat in the trunk, but not with percent 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 preadipocyte 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 as 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).

4.4 Depot-Specific Differences of WAT

4.4.1 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. 4.2).

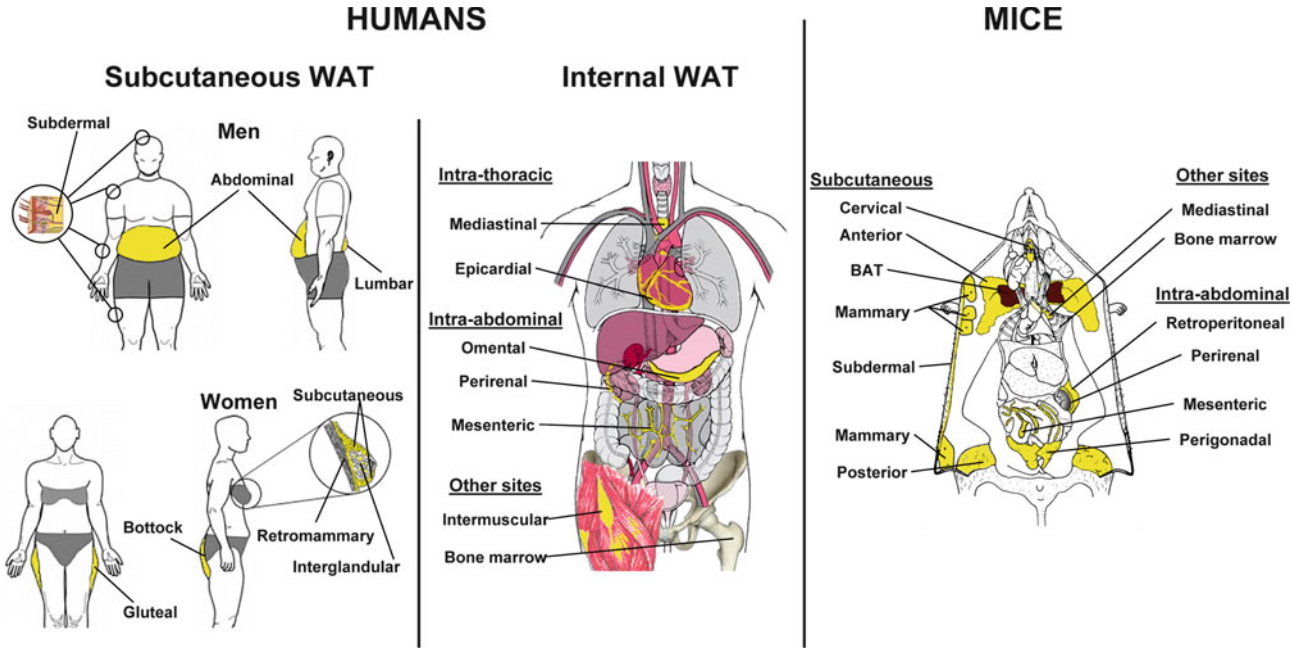

Fig. 4.2 White adipose tissue distribution in humans and mice. White adipose tissue is distributed throughout the body in both humans and mice. The two major compartments of WAT are located subcutaneously and intra-abdominally, although WAT can be found in other regions, such as the intra-thoracic region. 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

4.4.1.1 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 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.

4.4.1.2 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).

4.4.1.3 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).

4.4.1.4 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 strains 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 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).

4.4.1.5 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).

4.4.2 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.

4.4.3 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.

4.5 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 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 WAT. 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 differences 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

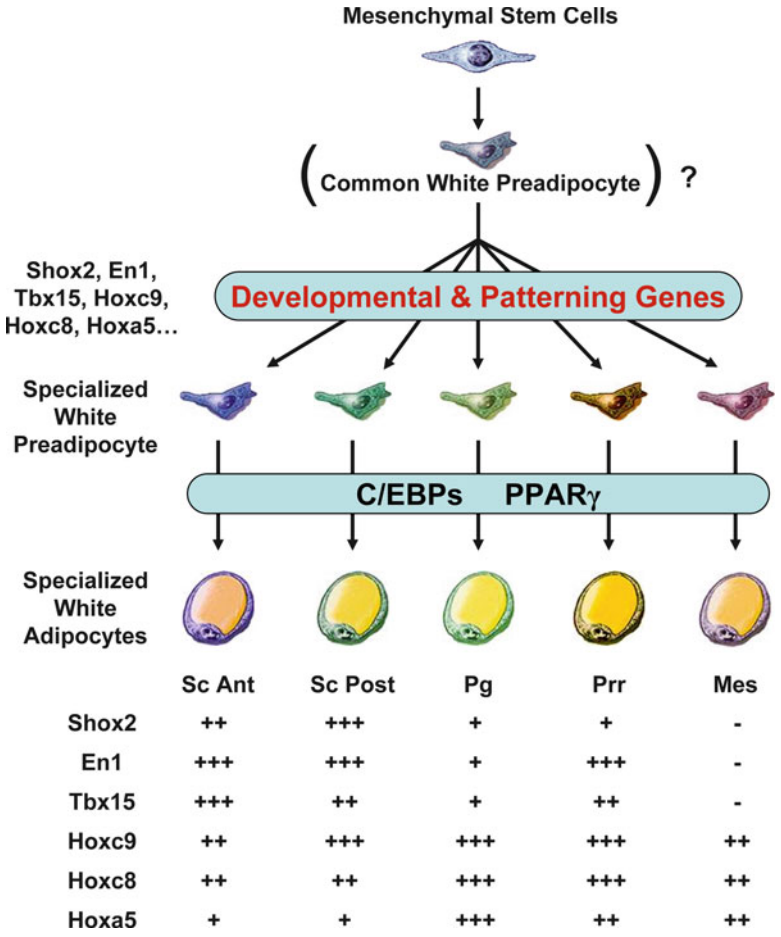

Fig. 4.3 Hypothetical scheme of the adipogenic lineage of the different WAT depots. Under the influence of developmental and patterning genes including *Shox2*, *En1*, *Tbx15*, *HoxC9*, *HoxC8*, and *HoxA5*, mesenchymal stem cells or a pool of common white preadipocyte precursors give rise to different 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

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. 4.3).

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-abdominal 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 has 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).

4.6 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.

4.7 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 5
Sex Differences in Body Fat Distribution

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. A marked sex dimorphism and large interindividual variations are observed in fat distribution, and excess accumulation of visceral fat is a strong predictor of cardiometabolic risk in both sexes. However, adipose tissue cellularity and function are distinctly related to obesity in women and men. 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. Proneness to 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, adipose tissue exposure to active androgens is known to inhibit adipogenesis and lipogenesis. Estrogens have important central effects 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

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5.1 Introduction

Epidemiological data have now shown that worldwide obesity rates have steadily increased over the 1980s and 1990s (Flegal et al. 1998, 2002; Katzmarzyk and Mason 2006; Kuczmarski et al. 1994; Seidell and Flegal 1997). Although several studies seem to show that obesity rates have now entered a period of relative stability, with smaller increases in adults and also possibly in children (Basu 2010; Flegal et al. 2010; Han et al. 2010; Rokholm et al. 2010; Schneider et al. 2010), the prevalence of obesity remains elevated in many countries and still represents a major issue from the scientific and clinical standpoints (Flegal et al. 2010; US Department of Health and Human Services and Carmona RHM 2003).

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), the latter which includes type 2 diabetes, dyslipidemic states, and alterations in several cardiovascular disease risk factors (Bray 1985; Després 1991; Després 1994a; Ford 1999; Garrison et al. 1987; Kissebah et al. 1989; NIH Consensus Conference 1985; Sims and Berchtold 1982). Among the metabolic disorders closely related to obesity, projected worldwide increases in the incidence of type 2 diabetes are of considerable concern (Ekoe et al. 2001; Flegal et al. 2002; Shaw et al. 2010).

The susceptibility to develop type 2 diabetes and cardiovascular disease in relation to excess body weight is highly variable among overweight and obese individuals. Some appear to be relatively protected from the development of medical problems in relation to their excess body fatness, even in the obese range (Andres 1980; Brochu et al. 2001; Primeau et al. 2010; Sims 1982). One of the critical determinants of disease in overweight or obese individuals of both sexes is the presence of a central pattern of fat distribution, more specifically, of large fat stores within intra-abdominal anatomical structures such as the mesentery and greater omentum (Arner 1995; Després et al. 1990; Wajchenberg 2000). This phenotype, which is also termed visceral obesity, has now clearly emerged as one of the most prevalent manifestation 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 human males and females relate to cardiometabolic disease risk.

5.2 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 7. After adrenarche, girls accumulate fat mass more rapidly and eventually reach slightly higher values than boys (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 vs. 16 kg respectively) (Van Loan 1996). Conversely, total fat mass increases proportionately more in girls (Van Loan 1996). 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 various populations (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. The amount of fat that accumulates at the abdominal level can be estimated using imaging techniques such as computed tomography (CT) 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).

A most striking feature is the very large interindividual 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 body mass index (BMI) range. As 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, 1994b; Despres and Lemieux 2006; Lemieux and Despres 1994). The following sections will review the metabolic alterations related to visceral obesity and describe the potential link between adipose tissue characteristics and disease development.

5.3 Linking Body Fat Distribution to Disease Risk

In both men and women, visceral adipose tissue accumulation has been positively associated with fasting insulin and C-peptide levels, as well as with the insulin response to an oral glucose challenge (Després et al. 1989; Pouliot et al. 1992). These associations appear to be independent from concomitant variations in total body fat mass (Lemieux and Despres 1994; Wajchenberg 2000). The negative correlation between CT-measured visceral fat accumulation and glucose disposal assessed using the hyperinsulinemic-euglycemic clamp technique is also a well-established phenomenon (Bonora et al. 1992; Brochu et al. 2000; DeNino et al. 2001; Dvorak et al. 1999; Goodpaster et al. 1997; Rendell et al. 2001; Sites et al. 2000; Wilson et al. 1987). In addition, prospective studies have shown that visceral obesity is associated with an increased risk of developing type 2 diabetes (Bergstrom et al. 1990; Boyko et al. 2000). The dyslipidemic state of visceral obesity includes increased serum levels of triglycerides and apolipoprotein B (Despres and Lemieux 2006; Lemieux et al. 2000). These alterations are associated with low high-density lipoprotein (HDL)-cholesterol concentrations and a higher proportion of small, dense low-density lipoprotein (LDL) particles (Despres and Lemieux 2006; Lemieux et al. 2000). Additionally, visceral obesity is associated with endothelial dysfunction, elevated blood pressure, low-grade, chronic inflammation, and prothrombotic defects (Couillard et al. 2005; Juhan-Vague and Alessi 1999).

Interestingly, sex differences in visceral adipose tissue accumulation account for an important portion of the well-known difference in cardiometabolic risk factors between men and women. For example, sex differences in glucose tolerance or plasma lipid and lipoprotein levels, including concentrations of apolipoprotein B and triglycerides were largely eliminated by statistical control for visceral adipose tissue accumulation (Lemieux et al. 1994). Similar studies have been performed on the contribution of body fat distribution patterns to sex differences in other markers such as the presence of small, dense LDL particles, HDL particle size, and circulating inflammatory markers (Cartier et al. 2008, 2009, 2010; Lemieux et al. 2002; Pascot et al. 2001, 2002). Consistent with the multifactorial etiology of the alterations found in the metabolic syndrome, a portion of the sex differences in some of these markers remained after statistical control for visceral fat accumulation. However, it can be concluded that visceral adipose tissue accumulation, along with other hormonal and genetic factors, is a significant contributor to sex-related differences in several metabolic parameters. The predominant association of excess

visceral fat accumulation with metabolic disease appears to emerge not only from the anatomic localization of this depot, but also from the intrinsic physiological and metabolic nature of each abdominal adipose tissue compartment. These characteristics are reviewed next.

5.3.1 Adipose Tissue Morphology

The size of each fat compartment results from the integration of adipocyte number and cell size. Important interindividual 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 small adipocytes (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. 5.1). This plateau suggests that the presence of large adipocytes triggers the generation of new

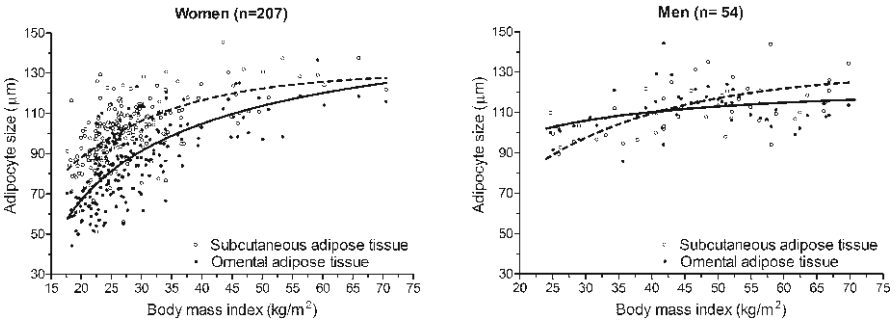

Fig. 5.1 Adipocyte size in subcutaneous and omental adipose tissue of women and men. Mean adipocyte diameter of subcutaneous and omental adipose tissue according to BMI in women (n = 207) and men (n = 54) undergoing abdominal elective surgery or biliopancreatic diversion. Women were 47.7 ± 5.5 years old (range: 30–68.3 years) with a mean BMI of 28.5 ± 8.8 kg/m^2 (range: 17.6–70.5 kg/m^2). Men were 44.3 ± 9.6 years old (range: 22.6–61.2 years) with a mean BMI of 47.3 ± 13.1 kg/m^2 (range: 24.6–69.9 kg/m^2). Adapted from Veilleux et al. (Veilleux et al. 2011 with permission) and Boivin et al. (Boivin et al. 2007)

adipocytes to store excess dietary fat in severely obese individuals. Accordingly, adipocyte number is positively associated with adiposity measures, 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). Indeed, a more pronounced and earlier elevation in cell number is observed in adipose tissue of children who had become obese before adulthood (Jaenicke and Waffenschmidt 1979). 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 adipocyte generation 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.

Sex and anatomical localization have been shown to be important determinants of mean adipose tissue cellularity (Boivin et al. 2007; Fried and Kral 1987; Salans et al. 1973; Tchernof et al. 2006). 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). Sex differences in visceral adipocyte size and number have not yet been systematically studied. We know that lean to moderately obese men tend to have larger omental adipocytes than women (Fried and Kral 1987) (Fig. 5.1). Conversely, studies tend to demonstrate that omental and subcutaneous adipocytes of massively obese women reach a higher maximal cell diameter value (approximately 130 μm) compared to massively obese men (approximately 120 μm) (Spalding et al. 2008) (Fig. 5.1). Moreover, maximal adipocyte size of both the abdominal subcutaneous and omental fat depots in women is reached at higher BMI values compared to men (Spalding et al. 2008) (Fig. 5.1). 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 preadipocyte 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).

Regional differences in adipose tissue cellularity are also sex-specific in humans. In women, omental adipocytes are 20–30% smaller than abdominal subcutaneous adipocytes for much of the spectrum of adiposity values (Boivin et al. 2007; Fried and Kral 1987; Ostman et al. 1979; Rebuffe-Scrive et al. 1990; Reynisdottir et al. 1997; Tchernof et al. 2006) (Fig. 5.1). In fact, omental and abdominal subcutaneous adipocytes tend to reach a similar size at very elevated BMI values (>60 kg/m^2) (Boivin et al. 2007; Ostman et al. 1979; Rebuffe-Scrive et al. 1990; Reynisdottir et al. 1997; Tchernof et al. 2006). Even in severely obese subjects, only a few individuals have omental adipocytes as large as their abdominal subcutaneous counterparts (Tchernof et al. 2006). Women also tend to have larger adipocytes in the lower body subcutaneous regions compared to the abdominal subcutaneous sites (Fried and Kral 1987; Tchoukalova et al. 2010). Mesenteric adipocytes are similar in size to those of abdominal subcutaneous fat (Fried and Kral 1987). Interestingly, as women reach menopause, depot differences in adipocyte size seem to be attenuated since the size of omental, but not of subcutaneous adipocytes, is increased (Tchernof et al. 2004). The presence of larger omental adipocytes along with increased visceral fat accumulation in postmenopausal women suggests that ovarian hormone deficiency may affect adipocyte hypertrophy in this depot (Tchernof et al. 2004).

In men, adipocytes of the visceral and abdominal subcutaneous fat compartments have similar sizes across the range of adiposity values (Boivin et al. 2007; Edens et al. 1993; Fried et al. 1993; Fried and Kral 1987; Marin et al. 1992c; Rebuffe-Scrive et al. 1990). Although differences are minimal, abdominal subcutaneous adipocytes appear to be slightly larger than omental adipocytes in men with a BMI lower than 40 kg/m^2. The opposite is observed in men with higher BMIs (Boivin et al. 2007; Edens et al. 1993; Fried et al. 1993; Fried and Kral 1987; Hoffstedt et al. 1997). This suggests that visceral adipocytes become larger than abdominal subcutaneous adipocytes in men, as opposed to women, who display larger subcutaneous adipocytes throughout the adiposity continuum. While omental and subcutaneous adipocyte cellularity is highly similar in men, adipocytes from the mesenteric compartment are those with the highest mean size. These cells are at least 30% larger than those of all other fat compartments (Fried and Kral 1987). 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.

5.3.2 Adipose Tissue Metabolism and Cardiometabolic Risk Factors

The association between increased adipocyte size and metabolic alterations is now well established (Arner et al. 2010; Bjorntorp et al. 1971; Ledoux et al. 2009; Lundgren et al. 2007; Weyer et al. 2000). Recent studies demonstrated that fat cell size is a critical determinant of adipose tissue function, independent of obesity itself (Arner et al. 2010; Bjorntorp et al. 1971; Hoffstedt et al. 2010; Ledoux et al. 2009; Lundgren et al. 2007; Weyer et al. 2000). Subcutaneous adipocyte size is related to measures of insulin resistance in both women and men (Arner et al. 2010; Hoffstedt et al. 2010; Ledoux et al. 2009; Lundgren et al. 2007; Weyer et al. 2000). In addition to cross-sectional analyses using whole body insulin resistance, two prospective studies showed that subcutaneous adipocyte hypertrophy is an independent risk factor for developing type 2 diabetes independent of adiposity and body fat distribution (Lonn et al. 2010; Weyer et al. 2000). A detailed characterization of subcutaneous adipocyte morphology revealed that adipose tissue hypertrophy, rather than absolute adipocyte size, is associated with higher fasting insulin levels and homeostatic model assessment of insulin resistance index independent of body fat mass (Arner et al. 2010). However, further adjustment for body fat distribution demonstrates that visceral fat accumulation is an important confounding factor in the relation between adipocyte morphology and insulin resistance (Veilleux et al. 2011). Previous observations of an association between fat cell size and hyperinsulinaemia or peripheral insulin resistance may therefore arise from differences in abdominal fat distribution rather than subcutaneous adipose tissue cellularity per se. In contrast to other studies, Ledoux et al. found that omental, but not subcutaneous adipocyte size is associated with alterations in glucose and insulin homeostasis (Ledoux et al. 2009). On the other hand, mean adipocyte size of the visceral fat compartment has been associated with lipid profile alterations. Visceral, but not subcutaneous adipocyte hypertrophy is associated with increased plasma and very-low-density lipoprotein (VLDL)-triglyceride levels as well as with a higher total cholesterol to HDL-cholesterol ratio (Hoffstedt et al. 2010). Moreover, visceral adipose tissue cellularity is a predictor of hypertriglyceridemia independent of body composition and fat distribution in women (Veilleux et al. 2011). Aside from alterations in whole body glucose homeostasis and the lipid profile, adipocyte sizes in both visceral and abdominal subcutaneous adipose tissues have also been associated with hypertension (Ledoux et al. 2009).

Associations of fat cell size with alterations in glucose and lipid homeostasis as well as with other cardiometabolic risk factors may emerge from adverse changes in the metabolic function of enlarged adipocytes. Cell size-related differences have been reported for lipolysis, insulin sensitivity, and adipokine secretion (Bjorntorp and Sjostrom 1972; Farnier et al. 2003; Franck et al. 2007; Jernas et al. 2006; Skurk et al. 2007; Zinder and Shapiro 1971). Most of these studies have separated mature adipocyte fractions according to cell size and directly compared large adipocytes to small adipocytes from the same individual (Bjorntorp and Sjostrom 1972; Farnier

et al. 2003; Franck et al. 2007; Jernas et al. 2006; Skurk et al. 2007; Zinder and Shapiro 1971). In this context, the independent impact of adipocyte size on cell metabolism can be studied without the confounding effect of overall adiposity and body fat distribution of the adipose tissue donor. While large adipocytes have higher protein level of the glucose transporter GLUT4 (Farnier et al. 2003), Frank et al. observed that insulin-induced GLUT4 translocation to plasma membrane was blunted in large adipocytes compared to small ones (Franck et al. 2007). Lipid uptake and lipid synthesis by mature adipocytes are also associated with cell size as suggested by higher in vitro lipoprotein lipase (LPL) and fatty acid synthase activities in large compared to small adipocyte populations (Farnier et al. 2003). Alterations in the maximal lipolytic capacity have also been observed in large adipocytes (Farnier et al. 2003). Finally, Skurk et al. have shown that adipocyte size is an important determinant of adipokine mRNA expression and in vitro secretion (Skurk et al. 2007). They observed higher leptin, interleukin-6 (IL-6), IL-8, monocyte chemoattractant protein-1, and granulocyte colony-stimulating factor, but lower IL-10 secretion, in large compared to small adipocytes (Farnier et al. 2003; Skurk et al. 2007). Thus, the adipokine secretory pattern of large adipocytes is shifted toward a proinflammatory profile.

Overall, available studies clearly suggest that cell size is an important determinant of adipocyte function independent of body composition and fat distribution. Adipose tissue is composed of mature adipocytes with wide size distributions, and a shift toward a higher mean adipocyte size may strongly influence the overall metabolic function of a given adipose tissue depot. As demonstrated below, this factor alone likely contributes in large part to sex-related differences in cardiometabolic disease risk.

Triglyceride-rich lipoprotein hydrolysis catalyzed by LPL and 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 and Jensen 2007). Similarly, LPL activity increases along with adipocyte size in thigh, abdominal subcutaneous and visceral adipose tissue of men (Edens et al. 1993; Votruba and Jensen 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 et al. 1993; Fried and Kral 1987; 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 of test meals with fatty acid tracers combined with adipose tissue sampling provided 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 gynoid 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 storage, adipose tissue blood flow during the postprandial period is suggested as an important determinant of sex- and depot-related differences in lipid accumulation (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 different fat compartments, which may consequently alter body fat distribution (Votruba and Jensen 2007).

Net lipid accumulation in a given fat depot reflects the balance between triglyceride synthesis and the rates of lipolysis at that site. As previously stated, fat cell size is a major determinant of lipolytic responsiveness. Analyses of mature adipocyte populations separated according to cell size have shown that larger adipocytes have higher basal and stimulated lipolytic rates (Farnier et al. 2003). Lower basal lipolysis in omental compared to abdominal subcutaneous adipose tissue of women is consistent with the observation that adipocytes are smaller in the former than in the latter depot (Edens et al. 1993; Lundgren et al. 2008; Reynisdottir et al. 1997; Richelsen et al. 1991; Tchernof et al. 2006). In normal-weight to morbidly

obese men, no difference in lipolysis is observed between omental and abdominal subcutaneous adipocytes, consistent with a similar cell size in both depots (Boivin et al. 2007; Lundgren et al. 2008). In both subcutaneous and visceral adipose tissue, basal lipolysis is positively correlated with mean adipocyte size, confirming again the strong impact of fat cell size on lipolytic rates.

Yet, subcutaneous and visceral adipose tissues display different intrinsic responsiveness to lipolytic regulators. Indeed, lipolysis in omental adipocytes is more responsive to β-adrenergic agonist stimulation compared to that of abdominal subcutaneous adipocytes (Edens et al. 1993; Reynisdottir et al. 1997; Richelsen et al. 1991; Tchernof et al. 2006), while it is less sensitive to insulin suppression (Bolinder et al. 1983; Mauriege et al. 1995; Meek et al. 1999; Zierath et al. 1998) in both sexes. Regarding lower body fat stores, lipolysis of these depots is almost completely blunted at high doses of insulin while visceral adipose tissue lipolysis is only suppressed by half in these conditions (Meek et al. 1999). Regional differences have also been found in basal and insulin-stimulated glucose uptake. Basal and insulin-stimulated glucose uptake rates are higher in omental than in subcutaneous adipocytes (Lundgren et al. 2004; Marette et al. 1997; Stolic et al. 2002; Westergren et al. 2005). However, while visceral adipocytes are resistant to the anti-lipolytic effect of insulin compared to subcutaneous adipocytes (Mauriege et al. 1995; Zierath et al. 1998), no obvious difference in glucose uptake sensitivity to insulin has been observed (Lundgren et al. 2004; Marette et al. 1997; Stolic et al. 2002; Westergren et al. 2005). These results suggest that insulin action could be differentially altered in each fat compartment of individuals with visceral obesity and would only weakly relate to regional differences in adipocyte size. Expression of insulin signaling genes in both adipose tissue compartments also shows an apparent dissociation between insulin effects on lipolysis and glucose uptake. While data remain inconsistent, insulin receptor substrate-1 (IRS-1) protein levels seem to be higher in subcutaneous adipose tissue than omental adipose tissue of normal-weight subjects (Bashan et al. 2007; Lundgren et al. 2004; MacLaren et al. 2008; Veilleux et al. 2009a; Zierath et al. 1998). Visceral adipose tissue accumulation is associated with a reduced IRS-1 protein level in subcutaneous, but not omental adipose tissue (Veilleux et al. 2009a). Moreover, omental adipose tissue GLUT4 protein content is higher compared to subcutaneous adipose tissue (Marette et al. 1997; Veilleux et al. 2009a). GLUT4 expression in both adipose tissue depots is reduced in abdominally obese individuals, but the decrease in omental GLUT4 expression seems steeper (Garvey et al. 1992; Veilleux et al. 2009a). Reduced subcutaneous and omental GLUT4 expression in abdominally obese individuals indirectly suggests lower glucose uptake in both adipose tissue depots. On the other hand, relatively low omental IRS-1 expression is consistent with reduced sensitivity of lipolysis and glucose uptake to insulin in omental fat of abdominally obese individuals (Stolic et al. 2002; Veilleux et al. 2009a).

Abundant studies by the Jensen group have now shown that whole body subcutaneous adipose tissue is the major source of circulating free fatty acids (FFA), as it contributes to more than 85% of systemic FFA release in various clinical conditions (Basu et al. 2001; Guo et al. 1999; Jensen 1995; Martin and Jensen 1991; Nielsen et al. 2004).

In lean women and men, as little as 5–10% of FFA released in the portal vein are predicted to originate from visceral adipose tissue lipolysis (Nielsen et al. 2004). Since excess visceral fat accumulation is associated with increased positive lipolytic stimulation and insulin resistance, FFA released by this highly responsive tissue is likely to be increased in such condition. As expected, visceral adipose tissue lipolysis increased with visceral fat mass, so that in visceral obese women and men, this tissue contributed to nearly 50% of portal vein FFA release (Nielsen et al. 2004). Consistent with these findings, visceral adipose tissue was the main correlate of elevated postprandial VLDL-triglyceride release in insulin-resistant individuals (Couillard et al. 1998; Hodson et al. 2007). Increased local FFA flux in the portal vein may, in consequence, contribute to alter liver function (Nielsen et al. 2004). In absolute values, men have more visceral fat and higher lipolytic activity than women, so that more FFA is released into the portal vein by visceral adipose tissues of men (Nielsen et al. 2004). This difference may contribute to increase cardiovascular disease risk in men compared to women (Boivin et al. 2007; Tchernof et al. 2006). In women, during menopause, increased lipid accumulation and enlargement of adipocytes in the visceral fat compartment is associated with higher lipolytic rates (Tchernof et al. 2004). Proportional increases of FFA released in the portal vein could also contribute to cardiometabolic risk in postmenopausal women (Carr 2003).

Overall, available studies on adipocyte morphology suggest that adipocyte size appears to be an important determinant of sex-related and depot-related differences in lipid metabolism. Adipocyte metabolism in men favors a relatively more efficient accumulation of lipids in the visceral fat compartments. On the other hand, the proportion of FFA released from visceral adipose tissues increases with visceral obesity, through the combination of a larger visceral adipocyte size as well as increased relative lipolytic responsiveness to positive lipolytic stimuli and reduced inhibition by insulin specifically in visceral adipose tissue. Accordingly, in vivo experiments demonstrated that while visceral adipose tissue lipolysis accounts for a small proportion of whole body FFA release, the contribution of this depot increases up to approximately 50% along with visceral fat accumulation (Nielsen et al. 2004).

5.3.3 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). Adipokines are mainly secreted by adipocytes or preadipocytes, but also, especially in obesity, by macrophages invading the tissue (Ferrante 2007; Neels and Olefsky 2006; Trayhurn and Wood 2005). Chronic, low-grade inflammation caused by altered adipokine secretion may alter glucose and lipid metabolism and contribute to the altered cardiometabolic risk of individuals with visceral obesity (Ferrante 2007; 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). In recent experiments (Drolet et al. 2009), we have examined adiponectin release by purified mature adipocyte suspensions from the omental and subcutaneous fat depots in women. We found that compared to subcutaneous adipocyte adiponectin release, omental adipocyte adiponectin release is reduced to a greater extent in visceral obese women and better predicts obesity-associated metabolic abnormalities (Drolet et al. 2009). Thus, reduced visceral fat adiponectin release may be an important contributor to hypoadiponectinemia in visceral obese individuals.

Leptin, a foremost 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 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). In addition, testosterone 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 proinflammatory adipokines such as tumor necrosis factor-alpha, plasminogen activator inhibitor -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 proinflammatory adipokines (Weisberg et al. 2003). The chronic low-grade inflammation triggered 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.

5.4 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 further 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, 1999, 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, 1999, 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 5.1–5.3.

5.4.1 Androgens

In men, low circulating levels of endogenous androgens are associated with abdominal/visceral obesity assessed by waist circumference or CT (Gapstur et al. 2002; Khaw and Barrett-Connor 1992; Nielsen et al. 2007; Pasquali et al. 1991; Phillips et al. 2003; Seidell et al. 1990a). 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). On the other hand, 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. 1995; Tsai et al. 2004). Individuals with elevated plasma SHBG and testosterone levels are also generally characterized by a lower number of metabolic syndrome features (Blouin et al. 2005b; Hajamor et al. 2003; Laaksonen et al. 2003;

Table 5.1 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), Elbers et al. (1999), Elbers et al. (2003)
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), Elbers et al. (1999), Elbers et al. (2003)
Insulin resistance	Men	Testosterone tx	↗/↘	Bonetti et al. (2008), Boyanov et al. (2003), Marin et al. (1992c), Marin (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)
Visceral fat accumulation	Men	Androstenedione, 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. (1990a), Tchernof et al. (1995)
	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. (1990c), 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)

(continued)

Table 5.1 (continued)

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 cat-echolamine-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 cat-echolamine -stimulated lipolysis	Rat and human primary adipocytes	Testosterone and DHT	↗	Anderson et al. (2002), Xu and Bjorntorp (1990)
Preadipocyte proliferation	3T3-L1 and human primary preadipocytes	Testosterone and DHT	NS	Dieudonne et al. (2000), Monjo et al. (2005)
Preadipocyte differentiation	3T3-L1 and rat primary preadipocytes	Testosterone and DHT	↘	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 5.2 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)
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 vitro catecholamine -stimulated lipolysis	Human primary adipocytes	Estradiol/Menopause	**NS**	Rebuffe-Scrive et al. (1987), Tchernof et al. (2004)
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 5.3 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. (1992b), Rosmond et al. (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. (1992b), Rosmond et al. (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 primary adipocytes	Cortisol	↗	Hauner et al. (1989), Ottosson et al. (1995)
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 sample and rat primary adipocytes	Dexamethasone	↗	Fain et al. (1965), Lacasa et al. (1988)
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 endoplasmic reticulum 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

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. 1995; 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). 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. 1999).

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 (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). 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). Recent 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 recent study showed 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, putting into question what had been considered as common knowledge in PCOS (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 (Dunaif 1997). Finally, 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 fat storage, even at high doses (Blouin et al. 2010). On the other hand, 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 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 failed to observe any correlation (Ivandic et al. 2002; Kaye et al. 1991). As 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; Tchernof et al. 1999; Tchernof and Labrie 2004). However, as opposed to men, DHEA concentrations are 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).

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 activity 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. 2010; 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, 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). Recently, increased androgen receptor expression was reported following induction of preadipocyte differentiation (Blouin et al. 2009; 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-keto reductase 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 (HSD) 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 is higher in subcutaneous compared to visceral adipose tissue in both women and men (Blanchette et al. 2005; Blouin et al. 2003, 2005b, 2006). In both sexes, AKR1C enzyme expression and androgen inactivation rates of visceral adipose tissue are positively correlated with measures of obesity including BMI, fat cell size, and visceral adipose tissue area assessed by CT (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. 2009). This increase is believed to occur early in the preadipocyte differentiation process as the result of glucocorticoid stimulation (Blouin et al. 2009). Increased androgen inactivation by AKRIC2 may lead to reduced local exposure of fat cells to active androgens. This may remove part of the inhibitory effect of this hormone on adipocyte differentiation and modulate fat accumulation in each fat depot. Future studies may eventually establish the contribution of this mechanism to body fat distribution patterns in humans.

5.4.2 Estrogens

Estrogens are involved in female sexual development and the reproductive cycle (Mattsson and Olsson 2007). In premenopausal women, estrogens are produced mainly in ovaries. 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 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). 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). 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).

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). Sex- and depot-related differences in estrogen receptor levels have been reported but remain unclear (Blouin et al. 2009; 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 normal genotype (Goulart et al. 2009; Nilsson et al. 2007; Okura et al. 2003).

Several studies reported that P450 aromatase, which generates estradiol from testosterone, is expressed in adipose tissue (Blouin et al. 2009; Cleland et al. 1983; Mackenzie et al. 2008). Aromatase mRNA expression and activity are increased during adipogenesis and are positively associated with adiposity in humans (Blouin et al. 2009; Wake et al. 2007). Involvement of this enzyme in body fat distribution is also suggested by the fact that aromatase-knockout mice display progressive visceral adipose tissue accumulation (Jones et al. 2000). In addition to increased estrogen levels, aromatase activity may provide another inactivation pathway for androgens in adipose tissue. However, it is important to note that most of the effects of testosterone on adipose tissue function have been repeated using the non-aromatizable androgen DHT (Blouin et al. 2010; Gupta et al. 2008). The independent impact of estrogens and the involvement of androgen aromatization in human fat distribution patterns remain to be clearly established.

5.4.3 Glucocorticoids

In addition to their involvement in the immune system, glucocorticoids regulate energy homeostasis especially under conditions of stress (Putignano et al. 2004). This hormone promotes 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 impeding cellular proliferation and promoting differentiation of preadipocytes to lipid-storing, mature adipocytes (Gregoire et al. 1991; Hauner et al. 1989).

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 alterations 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. 1992a; 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 an 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 11βHSD. In vitro, inactivation of cortisol to cortisone (11β-dehydrogenase 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). 11-HSD1 knock-out mice show attenuated hyperglycemia provoked by stress and by diet-induced obesity (Kotelevtsev et al. 1997). Conversely, 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.

Only a few 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 have been mainly performed in females, but higher 11β-HSD1 expression levels in both omental and subcutaneous adipose tissue were observed 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 measures 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). However, body fat distribution measures are more closely related to 11β-HSD1 expression and oxoreductase activity in visceral adipose tissue than the same measures 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).

Other genes may also be involved in the regulation of local adipose tissue cortisol levels in obese individuals. Expression of 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 lessened 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). Recent reports also indentified another key 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 nicotinamide adenine dinucleotide phosphate 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 current literature. Finally, 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 measures 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). Insufficient data on the role of 11β-HSD2, H6PDH, and GRα 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 involvement reinforces the hypothesis that visceral 11β-HSD1 expression may be the main determinant of local active glucocorticoid levels and a major etiological factor for human abdominal-visceral obesity.

5.5 Clinical Implications

As demonstrated 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). 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 disease.

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 an 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). On the other hand, 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; Labrie 2007). Inhibitors of local cortisol generation by 11βHSD1 are currently considered as a potentially important avenue for future drug development (Bujalska et al. 2008a; Gathercole and Stewart 2010). In a recent study (Rosenstock et al. 2010), addition of one these inhibitors (INCB13739) to metformin therapy in patients with inadequate glycemic control was efficacious and well-tolerated, showing for the first time, that decreasing local cortisol exposure through 11βHSD1 inhibition improves hyperglycemia over 12 weeks in patients with type 2 diabetes (Rosenstock et al. 2010). Thus, inhibition of local cortisol generation may offer a new potential approach to control abdominal obesity-related alterations and cardiometabolic risk factors in type 2 diabetes.

5.6 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 and breast-feeding) (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 6
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

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6.1 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.

6.2 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 6.1). 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 6.1 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)

6.3 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).

6.3.1 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. 6.1. 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

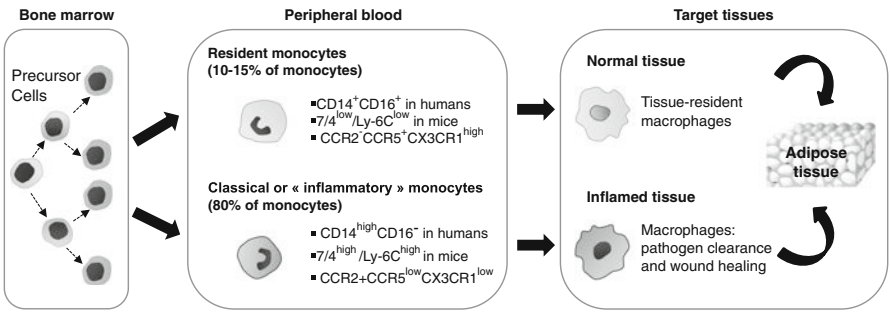

Fig. 6.1 Trafficking of monocytes from bone marrow to peripheral blood and target tissue. Under steady-state conditions, resident monocytes enter the tissues to replenish the pool of tissue-resident macrophages. Inflammatory monocytes immigrate into inflamed tissue and differentiate into so-called newly recruited macrophages

(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), 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).

6.3.2 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 over-expressing 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 upregulated 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).

6.3.3 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. 6.2. 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 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

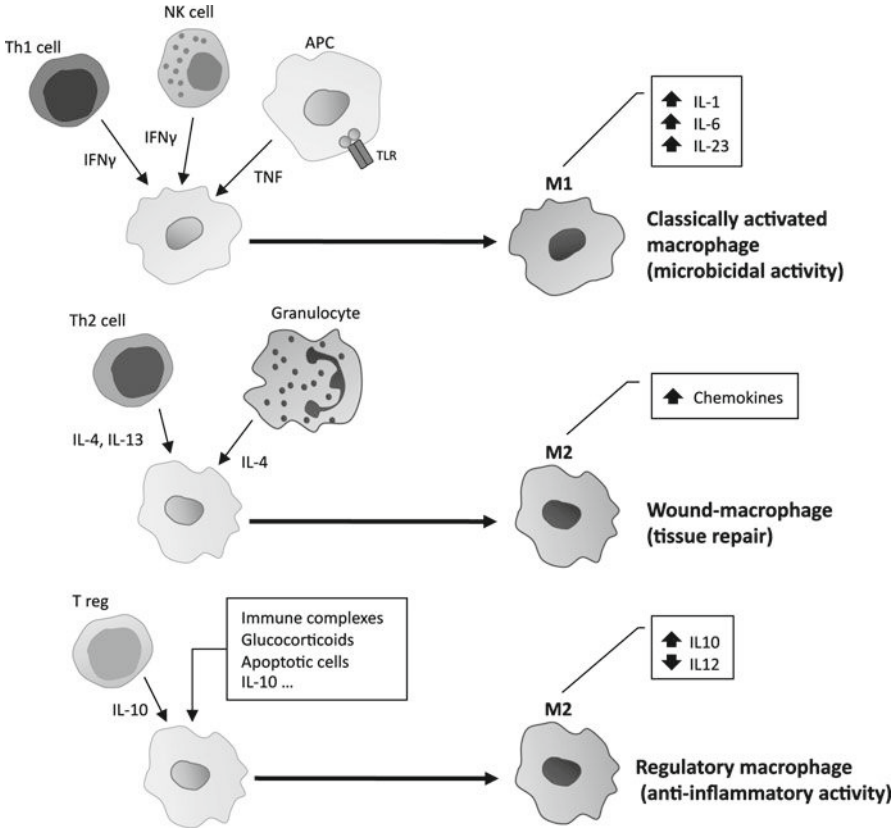

Fig. 6.2 Distinct phenotypes of tissue macrophages, depending of the microenvironment produced by surrounding cell cytokine release. Each macrophage subgroup is associated with typical secretory responses and functional characteristics (adapted from Mosser and Edwards 2008). *APC* antigen presenting cell; *T reg* regulatory T cell

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).

6.4 Macrophages in Adipose Tissue

6.4.1 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. 6.3). 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 "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).

6.4.2 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

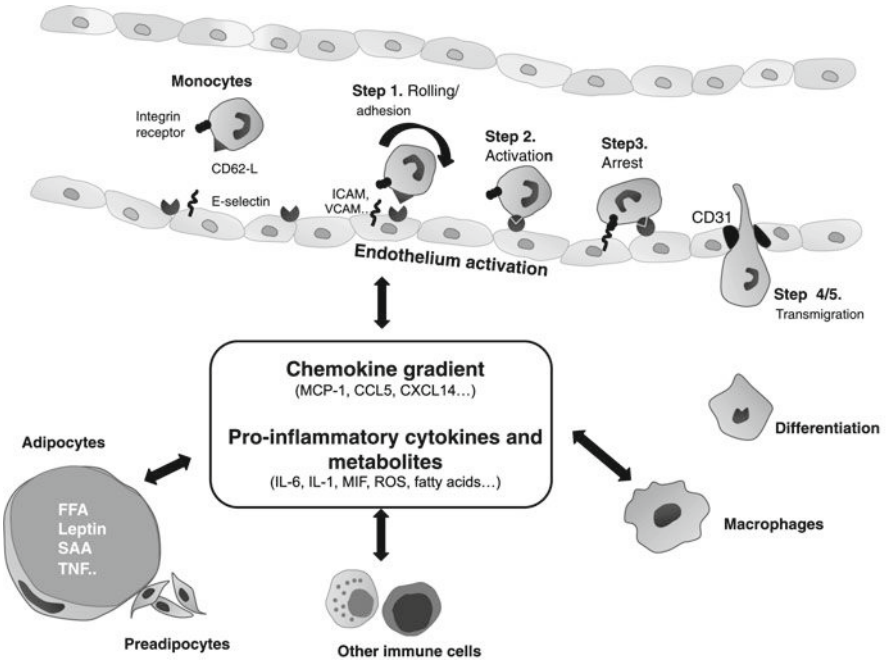

Fig. 6.3 Monocyte recruitment in the adipose tissue. Upon obesity, adipose tissue macrophages together with hypertrophic adipocytes, preadipocytes, and probably other immune cells produce a panel of chemokines, proinflammatory cytokines, and metabolites that participate into monocyte recruitment. Endothelium 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

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 6.2). In 2007, Lumeng et al. demonstrated that obesity induces a phenotypic switch in macrophages from an anti-inflammatory M2-polarized state to

Table 6.2 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 • Microsialin (CD68 mouse equivalent) • mCD14, a co-receptor for the detection of bacterial lipopolysaccharide (LPS)	• 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 • ArgII, arginase isoform II, a mitochondrial enzyme that transforms L-arginine into L-ornithine, a precursor of polyamines and proline	• 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
Human	• CD68, a member of the lysosomal-associated membrane protein family with a macrophage-specific mucin-like extracellular domain • mCD14, a co-receptor for the detection of bacterial LPS, a co-receptor for the detection of bacterial LPS	• CD11c, a dendritic cells marker, also called integrin alpha X • CD40, a costimulatory protein found on antigen presenting cells and is required for their activation and lymphocyte activation (ligand: CD154) • 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	• 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

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.

6.4.3 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.

6.4.4 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 profibrotic 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.

6.5 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. 6.4).

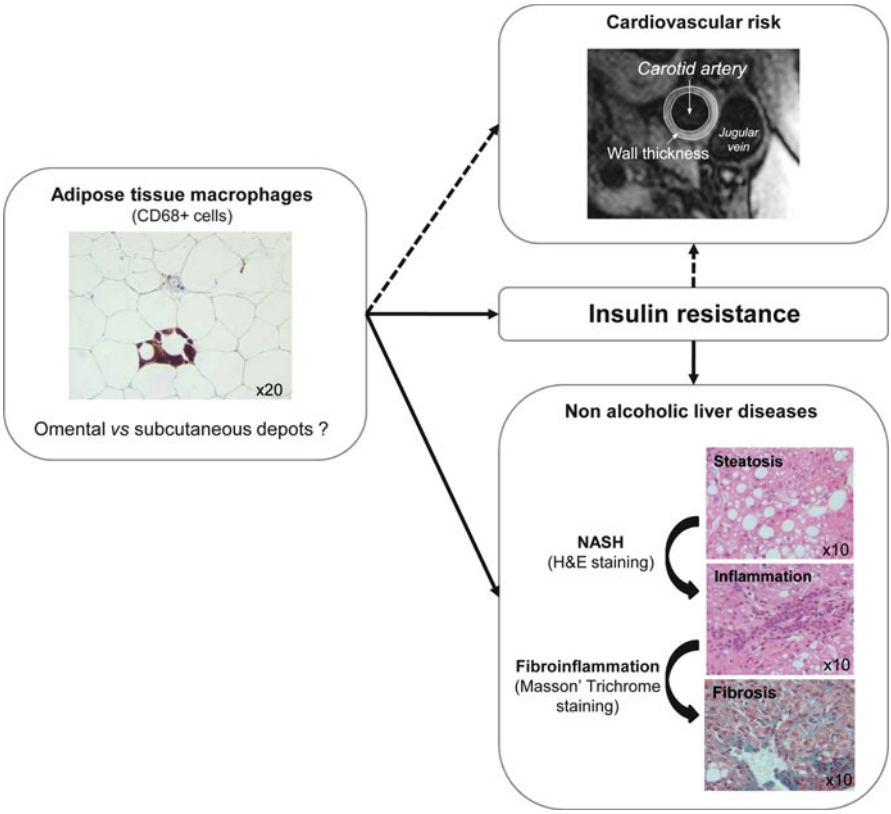

Fig. 6.4 Potential relationship between adipose tissue infiltrated macrophages and obesity comorbidities. Insulin resistance is a dependent factor implicated in the link between adipose tissue macrophages and non-alcoholic liver disease. The relative contribution of omental vs. subcutaneous inflammation 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)

6.5.1 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 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).

6.5.2 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).

6.5.3 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).

6.6 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.

6.7 Adipose Tissue Inflammation and Weight Loss

6.7.1 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.

6.7.2 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.

6.8 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. 6.5). 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

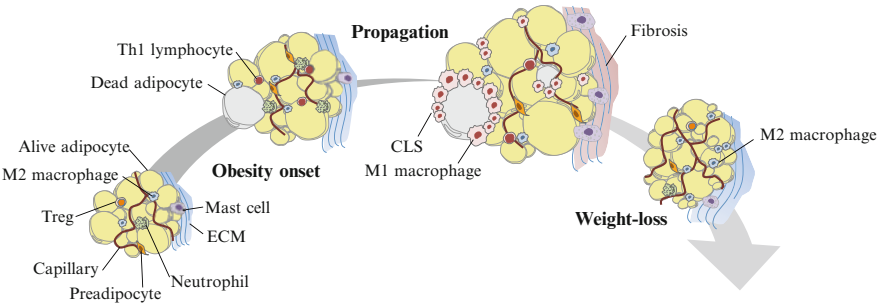

Fig. 6.5 Cellular and structural alterations in adipose tissue during obesity and weight loss. Healthy adipose tissue contains resident M2-polarized macrophages and other immune cells that help to maintain tissue homeostasis. During weight gain, hypertrophic adipocytes become inflammatory and/or necrotic 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)

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.

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Chapter 7
Adipocyte Growth and Factors Influencing Adipocyte Life Cycle

Srujana Rayalam and Clifton A. Baile

Abstract Adipose tissue growth occurs in the body at specific sites called adipose tissue depots. These fats depots form from the accumulation of adipocytes, the predominant cells of adipose tissue that are filled with triglycerides. Lately, adipose tissue is considered much more than an energy storage site. It is a source of hormones, growth factors, cytokines, and signaling molecules that regulate body metabolism. Furthermore, adipose tissue growth occurs through increases in size and number of adipocytes, which in turn is determined by a balance of lipolysis, lipogenesis, and adipocyte proliferation. Recently, a life cycle for adipocytes is acknowledged, which includes proliferation, growth arrest, clonal expansion, terminal differentiation, and apoptosis. Several factors affect adipocytes in their life cycle, and in the current chapter, major factors that influence the adipocyte life cycle are categorized into adipokines, transcription factors, hormonal factors, and nutritional and environmental factors. Studying these factors that influence and target different stages of the adipocyte life cycle might prove beneficial in understanding the physiological and pathophysiological mechanisms underlying adipose tissue development.

Keywords Adipocytes • Adipocyte life cycle • Adipogenesis • Apoptosis • Adipokines • Phytochemicals

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7.1 Background

The overall regulation of adipose tissue mass involves complex interactions between endocrine, paracrine, and autocrine systems. While body weight and fat distribution were once considered to be genetically determined to a significant extent (Stunkard et al. 1986), recent evidence suggest that hypothalamic centers play a major role in modulating adipose tissue mass by controlling food intake, metabolic rate, and activity in a coordinated manner (Prins and O'Rahilly 1997). The hypothalamic nuclei integrate peripheral signals, such as adiposity and caloric intake, to regulate important pathways within the central nervous system to control food intake. Thus, the hypothalamus acts as a "key controller" of food intake and energy homeostasis.

It is interesting to note that either excess fat in the body, as in case of obesity, or subnormal amounts of adipose tissue, as in the case of anorexia nervosa, contribute considerably to harmful metabolic consequences and represent significant medical and socioeconomic burdens in the world today. The research on understanding the pathophysiology of adipose tissue has been expanding lately, and adipose tissue is now recognized as an endocrine organ that is biologically active and dynamic in addition to its long-established role as a passive reservoir for energy storage.

Adipose tissue is of two types: white adipose tissue (WAT) and brown adipose tissue (BAT). While the development of WAT is well studied with regards to adipocyte biochemistry, very little is known about the ontogeny of BAT. Histologically, the number and size of mitochondria are much greater in BAT, compared to WAT indicating its primary role in energy dissipation. It should be noted that the functions of BAT and WAT are opposite, in that WAT mainly acts as an energy storing tissue while BAT is a thermogenic tissue. Rodents have a distinct BAT organ located in the interscapular region. However, in humans, it surrounds the heart and great vessels only in newborns and tends to disappear as humans mature. Lately, the research interest in brown fat has considerably increased due to the finding that humans retain metabolically active BAT depots postnatally and by stimulating brown adipogenesis it is possible to combat human obesity (Fruhbeck et al. 2009).

WAT can be broadly categorized into subcutaneous and visceral adipose tissues. Subcutaneous adipose tissue is located underneath the skin and also contributes to the physical functions including temperature regulation and thermal isolation. This type of adipose tissue is the main characteristic of the distinct body compositions of human males and females. Visceral adipose tissue occupies the body cavity surrounding the internal organs. As lipid is mobilized from visceral adipose depots, it is released directly into the portal circulation, which has been suggested as a contributing factor in cardiovascular disease (Smith et al. 2001). While there is a consensus that visceral fat has a strong association with cardiovascular risk factors, studies indicate that subcutaneous adipose tissue also has strong correlation with insulin sensitivity like visceral fat (Goodpaster et al. 1997). In the current chapter, the term adipose tissue will be used synonymously with WAT, unless otherwise specified.

Body fat distribution has been linked with several metabolic disorders. Usually, a predominant upper body fat distribution with increased visceral fat is associated

with an abnormal metabolic profile (Bjorntorp 1991). On the other hand, it is recognized that increasing amounts of lower body fat, which includes gluteal and leg depots, is associated with a reduced risk of metabolic complications (Snijder et al. 2004). In addition to these sites, muscle and bone marrow are important sites for adipocyte accumulation especially in an aging adult. Bone marrow adipocytes act as negative regulators for hematopoietic environment, and enhanced adipogenesis is observed in bone marrow with aging and in individuals with osteoporosis (Justesen et al. 2001). Similarly, fat cells infiltrate into muscle tissue with aging directly contributing to development of sarcopenia. Furthermore, fat accumulation in skeletal muscle adversely influences plasma insulin and lipid metabolism (Ryan and Nicklas 1999).

WAT secretes a variety of bioactive peptides, known as adipokines, which act both locally (autocrine/paracrine) and centrally (endocrine) (Kershaw and Flier 2004). It also expresses several receptors that mediate effects by responding to central and peripheral signals. A number of these adipokines and receptors significantly contribute to adipocyte growth and development. First, we will discuss the adipocyte life cycle, and in the second half of the chapter, the major adipokines and receptors, enzymes, environmental factors, and other exogenous factors like phytochemicals that affect adipocyte growth and development will be reviewed.

7.2 The Adipocyte Life Cycle

The metabolic properties of an adipocyte depend on its position within its own life cycle and the position within the life cycle of the organism. Although it is well established that the adipose tissue mass is determined by a balance of lipolysis, lipogenesis, adipocyte proliferation, and death (apoptosis), the metabolic consequences of obesity depend on whether expansion of adipose tissue is achieved primarily by an increase in adipocyte number or adipocyte size (Smith et al. 2006).

The adipocyte life cycle starts with differentiation of adipocytes from either committed embryonic stem cells or mesenchymal stem cells (MSCs) and includes a growth phase followed by growth arrest, clonal expansion, and a complex sequence of changes in gene expression leading to storage of lipid and finally cell death (Gregoire 2001) (Fig. 7.1). Adipocytes differentiated both *in vitro* and *in vivo* have many similarities. Furthermore, the subcutaneous injection of preadipose cells in nude mice resulted in the development of mature fat pads that are histologically identical to WAT (Green and Kehinde 1979). Nevertheless, primary cells, which are diploid, reflect the *in vivo* milieu better than the aneuploid cell lines, and moreover, primary cells can be derived from fat pads obtained from various depots. Since depot-related molecular and biochemical differences have been observed in fat pads, primary cells hold an advantage over cell lines in this aspect (Gregoire et al. 1998). In contrast, primary cells include a heterogeneous population of stromal vascular preadipocytes, while cell lines are usually a uniform population of preadipocytes, making the differentiation process uniform and consistent.

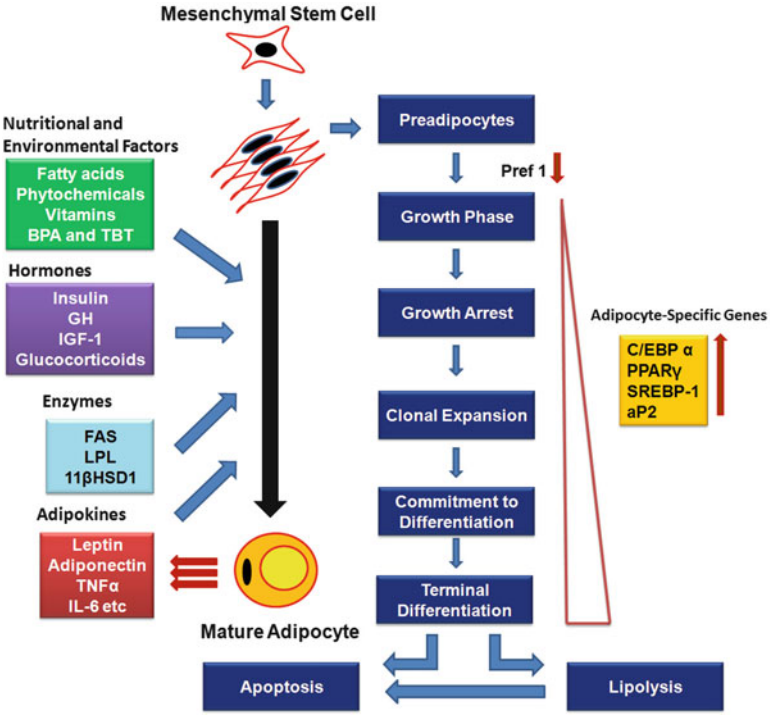

Fig. 7.1 Adipocyte life cycle. Mesenchymal stem cells are the precursors of preadipocytes. Once preadipocytes are triggered to mature, they proliferate and undergo growth arrest followed by a round of cell division known as clonal expansion and commitment to differentiation. Committed cells subsequently differentiate into mature adipocytes. This is accompanied by a dramatic increase in expression of adipocyte-specific genes. Mature adipocytes can continue storing lipid when energy intake exceeds output, and they can mobilize lipid through lipolysis when energy output exceeds input. Mature adipocytes can also undergo apoptotic cell death under certain conditions. Several adipokines, hormones, enzymes, nutritional, and environmental factors influence adipocyte life cycle contributing to overall adipose tissue growth and development

The morphological studies performed on several species including humans reveal that WAT formation begins before birth (Slavin 1979; Poissonnet et al. 1983). It is well accepted that adipocytes are derived from MSCs, which have the potential to differentiate into myoblasts, chondroblasts, osteoblasts, or adipocytes. In bone marrow, an inverse relationship exists between osteogenic and adipogenic differentiation of the MSCs, and studies have suggested that MSCs are by default programmed to differentiate into adipocytes (Kirkland et al. 2002). Thus, the presence of appropriate growth factors likely determines the developmental pathway being activated in the bone marrow, which is a major source of MSCs. Given that adipocytes secrete several adipokines and cytokines that inhibit osteoblastic activity (Maurin et al. 2000), once a certain level of bone marrow adiposity is reached, conditions may promote further adipogenesis at the expense of osteogenesis.

In this regard, it is interesting to note that compounds regulating lipid metabolism may also have a significant effect on bone formation in ovariectomized (Garrett et al. 2001), estrogen-deficient rats (Oxlund and Andreassen 2004), and in postmenopausal women (Lupattelli et al. 2004). There is also evidence that an embryonic stem cell precursor that has the capacity to differentiate into the mesodermal cell types like chondrocytes, osteoblasts, and myocytes may give rise to adipocyte lineage as well (Konieczny and Emerson 1984). However, the molecular events leading to the commitment of the embryonic stem cell precursor to the adipocyte lineage are not well characterized.

7.2.1 Growth Phase

During the growth phase, preadipocytes, both *in vitro* and *in vivo*, morphologically resemble fibroblasts. Preadipocyte factor 1 (pref-1), a preadipocyte secreted factor, serves as a marker for preadipocytes and is extinguished during adipocyte differentiation (Wang et al. 2006). The morphological modifications in preadipocytes are usually accompanied by changes in the extracellular matrix (ECM) and cytoskeletal components. A decrease in actin and tubulin expression is an early event in adipocyte differentiation that primarily contributes to morphological changes. These changes promote the expression of critical adipogenic transcription factors, including CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor-γ (PPARγ), which is a key factor for regulating adipogenesis. The change in cell shape is primarily mediated by the degradation of stromal ECM by plasminogen cascade, and plasminogen deficiencies cause an inhibition of adipocyte differentiation (Gregoire 2001).

7.2.2 Growth Arrest

Committed preadipocytes have to withdraw from the cell cycle before undergoing adipose conversion. Thus, growth arrest is required for adipocyte differentiation of preadipose cell lines and primary preadipocytes, which is normally believed to be achieved by contact inhibition. However, recent reports suggest that cell–cell contact per se is not a critical factor, and cells plated even at low density can differentiate into adipocytes. C/EBPα and PPARγ are involved in the preadipocyte growth arrest that is required for adipocyte differentiation (Umek et al. 1991). Several changes at the gene expression level take place during the preadipocyte to adipocyte transition phase. Other molecular markers like sterol regulatory element binding protein-1c (SREBP-1c), adipocyte determination and differentiation factor 1 (ADD1), and GATA binding transcription factors, GATA-2 and -3, play important roles in the molecular control of the preadipocyte–adipocyte transition (Gregoire et al. 1998; Gregoire 2001)

7.2.3 Clonal Expansion

Complex changes in gene expression patterns occur during clonal expansion. As preadipocytes enter into S phase, C/EBPα plays a vital role by functioning as a transcriptional activator of adipocyte genes (Lane et al. 1999). Notably, over 2,000 genes are associated with the acquisition of adipocyte phenotype, over 100 of which are not yet characterized (Guo and Liao 2000). During the mitotic clonal expansion, preadipocytes undergo one round of DNA replication, in order to differentiate into adipocytes. However, under *in vitro* conditions, primary preadipocytes derived from human adipose tissue do not require cell division to enter the differentiation process as these cells undergo critical cell divisions *in vivo* (Entenmann and Hauner 1996).

7.2.4 Commitment to Differentiation

Before entering the pathway of adipocyte differentiation, the preadipocytes must be committed to the adipocyte lineage. The committed cells always require inducers or some stimulus to initiate the cascade of biochemical events that lead to adipocyte differentiation. Although C/EBPα promotes withdrawal from the cell cycle during commitment, C/EBPα alone is not sufficient to promote differentiation in the absence of other inducing agents (Sadowski et al. 1992). *In vitro* differentiation is normally induced by supplementing the cell culture media with dexamethasone, 1-methyl-3-isobutylxanthin (IBMX), and insulin. Addition of these agents modulates both the mRNA and protein levels of more than 100 adipocyte-specific transcription factors. These rapidly induced proteins promote the terminal differentiation of adipocytes (Sadowski et al. 1992). A dramatic decrease in pref-1 expression, which is abundant in preadipocytes and is not detectable in mature adipocytes, also accompanies adipocyte differentiation.

7.2.5 Terminal Differentiation

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 including ATP citrate lyase, malic enzyme, acetyl-CoA carboxylase, stearoyl-CoA desaturase (SCD1), glycerol-3-phosphate dehydrogenase (GPDH), fatty acid synthase (FAS), and glyceraldehyde-3-phosphate dehydrogenase (Spiegelman et al. 1983). Adipocytes markedly increase de novo lipogenesis and acquire sensitivity to insulin in addition to increasing glucose transporters and insulin receptor numbers. Other adipose tissue-specific proteins like ap2, an adipocyte-specific fatty acid binding protein (FABP), fatty acid

translocase/cluster of differentiation 36 (CD36), a putative fatty acid transporter, and perilipin, a lipid droplet-associated protein, are also synthesized during this phase. Synthesis of adipocyte-secreted products including leptin, adipsin, resistin, and other cytokines also begins during the late phase of differentiation (Gregoire et al. 1998).

7.2.6 Dedifferentiation

A hallmark for terminal differentiation of adipocytes is not clearly defined. There is evidence that the partially differentiated human preadipocytes could still undergo cell division leading to dedifferentiation. The dedifferentiated adipocytes look like fibroblasts, with long and spindle-shaped cytoplasmic extensions. Tumor necrosis factor-α (TNFα) promotes dedifferentiation in 3T3-L1 mature adipocytes or primary human adipocytes, resulting in loss of lipids and morphological changes. It should be noted that although preadipocytes and TNFα-treated adipocytes share many characteristics in terms of cell morphology and gene expression, pref-1 expression levels are not restored by TNFα, and thus, preadipocytes are likely to be different from dedifferentiated cells (Gregoire et al. 1998).

7.2.7 Apoptosis

Mature adipocytes after terminal differentiation undergo apoptosis (Rayalam et al. 2008a). Apoptosis, also called "programmed cell death," is a self-directed-cellular "suicide" and is different from other types of cell death. Cell shrinkage, chromatin condensation, cellular budding, and rapid phagocytosis by macrophages or adjacent cells are the typical events of apoptosis that occur in fixed sequence (Hacker 2000). The process of apoptosis involves a cascade of molecular events, and two apoptotic pathways are identified to date, the extrinsic, or death receptor pathway, and the intrinsic, or mitochondrial pathway (Igney and Krammer 2002). The extrinsic pathway is triggered by the binding of an extracellular ligand to a death receptor which belongs to TNF receptor gene superfamily (Locksley et al. 2001). Apoptosis through the intrinsic pathway is not mediated by receptors but the signals produced in the cell lead to mitochondrial events, ultimately leading to DNA fragmentation and condensation of peripheral nuclear chromatin (Joza et al. 2001). Recent findings however suggest that, apart from induction of apoptosis in mature adipocytes, it is possible to selectively eliminate either preadipocytes or maturing preadipocytes by treating 3T3-L1 cells with certain phytochemicals (Rayalam et al. 2008a).

It should be noted that in contrast to necrosis, apoptosis is executed in a precise manner without generating inflammation. In obese mice, necrosis of adipocytes is

seen with formation of "crown-like structures" characterized by dead adipocytes surrounded by macrophages (Murano et al. 2008). Conditions like hypoxia or increased production and release of certain chemikines by adipocytes have been implicated in the cause of necrosis (Pang et al. 2008). While necrotic cell death is often associated with tissue damage resulting in inflammatory response, apoptosis leads to apoptotic bodies which are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells causing no inflammatory response.

7.2.8 Lipolysis

Lipolysis is defined as a process in which triacylglycerides, a major source of energy reserve in adipocytes, are hydrolyzed to generate fatty acids and glycerol. Mature adipocytes undergo lipolysis in an orderly manner with lipolytic enzymes acting sequentially at each step, during which triacylglycerol is hydrolyzed to form diacylglycerol, and then monoacylglycerol, with the liberation of fatty acids and glycerol (Duncan et al. 2007). Obesity is associated with an increase in basal rates of lipolysis, contributing to the development of insulin resistance. Alterations in lipolysis are frequently associated with obesity, including an increase in basal rates of lipolysis that may contribute to the development of insulin resistance, as well as an impaired responsiveness to stimulated lipolysis (Large et al. 1999). Hormone-sensitive lipase was once believed to control the process of lipolysis, but recent studies suggest the interplay of several novel adipose tissue lipases and lipid droplet-associated proteins in triacylglycerol hydrolysis (Duncan et al. 2007).

7.3 Factors Influencing the Adipocyte Life Cycle

It is well established that adipose tissue is a complex and highly active metabolic and endocrine organ (Ahima and Flier 2000). In addition to adipocytes, adipose tissue contains connective tissue matrix, nerve tissue, stromal vascular cells, erythrocytes, endothelial cells, macrophages, and other immune cells (Frayn et al. 2003). While most of the bioactive proteins are secreted from adipocytes, several secreted proteins are derived from the non-adipocyte fraction of the adipose tissue as well.

The factors that influence adipocyte growth and development are divided into four categories in this chapter: (1) proteins secreted by adipose tissue that have metabolic effects on distant cells or tissues, (2) transcription factors, (3) enzymes involved in the metabolism of lipids, (4) hormonal factors, and (5) environmental and nutritional factors (Table 7.1).

Table 7.1 Summary of factors influencing adipocyte life cycle

	Effect on adipocyte life cycle	References
Adipokines		
Leptin	Reduced lipid accumulation in maturing 3T3-L1 adipocytes and induced adipocyte apoptosis and lipolysis *in vivo*	Ambati et al. (2007) and Della-Fera et al. (2001)
Adiponectin	Reduced circulating free fatty acid levels and increased insulin sensitivity in obese mice	Fruebis et al. (2001)
TNFα	Inhibited adipocyte differentiation and intracellular lipid accumulation	Xing et al. (1997)
IL-6	Inhibited adipogenesis and lipoprotein lipase activity *in vitro* and increased free fatty acid concentrations *in vivo*	Greenberg et al. (1992) and Fernandez-Real and Ricart (2003)
Visfatin	Facilitated adipocyte differentiation	Sethi and Vidal-Puig (2005)
Resistin	Plays a role in obesity-related insulin resistance and in adipocyte differentiation	Banerjee and Lazar (2003)
Receptors and transcription factors		
PPARs	PPARγ, master regulator of adipogenesis. Synthetic ligands for PPARγ are potent inducers of adipocyte differentiation	Spiegelman et al. (1993), Morrison and Farmer (2000), and Viswakarma et al. (2010)
C/EBPs	Ectopic expression of C/EBPα or C/EBPβ-induced adipogenesis *in vitro* and mouse models with deletion of C/EBP genes resulted in severe abnormalities in fat	Morrison and Farmer (2000), Lefterova and Lazar (2009), and White and Stephens (2010)
SREBP-1	*In vitro* studies support a role in adipogenesis, while *in vivo* studies suggest that SREBPs are not required for adipose tissue expansion of adipose tissue	Kim et al. (1998), Morrison and Farmer (2000), and White and Stephens (2010)
STATs	Promoted adipogenesis *in vitro*	Teglund et al. (1998) and White and Stephens (2010)
Enzymes and transporters		
11βHSD1	Increased lipid accumulation and visceral adiposity	Stewart and Tomlinson (2002)
FAS	Key enzyme in de novo lipogenesis and catalyzes the synthesis of saturated fatty acids	Schmid et al. (2005)
LPL	Is required for efficient fatty acid uptake and storage	Wang and Eckel (2009)
aP2	A marker of terminal cell differentiation	Spiegelman et al. (1983) and Shen et al. (1999)
GLUT-4	Is needed for optimal glucose metabolism	Fernyhough et al. (2007)
Hormones		
Insulin	Plays a central role in the regulation of adipocyte metabolism and presence of the insulin receptor is required for adipocyte differentiation	Rosen and Spiegelman (2000) and Accili and Taylor (1991)

(continued)

Table 7.1 (continued)

	Effect on adipocyte life cycle	References
GH	Stimulates lipolysis in mature adipocytes and primary preadipocytes while promotes adipogenesis in preadipocyte cell lines	Etherton (2000) and Kawai et al. (2007)
IGF-1	Implicated in the regulation of adipocyte differentiation and lipid accumulation *in vitro*	Kloting et al. (2008)
Glucocorticoids	Used to induce optimal differentiation of preadipocytes in culture	Gregoire (2001)
Nutritional factors		
Fatty acids	Dietary polyunsaturated fatty acids decreased lipid droplet size both *in vitro* and *in vivo*	Madsen et al. (2005)
Vitamins	Vitamins C, A, E, and D decreased adipocyte proliferation and inhibited lipid accumulation	Campion et al. (2006), Viroonudomphol et al. (2003), Bonet et al. (2003), and Kong and Li (2006)
Phytochemicals	Several phytochemicals like genistein, resveratrol, quercetin, xanthohumol, and guggulsterone decreased adipocyte proliferation and inhibited lipid accumulation *in vitro*	Yang et al. (2007, 2008), Park et al. (2008, 2009), and Rayalam et al. (2008b)
Environmental factors		
BPA	Induced differentiation and stimulated lipid accumulation in 3T3-L1 adipocytes	Kidani et al. (2010)
TBT	Promotes adipocyte differentiation and adipogenesis both *in vitro* and *in vivo* via PPARγ	Inadera and Shimomura (2005)

7.3.1 Adipokines

Well-studied adipokines that influence adipocyte growth through both central and peripheral mechanisms include leptin, adiponectin, TNFα, interleukin-6 (IL-6), visfatin, and resistin. A majority of these adipokines are implicated in insulin sensitivity and appetite regulation (Fig. 7.2).

7.3.1.1 Leptin

Leptin was originally discovered as the missing protein in the genetically obese ob/ob mouse (Halaas et al. 1995) and later was widely recognized for its ability to regulate adipose tissue mass by influencing food intake and energy expenditure. It is a 16-kDa polypeptide containing 167 amino acids. Leptin expression and secretion in turn are regulated by several factors including insulin, glucocorticoids, TNFα, estrogens, and C/EBPα and decreased by β3-adrenergic activity, androgens, free fatty acids, growth hormone (GH), and PPARγ agonists (Margetic et al. 2002).

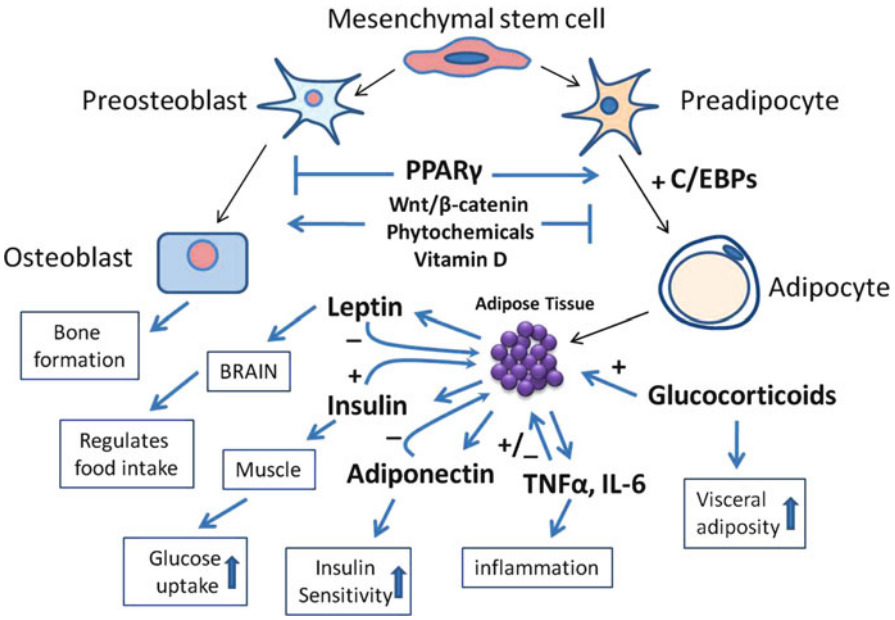

Fig. 7.2 An overview of major factors influencing adipocyte differentiation and adiposity. While PPARγ is considered a master regulator of adipogenesis, it negatively regulates bone formation in bone marrow. In contrast, vitamin D and Wnt/β-catenin promote bone formation at the expense of adipogenesis. Transcription factors like C/EBPs promote the adipocyte differentiation. Adipokines leptin and adiponectin act as both endocrine and paracrine hormones and negatively regulate adipogenesis, while the effect of inflammatory cytokines (TNF-α and IL-6) on adipose tissue growth is not clear. Glucocorticoids like dexamethasone promote adipogenesis *in vitro* and increase visceral adiposity *in vivo*

Leptin's effects are mediated through its receptors, which are members of the cytokine receptor class I superfamily and are expressed in both the central nervous system and periphery. These receptors orchestrate complex metabolic changes in a number of organs and tissues, altering nutrient flux to favor lipid mobilization over lipid storage (Baile et al. 2000). The effects of leptin on energy homeostasis are well documented, and recent studies suggest that leptin can also decrease adiposity by triggering apoptosis and lipolysis in adipocytes (Qian et al. 1998; Della-Fera et al. 2001). Adipose tissue from rats given cerebral ventricular injections of leptin demonstrated features of apoptosis, including internucleosomal fragmentation of genomic DNA, elevated levels of DNA strand breaks, reduction in total DNA content, and cellular volume (Qian et al. 1998).

The mechanisms of leptin-induced adipose tissue apoptosis are not fully understood. Although most of the leptin's effects on adipocyte apoptosis and lipolysis are believed to be centrally mediated by stimulation of the sympathetic nervous system, the possibility that leptin can act directly on adipocytes to induce apoptosis has not been thoroughly investigated, although leptin did not directly stimulate apoptosis in

3T3-L1 adipocytes *in vitro* (Ambati et al. 2007). Nevertheless, leptin significantly reduced lipid accumulation and GPDH activity in maturing 3T3-L1 preadipocytes, indicating that leptin may not act directly to induce adipocyte apoptosis, but can act directly to inhibit maturation of preadipocytes (Ambati et al. 2007). Further, leptin can act to regulate the lipid storage characteristics and potential thermogenic functions of fat even before birth (Yuen et al. 2003).

7.3.1.2 Adiponectin

Adiponectin is an approximately 30-kDa polypeptide specifically expressed in differentiated adipocytes, and its expression is higher in subcutaneous than visceral adipose tissue (Fain et al. 2004). Adiponectin receptors are G-protein coupled receptors and are primarily expressed in muscle and liver. Accordingly, adiponectin has also been shown to augment lipid oxidation in skeletal muscle and to reduce hepatic glucose production in liver. Likewise, a close correlation exists between reduced plasma levels of adiponectin and obesity, insulin resistance, and cardiovascular disease. Before the onset of obesity, plasma adiponectin levels decline and administration of adiponectin improves insulin sensitivity, suggesting that hypoadiponectinemia might be a contributing factor for obesity (Hotta et al. 2001). Furthermore, administration of adiponectin to obese mice reduces circulating free fatty acid levels by enhanced skeletal muscle fat oxidation and enhances insulin sensitivity (Fruebis et al. 2001). In addition, lack of adiponectin induces glucose intolerance and insulin resistance and increases serum unsaturated fatty acids without a significant effect on food intake or body weight (Maeda et al. 2002).

Although cellular mechanisms regulating adiponectin are not completely understood, it is believed that most of adiponectin's effects are mediated via increased phosphorylation of the insulin receptor, activation of 5′ adenosine monophosphate-activated protein kinase (AMPK), and modulation of the nuclear factor κB pathway (Chandran et al. 2003). Recently, adiponectin receptors were shown to be expressed in adipocytes, and overexpressing adiponectin in adipocytes leads to the downregulation or desensitization of receptors as a result of negative feedback effects and to lipid accumulation in adiponectin overexpressing cells. Thus, the regulation of adipocyte metabolism and adipose tissue mass *in vivo* by adiponectin may be due in part to its actions as an autocrine or paracrine factor (Fu et al. 2005).

7.3.1.3 TNFα

TNFα is a 26-kDa transmembrane protein with pleiotropic effects on cellular proliferation and differentiation. TNFα exerts its effects by binding as a trimer to type I or type II TNFα receptors (also called CD120a and CD120b respectively), which belong to TNF receptor super family that includes Fas receptor, CD40, CD27, osteoprotegerin, and receptor activator of nuclear factor kappa B. Both TNFα and its receptors are expressed in adipocytes, and like adiponectin, the expression levels

of TNFα are greater in subcutaneous compared with visceral adipose tissue (Fain et al. 2004).

Adipose tissue expression of TNFα is increased in obese rodents and humans, and plasma levels of TNFα have been positively correlated with obesity and insulin resistance. Accordingly, TNFα has been implicated in the pathogenesis of obesity and metabolic syndrome. Furthermore, chronic exposure to TNFα inhibits adipocyte differentiation and intracellular lipid accumulation and induces insulin resistance (Xing et al. 1997). One possible mechanism by which TNFα indirectly impairs insulin signaling is by increasing serum-free fatty acids. While treatment with soluble TNF receptors improves insulin sensitivity in rodents, insulin resistance in humans is unaltered (Ruan and Lodish 2003).

Potential mechanisms through which TNFα acts have been investigated by several groups. Adipocytes treated with TNFα fail to express adipocyte-specific genes leading to diminished lipid accumulation. Regardless, expression of Pref-1, a preadipocyte marker, is not altered by TNFα and rather decreases in a manner indistinguishable from that of cells not treated with TNFα. On the contrary, a significant decrease in the expression of PPARγ expression was observed with TNFα treatment (Xing et al. 1997). Also, in adipose tissue, TNFα represses genes involved in uptake and storage of unsaturated fatty acids and glucose and changes expression of several adipocyte-secreted factors including adiponectin and IL-6 (Ruan et al. 2002). Thus, TNFα affects multiple metabolic processes and is responsible for a diverse range of signaling events within adipocytes.

7.3.1.4 IL-6

IL-6 is a 22–27 kDa immune-modulating adipokine associated with obesity and insulin resistance. Both circulating and adipose tissue IL-6 levels are positively correlated with obesity and insulin resistance (Fernandez-Real and Ricart 2003). The receptor for IL-6 is homologous to that for leptin, and like IL-6, its receptor is also expressed by both adipocytes and adipose tissue matrix. One third of total circulating concentrations of IL-6 originate from adipose tissue. While the isolated adipocytes contribute to only 10% of IL-6 secretion, the stromal vascular cellular fraction, including stromal preadipocytes, is a major source for IL-6. Thus, in contrast to adiponectin and TNFα, expression and secretion of IL-6 is two to three times greater in visceral relative to subcutaneous adipose tissue (Fain et al. 2004).

In vitro, IL-6 inhibited adipogenesis and lipoprotein lipase (LPL) activity (Greenberg et al. 1992). In addition, induction of differentiation in human preadipocytes markedly reduced IL-6 mRNA levels (Wang et al. 2005). *In vivo*, IL-6 infusion led to increased free fatty acid concentrations and fasting triglycerides (Fernandez-Real and Ricart 2003). These peripheral effects of IL-6 confirm a causal role for IL-6 in obesity and insulin resistance. On the contrary, the central effects of IL-6 are quite complex. Intracerebroventricular administration, but not intraperitoneal administration, of IL-6 in rats resulted in enhanced energy expenditure leading to weight loss. Since IL-6 is expressed in both adipose tissue and hypothalamic

nuclei, the authors concluded that IL-6 acting centrally, but not peripherally, exerts anti-obesity effects in rodents (Wallenius et al. 2002). Thus, IL-6 has different effects on lipid metabolism and energy homeostasis in the periphery and in the central nervous system.

7.3.1.5 Visfatin

Visfatin (pre-B-cell colony-enhancing factor, PBEF) is a novel adipokine that is preferentially produced in the intra-abdominal adipose tissue of obese mice and humans and has insulin-mimetic actions (Fukuhara et al. 2005). It is interesting to note that adipokines that promote insulin resistance like TNF α and IL-6 regulate the expression of visfatin (Ognjanovic et al. 2001). Plasma visfatin concentrations correlate with intra-abdominal fat mass but not with subcutaneous fat mass and/or type 2 diabetes mellitus in humans. Visfatin acts by binding to the insulin receptor at a site distinct from insulin and exerts hypoglycemic effect by reducing glucose release and stimulating glucose utilization in peripheral tissues (Beltowski 2006). Owing to these effects, visfatin is considered to possess both endocrine and paracrine effects. In the visceral adipose tissue, visfatin facilitates the differentiation of the adipose tissue through its pro-adipogenic and lipogenic actions (Sethi and Vidal-Puig 2005). In porcine preadipocytes, visfatin upregulated LPL expression facilitating lipid uptake and increased the gene expression of FAS in differentiated adipocytes to enhance lipogenic activity. In addition, the overexpression of visfatin in a preadipocyte cell line facilitated its differentiation to mature adipocytes and promoted the accumulation of fat through the activation of glucose transport and lipogenesis (Sethi and Vidal-Puig 2005). Thus, as an endocrine hormone, visfatin modulates insulin sensitivity in peripheral tissues, and as an autocrine/paracrine hormone, it promotes adipocyte proliferation and fat deposition in visceral adipose tissue (Sethi and Vidal-Puig 2005).

7.3.1.6 Resistin

Resistin, also known as adipocyte secreted factor and "found in inflammatory zone 3," is a recently described protein whose expression is adipocyte specific and downregulation by thiazoladinediones in rodents (Banerjee and Lazar 2003). Resistin expression is 15-fold greater in visceral compared with subcutaneous adipose tissue. The function of resistin is not well understood, but there is evidence that it plays a role in obesity-related insulin resistance as well as in adipocyte differentiation (Banerjee and Lazar 2003). The expression of the resistin gene is promoted by C/EBPα binding, which leads to the recruitment of transcriptional coactivators in murine adipocytes (Hartman et al. 2002). It should be remembered, however, that human resistin shares only 64% homology with murine resistin and is expressed at very low levels in adipocytes (Banerjee and Lazar 2003). Hence, studies in humans have failed to provide a link between resistin expression in adipose tissue or circulating resistin levels and adiposity or insulin resistance. Recent clinical studies,

however, demonstrated that resistin was more strongly associated with inflammatory and fibrinolytic markers than with obesity or insulin resistance, and suggested that the associations of resistin with insulin resistance and metabolic syndrome could be due to resistin's effects on inflammatory markers like plasminogen activator inhibitor 1 (PAI-1) levels (Qi et al. 2008).

7.3.1.7 Plasminogen Activator Inhibitor

PAI-1 is the primary physiological inhibitor of fibrinolysis by inhibiting tissue-type plasminogen activation *in vivo*. It is expressed by many cell types within adipose tissue including adipocytes, and its expression and secretion are greater in visceral relative to subcutaneous adipose tissue (Fain et al. 2004). Fully differentiated 3T3-L1 adipocytes in culture produce significant levels of PAI-1 mRNA and protein. *In vivo*, plasma PAI-1 activity is approximately fivefold higher in obese mice than in their lean counterparts, and in humans, PAI-1 levels are drastically upregulated in obesity associated with glucose intolerance, insulin resistance, hyperinsulinemia, and type 2 diabetes mellitus (Loskutoff and Samad 1998). Studies indicate that the increased levels of TNFα in the adipose tissues in obesity act through an autocrine manner to stimulate PAI-1 biosynthesis by the adipocyte and other cells in the adipose tissue (Loskutoff and Samad 1998).

7.3.2 Receptors and Transcription Factors

In addition to adipokines that act both locally and centrally, adipocytes also express several receptors to respond to afferent signals from periphery and central nervous system. Moreover, the structural and functional changes associated with adipocyte growth and differentiation involve changes in the expression levels of several hundred proteins. A complex network of transcription factors, together with specific transcriptional coactivators and corepressors, respond to afferent stimuli in the process of controlling the conversion of progenitor mesenchymal cells into fully functional adipocytes. Most of these changes occur at the level of gene expression through a series of molecular events involving several receptors and transcription factor families. Major adipocyte-specific factors that play a regulatory role in the process of adipogenesis are peroxisome proliferator-activated receptors, C/EBP binding proteins, sterol regulatory element binding protein (SREBP) -1c, and signal transducers and activators of transcription (STATs).

7.3.2.1 Peroxisome Proliferator-Activated Receptors

These are nuclear hormone receptors, which play a central role in the control of adipocyte gene expression and differentiation. The ligand–receptor interactions modulate the transcriptional regulation of adipogenesis. PPARα, PPARγ, and PPARδ

are the three known receptors in this family, and they exhibit diverse functions, despite the fact that they all bind to the same response elements. PPARγ is expressed predominantly in adipose tissue, and its expression is induced very early in the adipocyte differentiation process as discussed in the earlier sections. PPARα is expressed in liver, kidney, small intestine, heart, and skeletal muscle, and plays a prominent role in regulating lipid catabolism. On the other hand, PPARδ is ubiquitously expressed with relatively higher levels found in brain, adipose tissue, and skin. Activation of PPARδ also induces expression of genes required for fatty acid oxidation and energy dissipation in skeletal muscle and adipose tissue (Braissant et al. 1996; Brun and Spiegelman 1997).

PPARγ is considered the master regulator of adipogenesis. Compelling evidence from both *in vitro* and *in vivo* studies supports this fact. Thiazolidinediones, the potent inducers of adipocyte differentiation, are high affinity synthetic ligands for PPARγ. Also, ectopic expression of PPARγ in multiple cell lines under adipogenic conditions caused enhanced induction of adipocyte differentiation, suggesting that PPARγ is an essential regulator for adipocyte differentiation and promotes lipid storage in mature adipocytes (Barak et al. 1999). Recently, 15-deoxy-$\Delta^{12,14}$-prostaglandin J2, a derivative of prostaglandin J2, has also been identified as a naturally occurring ligand for PPARγ (Forman et al. 1995). While even a slight activation of transcriptional activity with these natural and synthetic compounds leads to robust adipocyte differentiation in cells expressing PPARγ, PPARα expressing cells require significant activation. On the contrary, adipocyte cells expressing PPARδ did not differentiate, even when treated with strong activators of PPARδ (Brun et al. 1996).

PPARs exist as heterodimers in the nucleus with retinoid X receptor-α bound to DNA with corepressor molecules, nuclear receptor corepressor, and silencing mediator of retinoid and thyroid hormone receptor. Upon activation with specific ligands, PPARs undergo conformational changes, dissociate with corepressor molecules, and recruit transcription cofactors that increase the gene transcription (Spiegelman et al. 1993; Morrison and Farmer 2000; Viswakarma et al. 2010).

7.3.2.2 CCAAT/Enhancer-Binding Proteins

C/EBPs were the first transcription factors shown to play a critical role in adipocyte differentiation. These proteins belong to a highly conserved family of leucine zipper transcription factors, which function through homo- and heterodimeric complexes with C/EBP family members. Although six members have been identified in this family, only C/EBPα, C/EBPβ, and C/EBPδ have been studied extensively for their roles in regulating adipogenesis. Ectopic expression of C/EBPα or C/EBPβ induces adipogenesis in nonprogenitor fibroblasts, and mouse models with deletion of C/EBP genes resulted in severe abnormalities in fat. While whole body ablation of C/EBPα leads to reduced WAT in adult mice, it should be noted that C/EBPs β and δ are responsible for inducing C/EBPα expression. Of note, ectopic PPARγ expression stimulated adipogenesis in C/EBPα-deficient murine fibroblasts suggesting that

C/EBPα is dispensable in adipocytes. Furthermore, combined loss of C/EBPα and -β dramatically reduces the expression of many genes, including FABP, adiponectin, and hormone sensitive lipase. Collectively, these data demonstrate a substantial role for all the three C/EBPs in the transcriptional activation of adipocyte-specific genes during the development of adipocyte differentiation, *in vitro* and *in vivo* (reviewed in Morrison and Farmer 2000; Lefterova and Lazar 2009; White and Stephens 2010).

7.3.2.3 Sterol Regulatory Element Binding Proteins (SREBPs)

SREBPs are basic helix-loop-helix transcription factors that are expressed in adipocytes and regulated during adipogenesis. Initially SREBP was called ADD1 for adipocyte differentiation and determination. The SREBP family consists of three proteins, designated SREBP-1a, -1c, and -2. Regulation of fatty acid biosynthesis is mediated primarily by SREBP-1a and -1c, and SREBP-2 is relatively selective and mediates transcriptional activation of cholesterol biosynthetic genes. *In vivo*, adipose tissue expresses predominantly SREBP-1c over other forms of SREBP, and ectopic expression of SREBP-1c enhances adipocyte gene expression in nonprogenitor murine fibroblasts under adipogenic conditions. However, expression of SREBP-1c alone is only capable of inducing adipogenesis to a limited extent, and additional studies suggest that SREBP-1c contributes to the production of PPARγ ligands, thereby facilitating the action of PPARγ. Collectively, *in vitro* studies support a role for SREBP-1 in adipogenesis, while *in vivo* studies suggest that SREBPs are not required for the production or expansion of adipose tissue (Kim et al. 1998; Morrison and Farmer 2000; White and Stephens 2010).

7.3.2.4 Wnt and β-Catenin

Wnts comprise a family of highly conserved secreted glycoproteins that act in a paracrine or autocrine manner by binding cell-surface receptors. The canonical Wnt signaling cascades activate the transcriptional regulator β-catenin. In the absence of Wnts, β-catenin undergoes proteosomal degradation. However, when Wnts bind to Frizzled receptors and low density lipoprotein receptor-related protein co-receptors, degradation complex that drives β-catenin degradation is inactivated, resulting in the translocation of β-catenin to the nucleus where it binds to other transcription factors to activate Wnt target genes (Cadigan and Nusse 1997; Miller et al. 1999).

Wnt pathway activation in 3T3-L1 preadipocytes caused impaired differentiation, while inhibition of β-catenin activity caused enhanced adipogenesis in preadipocytes. Furthermore, a reciprocal relationship exists between Wnt signaling and C/EBPβ or PPARγ, as activation of these factors led to a substantial reduction in β-catenin levels. Wnt signaling has been implicated in regulating MSC maintenance, proliferation, fate determination, and preadipocyte differentiation.

Thus, Wnt/β-catenin signaling acts as a molecular switch that when activated, represses adipogenesis (Ross et al. 2000; Moldes et al. 2003).

7.3.2.5 Signal Transducers and Activators of Transcription (STATs)

STATs comprise a family of cytoplasmic proteins that are activated by and mediate gene expression in response to extracellular stimuli that target mainly cytokine receptors. Ligand-mediated dimerization of the receptor results in phosphorylation of tyrosine residues on STATs causing their translocation to the nucleus to mediate specific gene expression. Induction of differentiation in murine and human preadipocytes causes upregulation of STATs 1, 5A, and 5B.

Further, STATs 5A and 5B are coordinately regulated by both PPARγ and C/EBPα in differentiating 3T3-L1 cells under a variety of conditions. Ectopic expression of STAT5A confers adipogenesis in 3T3-L1 preadipocytes and transgenic deletion of STATs 5A and 5B in mice resulted in significantly reduced fat pad sizes compared to wild-type mice. Taken together, these studies demonstrate that STAT proteins play a significant role in adipogenesis (Teglund et al. 1998; White and Stephens 2010).

7.3.2.6 Kruppel-Like Factors (KLFs)

Kruppel-like factors (KLFs) are DNA binding transcriptional regulators that play diverse roles in cell proliferation, differentiation, and development in mammals. Recent studies indicate that KLFs also have important roles in adipogenesis. KLF4 transactivates the C/EBPβ promoter and is now considered to be among the initiators of the adipogenic program. One protein in the KLF family, KLF15, conferred adipogenesis in non-precursor cells and resulted in the induction of PPARγ expression. In addition to promoting lipid accumulation, KLF15 also increases the expression of glucose transporter type 4 (GLUT4). Interestingly, KLF proteins that have been implicated in adipogenesis can either promote or impair the process and have different expression patterns. While KLFs 4, 15, and 6 favor adipogenesis, other members of the KLF family exhibited negative effects on adipogenesis. Although there is enough evidence that KLFs play an essential role in adipogenesis, the cross talk between these transcription factors with PPARγ and C/EBPα needs further investigation to fully elucidate the roles of KLFs in adipocyte differentiation (Mori et al. 2005; Lefterova and Lazar 2009).

7.3.3 Enzymes and Transporters

A number of enzymes are involved in lipid metabolism and indirectly influence the growth and development of adipose tissue.

7.3.3.1 11β-Hydroxysteroid Dehydrogenase

11β-Hydroxysteroid dehydrogenase type 1 and 2 (11βHSD1 and 2) are involved in the conversion of cortisone to cortisol. 11βHSD1 is highly expressed in adipose tissue, and overexpression of the enzyme and increased cortisol levels resulted in lipid accumulation and an increase in visceral adiposity (Stewart and Tomlinson 2002). Further, pharmacological inhibition of 11βHSD1 in humans increases insulin sensitivity, suggesting a potential therapeutic role for 11βHSD1 inhibition in the treatment of obesity and insulin resistance (Walker et al. 1995).

7.3.3.2 Acyl Coenzyme A: Diacylglycerol Acyltransferase 1 (DGAT1)

Diacylglycerol acyltransferase 1 (DGAT1) acts as a key enzyme in the synthesis of triglycerides, the main form of excess calorie storage in fat. DGAT1 is a microsomal enzyme with high expression in WAT, and overexpression causes increased triglyceride levels. The mRNA and protein levels of DGAT1 markedly increase after induction of differentiation in 3T3-L1 adipocytes in parallel with DGAT activity. DGAT1-deficient mice are resistant to diet-induced obesity due to increased energy expenditure, while overexpression of DGAT1 in adipose tissue results in increased adiposity but is not accompanied by loss of insulin sensitivity. It may be relevant that in 3T3-L1 adipocytes, overexpression of DGAT1 results in increased secretion of TNFα which is known to interfere with insulin signaling (Yu et al. 2002).

7.3.3.3 Aromatase and 17βHSD

Enzymes cytochrome P450-dependent aromatase and 17βHSD are highly expressed in adipose tissue stromal cells and preadipocytes. While aromatase mediates the conversion of androgens to estrogens, 17βHSD mediates the conversion of weak androgens/estrogens to their more potent counterparts. The ratio of 17βHSD to aromatase is positively correlated with central adiposity, implicating increased local androgen production in visceral adipose tissue. Moreover, targeted deletion of aromatase in mice and naturally occurring mutations in aromatase in humans caused increased visceral adiposity, dyslipidemia, and insulin resistance (Meseguer et al. 2002).

7.3.3.4 Fatty Acid Synthase (FAS)

FAS is the key enzyme in de novo lipogenesis and catalyzes the synthesis of saturated fatty acids, predominately palmitate, from acetyl-CoA and malonyl-CoA precursors. FAS is expressed at high levels in adipose tissue, liver, and lung. Since exogenous saturated fatty acids are abundantly available through diet in humans, FAS is considered an enzyme of minor importance. However, recently, central

effects of FAS on inhibition of food intake were revealed, indicating that FAS is not only involved in providing metabolic substrates, but also plays a role in satiety signaling. In addition, the substrates provided for triacylglycerol synthesis by FAS upregulation during differentiation of preadipocytes maintain the signaling for the differentiation process to complete, and an inhibition of FAS activity results in inhibition of preadipocyte differentiation and possibly reduction of adipose tissue (Schmid et al. 2005).

7.3.3.5 Lipoprotein Lipase (LPL)

LPL is one of the first genes induced during the process of adipocyte differentiation. LPL is a multifunctional enzyme and is expressed at high levels in adipose tissue and muscle. It is a rate-limiting enzyme that hydrolyzes triglyceride-rich lipoproteins to generate fatty acids for uptake in peripheral tissues. During adipocyte differentiation, insulin has a major effect on both LPL expression and activity by increasing its gene transcription, affecting posttranscriptional and posttranslational mechanisms. LPL activity has also been reported to increase as a function of fat cell size. Studies using 3T3-L1 adipocytes further establish that adipocyte-derived LPL is required for efficient fatty acid uptake and storage (Wang and Eckel 2009).

7.3.3.6 Fatty Acid Binding Proteins (FABPs)

FABPs belong to a family of low-molecular-weight cytoplasmic proteins involved in intracellular transport and metabolism of fatty acids (Storch and Thumser 2000). Adipocyte FABP, also called FABP4 or aP2, is the predominant FABP and is expressed in much higher levels compared to other forms of FABPs in adipocytes. aP2 protein is implicated in aiding the transport of hormone-sensitive lipase to the lipid droplet. aP2 expression is believed to be involved in upholding the balance between lipogenesis and lipolysis in differentiating preadipocytes. Most importantly, the expression of aP2 is highly regulated during adipocyte differentiation and is regarded as a marker of terminal cell differentiation (Spiegelman et al. 1983; Shen et al. 1999).

7.3.3.7 Glucose Transporter Type 4 (GLUT4)

Glucose transport occurs relatively late in the differentiation program after the expression of PPARγ and C/EBPs and involves a family of integral membrane proteins called glucose transporters. Of all the members of this family, GLUT4, a high affinity glucose transporter, is highly expressed in adipose tissue and muscle (Pessin et al. 1999). In the basal state 2–5% of GLUT4 protein is at the plasma membrane and remainder is localized to intracellular compartments. In response to insulin stimulation, about 50% of the protein is upregulated and localized in plasma membrane. The expression of GLUT4 is greatly increased during differentiation of

the preadipocytes, and both PPARγ and C/EBPα upregulate GLUT4 expression. PPARγ also directly modulates insulin-signaling pathway for GLUT4 to function within the cell. GLUT4 expression, or the expression of other metabolic markers, is needed for optimal glucose metabolism in monogastric animals, but may be depot dependent in some species like ruminants. Thus, GLUT4 expression is needed for optimal glucose metabolism (Fernyhough et al. 2007).

7.3.4 Hormones

Adipose tissue growth and development are controlled by a complex cross talk between central, hormonal, environmental, and nutritional stimuli. Studies regarding the endocrine regulation of adipose tissue development *in vivo* are not extensive. On the other hand, numerous studies have been reported on the hormonal regulation of adipogenesis at cellular and molecular levels *in vitro*. Clearly, the hormonal regulation is intricately required for adipose tissue development.

7.3.4.1 Insulin

Insulin is the most important hormonal factor influencing adipogenesis and lipogenesis. Efficient differentiation of adipocytes *in vitro* requires insulin. Insulin further increases the percentage of cells that differentiate and also increases the amount of lipid accumulation in each fat cell (Girard 1994). Insulin promotes lipogenesis by increasing the uptake of glucose in the adipocyte via recruitment of glucose transporters to the plasma membrane, as well as by activating lipogenic enzymes. The effects of insulin in adipocytes are achieved by the binding of insulin to the insulin receptor and thus activating it via tyrosine phosphorylation (Lane et al. 1990).

The presence of the insulin receptor is required for adipocyte differentiation (Accili and Taylor 1991). Inhibition of phosphatidyl inositol 3-kinase has been shown to block insulin-induced differentiation of 3T3-L1 preadipocytes. Insulin also plays a central role in the regulation of adipocyte metabolism, by acting as a potent inhibitor of lipolysis. It is interesting to note that although preadipocytes express few insulin receptors, the effects of insulin on differentiation have been shown to occur through cross-activation of the insulin-like growth factor 1 (IGF-1) receptor (Rosen and Spiegelman 2000).

Hyperinsulinemia, induced either by administration of exogenous insulin or an increase in the production of endogenous insulin, is associated with significant weight gain and is a characteristic feature of obesity. Several molecules like TNFα, leptin, and resistin interfere with insulin signaling both *in vitro* and *in vivo* and antagonize its effects at multiple levels in adipocytes. Conversely, some adipokines like adiponectin and omentin act as insulin-sensitizing agents by promoting insulin-stimulated glucose uptake (Karastergiou and Mohamed-Ali 2010).

7.3.4.2 Growth Hormone (GH)

Another hormone that has an important influence on adipocyte development and lipogenesis is GH. There are disparities in the effects of GH on adipogenesis and lipogenesis, *in vivo* and *in vitro*. GH affects both proliferation and differentiation of preadipocytes, although this varies between clonal cell lines and preadipocyte cultures. While GH stimulates lipolysis in mature adipocytes and primary preadipocytes, it promotes adipogenesis in preadipocyte cell lines. In contrast, GH inhibits lipogenesis by decreasing insulin sensitivity leading to downregulation of FAS *in vivo*. *In vitro* effects of GH on stimulating adipogenesis are mediated via GH-STAT 5A/5B signaling pathway in cooperation with C/EBPs and PPARγ, and the effect of GH on promoting lipolysis *in vitro* in mature adipocytes is through inhibition of LPL. Some effects of GH are indirectly mediated through the GH-mediated secretion of IGF-1 within adipose tissue (Etherton 2000; Kawai et al. 2007).

7.3.4.3 Insulin-Like Growth Factor 1 (IGF-1)

Initially, IGF-1 was considered a hepatic derived factor produced in response to GH; however, it was subsequently found that IGF-1 is locally produced by several other tissues, and adipose tissue is a major source. Importantly, GH is the main regulator of IGF-I mRNA expression in adipose tissue. Both IGF-1 and its receptor, IGF-1 receptor (IGF-1R), have been implicated in the regulation of adipocyte differentiation and lipid accumulation *in vitro*. In 3T3-L1 cells and human MSC, activation of IGF-1R, either by IGF-1 or insulin, leads to the modulation of several signal transduction pathways, including Akt, which stimulates cell growth and lipogenesis. Yet, recent findings indicate that IGF-1R signaling in adipocytes is not crucial for the development and differentiation of adipose tissue *in vivo* (Kloting et al. 2008).

7.3.4.4 Thyroid Hormones

Thyroid hormones regulate the expression of several adipocyte-specific genes and markers of differentiation like those involved in adipogenesis, lipogenesis, and lipolysis. In rodents, hypothyroidism induces a transient hypoplasia, whereas hyperthyroidism induces a transient hyperplasia of retroperitoneal and epididyma1 fat tissues. Triiodothyronine (T3) regulates the expression of PPARγ through specific thyroid response elements. The availability of T3 in turn depends on deiodinases D1, D2, and D3, and of these, D2 plays an important role in adipocyte differentiation. Cross talk between ligand-activated thyroid hormone receptors and other nuclear hormone receptors like PPARγ, liver X receptor alpha, and farnesoid X receptor (TR) is suggested to influence the diverse effects on adipocyte differentiation and lipid metabolism (Levacher et al. 1984; Sasaki et al. 2006).

7.3.4.5 Glucocorticoids and Sex Steroids

Glucocorticoids are used to induce optimal differentiation of preadipocytes in culture. Dexamethasone is the most commonly used glucocorticoid *in vitro*, which acts through the activation of its receptor, glucocorticoid receptor. Dexamethasone and its receptor influence the expression of transcription factors, PPARγ and C/EBPδ, and further, dexamethasone reduces the expression of pref-1, which is a negative regulator of adipogenesis (Smas et al. 1999). In humans, elevated circulating glucocorticoids, as in the case of Cushing's syndrome, lead to visceral obesity.

Although obvious differences in body fat distribution can be seen between males and females, the role played by sex hormones in adipogenesis is poorly understood. Estrogens modulate adipogenesis by increasing preadipocyte replication, without influencing the differentiation process. On the contrary, progesterone stimulates terminal differentiation in preadipose cell lines. Androgens block conversion of preadipocytes to mature adipocytes by decreasing the activity of glycerol-3 phosphate dehydrogenase. These actions are mediated by specific intracellular receptors, and both pre- and mature adipocytes in humans and rodents express estrogen and androgen receptors (Dieudonne et al. 2000).

7.3.5 Nutritional Factors

Food components with beneficial effects on decreasing adiposity have attracted increased attention lately. The dietary agents discussed below affect the adipocyte life cycle and lipogenesis by either inhibiting adipocyte specific transcription factors or by inhibiting key enzymes responsible for lipid synthesis. Furthermore, these compounds might act by enhancing lipid oxidation or by preventing free fatty acids from entering adipocytes.

7.3.5.1 Fatty Acids

Both natural and nonmetabolized long chain fatty acids per se behave as hormones and regulate the expression of various lipid-related genes in adipocytes at a transcriptional level. Long-chain, saturated, and polyunsaturated fatty acids have been shown to regulate transcription factors, such as C/EBPs, PPARs, and other adipose-specific genes, very early in adipocyte development. These effects not only affect adipocyte size, but also fat cell number. In particular, there is evidence that the fatty acids in fish oil, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), decrease preadipocyte proliferation in cell lines and reduce adiposity in rodents. These two omega-3 fatty acids have by far received the most attention, and EPA and DHA are also extensively studied for other health benefits.

In vitro and *in vivo* studies show that DHA inhibits mitotic clonal expansion, decreases GPDH activity, and reduces body fat in rodents (Buckley and Howe 2009).

It is noteworthy that the lipid droplets in cells induced to differentiate in the presence of polyunsaturated fatty acids like DHA and EPA are smaller in size than the lipid droplets formed in the presence of saturated fatty acids and monounsaturated fatty acids. Thus, dietary polyunsaturated fatty acids decrease lipid droplet size both *in vitro* and *in vivo* (Madsen et al. 2005).

Another fatty acid, t10,c12 conjugated linoleic acid (CLA) has pronounced effect on inhibiting adipocyte differentiation and inducing adipocyte apoptosis and decreasing body fat (Wang and Jones 2004). The isomers of CLA are also ligands for PPAR indicating that the effects of CLA on adipose development and gene expression might be due in part to its binding with PPAR.

7.3.5.2 Vitamins

Blood levels of antioxidants like vitamins E, C, and A have been found to be lower in obese people, and consuming multivitamin supplements reduced body weight and adiposity in obese individuals (Li et al. 2010). Ascorbic acid decreased total weight gain and adipose depots by downregulating genes involved in adipogenesis and adipocyte differentiation (Campion et al. 2006). Likewise, supplementation of vitamin E and selenium reduced insulin resistance in obese rats and in humans, and *in vitro* vitamin E suppresses adipocyte differentiation to exert anti-adipogenic effects (Viroonudomphol et al. 2003).

Vitamin A is mainly known for its involvement in vision, but it also plays an important role in regulating adiposity. The anti-adipogenic effects of vitamin A are exerted by blocking C/EBPβ-mediated induction of downstream genes, notably PPARγ, preventing entry of the preadipocytes into the growth-arrested phase and interacts with activators of PPARγ through retinoic acid receptors (Bonet et al. 2003).

Vitamin D status is strongly associated with variation in subcutaneous and visceral adiposity (Cheng et al. 2010). In 3T3-L1 adipocytes, vitamin D markedly suppressed the expression of PPARγ and C/EBPβ, antagonized PPARγ activity, and stabilized the inhibitory vitamin D receptor protein leading to decreased lipid accumulation and apoptosis induction (Kong and Li 2006) (Fig. 7.3).

7.3.5.3 Phytochemicals

A number of phytochemicals have been investigated for their effects on adipocyte life cycle. Flavonoids like green tea, genistein, quercetin, and xanthohumol have been studied *in vitro* using either 3T3-L1 cells or human adipocytes and *in vivo* using rodent animal models and human clinical studies. Epigallocatechin-3-gallate decreased lipid accumulation and the expression of PPARγ, and C/EBPα in maturing 3T3-L1 adipocytes and in mature adipocytes induced apoptosis (Lin et al. 2005). Likewise, genistein, a soy isoflavone, inhibited adipocyte proliferation, differentiation, adipogenesis, and induced apoptosis in 3T3-L1 adipocytes and in rodents (Kim et al. 2006). The inhibitory effects of genistein on adipogenesis are believed to

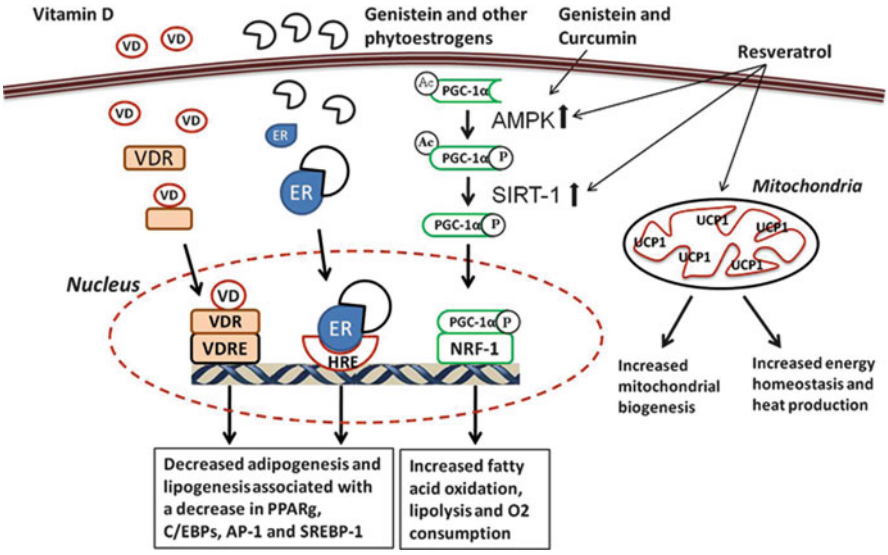

Fig. 7.3 Effects of vitamin D and phytochemicals on adipocyte biochemistry. Vitamin D binds to its nuclear receptor, gets translocated into nucleus, and binds to its responsive element. Likewise, certain phytoestrogens like genistein and quercetin bind to ERβ with high affinity and get translocated into nucleus. Both vitamin D and phytoestrogens cause changes in gene expression leading to a decrease in adipogenesis. Since majority of phytochemicals like genistein, resveratrol, and curcumin have multiple targets, they exert their anti-adipogenic effects by activating enzymes like AMPK and SIRT-1. Further, resveratrol also activated UCP1 expression in mitochondria causing increased energy expenditure and mitochondrial biogenesis. *AMPK* adenosine monophosphate kinase; *ER* estrogen receptor; *HRE* hormone response element; *NRF-1* nuclear respiratory factor 1; *PGC-1α* peroxisome proliferator-activated receptor γ coactivator-1 alpha; *RSV* resveratrol; *SIRT-1* sirtuin 1; *UCP1* uncoupling protein 1; *VD* vitamin D; *VDR* vitamin D receptor; *VDRE* vitamin D responsive element

involve both PPARγ-dependent and -independent pathways. Several studies show that genistein binds to PPARs and induces transcriptional activities. Furthermore, genistein may inhibit multiple signaling molecules including p38 and JAK/STAT signaling pathways through tyrosine phosphorylation (Dang 2009). Recent studies on xanthohumol, a flavonoid found in beer hops, indicate that this compound is more potent than genistein in inhibiting adipogenesis and inducing apoptosis and lipolysis in murine adipocytes (Rayalam et al. 2009).

One of the most investigated phytochemicals that is thought to be responsible for the beneficial effect of moderate wine consumption is the resveratrol. In 3T3-L1 preadipocytes, resveratrol decreased viability, lipid accumulation, and the expression of PPARγ, C/EBPα, HSL, LPL, and FAS genes (Rayalam et al. 2008b). Further, in rodents, resveratrol decreased body weight, body fat content, and adipose tissues mass (Lagouge et al. 2006).

Curcumin, a phenolic acid which is a popular spice in Asia, has been studied extensively for its anti-inflammatory and anti-carcinogenic effects. More recently,

curcumin has also been demonstrated to possess potent anti-adipogenic effects. Especially in 3T3-L1 preadipocytes, curcumin decreased proliferation and lipid accumulation but did not affect the expression of PPARγ and C/EBPα. In mature adipocytes, curcumin activated AMPK and caused fatty acid oxidation and also induced apoptosis. Further, in rodents fed a high-fat diet, curcumin reduced body weight gain and adipose tissue mass (Ejaz et al. 2009) (Fig. 7.3).

7.3.6 Environmental Pollutants

Since the molecular mechanisms underlying the development of obesity are not completely understood, recently, scientific attention has been drawn to the potential contributions of environmental pollutants that act as endocrine disrupting chemicals (EDCs) in the pathogenesis of metabolic diseases.

7.3.6.1 Bisphenol A (BPA)

Bisphenol A (BPA) is used commercially in products containing polycarbonate plastics such as food and water containers, and BPA is also present in microgram quantities in the liquid of preserved food in cans (Brotons et al. 1995). Thus, BPA is present ubiquitously in the environment and is ingested by humans routinely. BPA is considered an EDC because of its estrogenic actions.

Studies indicate that BPA is associated with induction of obesity, and serum BPA levels are higher in obese individuals compared to their non-obese counter parts (Takeuchi et al. 2004). *In vitro*, BPA induced differentiation and stimulated lipid accumulation in 3T3-L1 adipocytes through the downregulation of Akt signaling pathway and inhibition of adiponectin (Kidani et al. 2010).

Other EDCs, namely dicyclohexyl phthalate, endrin, and tolylfluanid, also stimulated adipogenesis in 3T3-L1 adipocytes. One recent study showed that these EDCs along with BPA significantly stimulated the glucocorticoid receptor without significant activation of the PPARγ. Interestingly, these compounds did not induce adipogenesis when preadipocytes were treated with compounds alone. However, the EDCs promote adipocyte differentiation by synergizing with agents present in the differentiation cocktail like either dexamethasone or insulin. Thus, it is possible that the pro-adipogenic effects of EDCs are mediated through the activation of the glucocorticoid receptor (Sargis et al. 2010).

7.3.6.2 Tributyltin (TBT)

Tributyltin (TBT) is an organotin compound used worldwide in agriculture and industry as biocide. Recently, it was shown that chronic and repeated exposure to this compound could lead to obesity and hepatic steatosis (Zuo et al. 2011).

In 3T3-L1 adipocytes, TBT induced morphological changes accompanied by the expression of adipocyte differentiation marker and enhanced lipid accumulation when supplemented with dexamethasone and insulin. Moreover, TBT acts as a ligand for PPARγ and promotes adipocyte differentiation and adipogenesis both *in vitro* and *in vivo* via PPARγ (Inadera and Shimomura 2005).

7.4 Conclusions

Adipose tissue growth involves an increase in adipocyte size and number. The cellular and molecular mechanisms that influence the adipocyte life cycle have been extensively studied. Adipocytes are derived from MSC, which have the potential to differentiate into myoblasts, chondroblasts, osteoblasts, or adipocytes. The life cycle of adipocytes includes alteration of cell shape and growth arrest, clonal expansion, and a complex sequence of changes in gene expression leading to terminal differentiation accompanied with the storage of lipid and finally apoptosis. Growth and differentiation of adipocytes are controlled by various hormones, enzymes, transcription factors, and growth factors. It is likely that the multitude of diverse regulatory signals and factors converge at a few classic signal transduction pathways leading to a synergistic enhancement in adipogenesis and lipogenesis or synergic increase in lipolysis or adipocyte apoptosis. Understanding the adipocyte life cycle and how the various molecules affect the adipocyte life cycle, which in turn regulate adiposity and energy balance, in physiological situations may lead to the development of novel therapeutic approaches to obesity.

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Chapter 8
The Evolution of Mammalian Adipose Tissue

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Abstract Gene products and metabolic pathways comprising and controlling adipose tissues are traced from their invertebrate origins through lower vertebrates to mammals and birds. Many functions of the mammalian liver and pancreas take place in adipose-like tissues in lower animals. Mammalian white adipose tissue is split into numerous depots, many with site-specific properties adapted to paracrine interactions, insulation, or structural roles. Paracrine provision of lipids to the immune system with fatty acid sorting optimizes cellular nutrition even during fasting or on deficient or imbalanced diets, averts competition with other tissues and utilizes scarce resources efficiently. Non-shivering thermogenesis occurs in avian muscles and in mammalian brown adipose tissue, recently as well as metabolic regulation and lipid storage shown to be developmentally related to muscle, not white adipose tissue. The biochemical mechanisms of thermogenesis evolved separately in birds and mammals utilizing several gene families, including uncoupling proteins, present in lower vertebrates. Mammalian thermogenic tissue lost contractile functions and expanded its lipid storage capacity, probably to improve function at hibernation temperatures, thus generating confusing resemblances to white adipose tissue. As well as storage and endocrine functions, adipose tissues' capacities for paracrine interactions, fatty acid sorting and thermogenesis are important in the evolution of mammalian heterothermy, lactation and predominance as herbivores able to thrive on indigestible, poor quality, nutritionally imbalanced diets. Some mammals tolerate high levels of obesity without metabolic impairment, but humans and other apes are not so adapted.

Keywords Comparative • Lower vertebrates • Mammals • Birds • Paracrine interactions • Immune system • Fatty acids • Hibernation • Diet-induced thermogenesis • Herbivory • Lactation

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8.1 Introduction

Advances in developmental biology and evolutionary theory are elucidating the origins and evolution of tissues and cell types, complementing the long-established discipline of comparative anatomy, functionality and adaptation. Both white and brown adipose tissues have been largely omitted from studies of the genetic and developmental bases of comparative anatomy during the past 30 years 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. Recent recognition of white adipose tissue's endocrine roles, its value as a source of stem cells and in reconstructive surgery as well as lipid storage have revived interest in its origins and evolution.

The comparative anatomy and histology of white adipose tissue were studied in detail (Hoggan and Hoggan 1879) 40 years before brown "adipose tissue" received similar attention (Rasmussen 1922, 1923). The similarities between the names of these tissues and their contrasting but apparently complementary contributions to obesity prompted biologists to emphasize their resemblances, an attitude that recent molecular and developmental findings reveal to be misleading.

Meanwhile, obesity and adipose tissues, once almost synonymous, have drifted apart as the focus shifts to appetite control and inheritance. Of several recent attempts to account for the evolution of obesity in humans, some hardly mention current understanding of the organization and basic properties of adipose tissues (Power and Schulkin 2009), while others recognize their central, distinctive roles in human appearance, social and sexual behavior, 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. However, in most wild animals, obesity is transient and controlled: hyperphagia and fat deposition are followed by periods of anorexia and/or intensive exercise, leading to weight loss. 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.

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 functions and structure of adipose tissues.

8.1.1 Storage Tissues in Invertebrates

Most animal cells contain small quantities of triacylglycerols that serve as energy reserves. Many invertebrates, especially those that undergo diapause or metamorphosis, have specialized 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 maximizes contact with the hemolymph 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). 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 itself seems to be specific to vertebrates, probably appearing early in the evolution of fish (Gorissen et al. 2009), but insects have analogous peptides that signal peripheral energy stores to the nervous system (Al-Anzi et al. 2009).

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). This capacity has proved very difficult to induce in mammalian cells (Samson and Chan 2006), though adipose stem cells are among the most promising (Kim et al. 2010).

8.2 Vertebrate Adipose Tissues

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 mobilization of

the lipid stores that support abrupt transitions between dormancy and vigorous activity. 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 mobilized 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 advances in the immune system such as mast cells (Crivellato and Ribatti 2010).

8.2.1 Lower Vertebrates

Many extant fish, especially the primitive groups, store large quantities of triacylglycerols in the liver and/or skeletal muscle, with sporadic occurrence of adipose tissue. 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 (Todorcevic 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 chelonians (tortoises and turtles), adipose tissue is partitioned into numerous small depots that superficially resemble those of mammals (Pond and Mattacks 1984; Davenport et al. 2009), an arrangement that may maximize storage capacity while minimizing distortion of contiguous tissues. 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. 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). Nutritionally imbalanced diets seem to be the main cause of pathology arising from severe obesity in captive reptiles (Frye 1981).

8.3 White Adipose Tissue in Mammals and Birds

The embryonic development of mammalian white adipocytes has been elucidated (Gesta et al. 2007; Rodeheffer et al. 2008; Poulos et al. 2010) and its structure and properties are fully described in other chapters and need not be discussed here.

White adipose tissue comprises >0.5 to 50% of the live body mass of free-ranging wild mammals, with an average of about 7% (Pond and Mattacks 1985b). 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 1985b) and birds (Pond and Mattacks 1985c). The topic has not been thoroughly investigated in reptiles or any other lower vertebrates. 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 mobilization 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 recently very controversial relationship between body mass and basal metabolic rate (Kolokotrones et al. 2010).

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). 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 uptake and release (Digby 1998). Comparative data are too sparse to establish how many other hepatic functions have been "taken over" by adipose tissue in mammals.

Birds have white adipose tissue that closely resembles that of mammals; species with larger adult body size have fewer, larger adipocytes (relative to fatness) than smaller species, the tissue forms early in development and is consistently arranged in several discrete depots that merge only when greatly expanded (Pond and Mattacks 1985a). White adipose tissue metabolism and its neural and endocrinological controls are also impressively similar in both groups (Price et al. 2008).

8.3.1 Anatomical Distribution

White adipose tissue of mammals, and to a lesser extent that of birds, is partitioned into a few large and numerous small depots. 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 epididymal depots are exceptionally large and easily dissected out in murid rodents (rats, mice and 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. In all mammals, white adipose tissue is distributed to a common pattern, characterized by site-specific differences in relative adipocyte volume and various biochemical features (Pond 1992). In pigs (Hausman et al. 2007; Klein et al. 2007) and humans (Ardilouze et al. 2004), site-specific differences can be identified in layers of subcutaneous adipocytes, only some of which can be attributed to functional relationships to hair.

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 distribution of adipose tissue (Piersma et al. 1999; Battley et al. 2000). Measurements on such "adaptively obese" animals reveal surprisingly low, sometimes undetectable energetic costs of the

additional body mass, both 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 reaches 32% body mass in Svalbard rock ptarmigans (*Lagopus muta hyperborea*) (Lees et al. 2010).

Many large, naturally obese mammals occur in areas that are seasonally cold, giving rise to the long-standing and widely disseminated belief that superficial adipose tissue is an adaptation to thermal insulation. However, comparative data on the partitioning of white adipose tissue between superficial and internal depots in mammalian carnivores 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. Abdominal volume and body surface area decrease relative to body mass with increasing size, so superficial adipose tissue can be impressively thick in large, naturally obese mammals regardless of habits and habitats (Pond and Ramsay 1992).

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, not thermal insulation, is limiting (Król et al. 2007). Additional superficial adipose tissue would exacerbate the problem. In experimentally overfed mice, too much superficial adipose tissue decreases skin thickness and elasticity (Ezure and Amano 2010). Such effects would be unlikely if subcutaneous adipose tissue naturally served as body insulation in their wild ancestors. The only exceptions are the three extant groups of marine mammals (Cetacea, Pinnipedia and Sirenia), in which hair reduction and efficient control of blood flow through the superficial adipose tissue enable it to serve as adjustable thermal insulation.

8.3.2 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 1985b). Carnivorous mammals and ruminants have about four times more white 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, which are large omnivores on a high-fat diet.

Wild mammals that naturally become obese have up to five times, usually only two to three 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. 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.

8.3.3 Structural Adipose Tissue

Some small depots are mainly or entirely structural, consisting of large quantities of extracellular material enclosing pockets of metabolically inert adipocytes. The firm, resilient tissue absorbs impact forces during locomotion and distributes weight in the feet, especially those of large terrestrial species such as elephants (Weissengruber et al. 2006). 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 have recently been studied in detail. As well as acting as shock absorbers, the adipose tissue protects blood vessels and facilitates movement (Theobald et al. 2006). 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. A recent review describes recent studies on their development, functional anatomy and surgery (Yousuf et al. 2010).

The white adipose tissue in the orbit behind and around the eye is also primarily structural (Wolfram-Gabel and Kahn 2002) but recent research has revealed it to be less metabolically inert and to have more in common with "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 1986) as in the more abundant metabolically active depots (Pond and Mattacks 1985b). 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 recent increase in the use of such material, both whole tissue and the stem cells derived from it, for reconstructive and cosmetic surgery has reinvigorated the study of previously neglected tissues (Clauser et al. 2008; Stillaert et al. 2010).

8.4 Brown Adipose Tissue

Brown adipose tissue (though not non-shivering thermogenesis) is unique to mammals (Cannon and Nedergaard 2004). As their names imply, brown and white adipose tissues were believed to closely similar, alternative versions of the same tissue. Their triacylglycerols are concentrated enough to form droplets that are clearly visible in intact tissues but are dissolved away completely by histological reagents, leaving blank spaces in preserved, sectioned tissue. The apparent similarity of their neural and endocrinological controls also suggests a common origin. Many depots are mixtures of brown and white adipocytes, and the two types of cell seem to be interconvertible (Cinti 2007), possibly under the influence of endocannabinoids (Perwitz et al. 2010). The principal differences between them are that white adipocytes release almost all of their lipolytic products for use by other tissues while brown adipocytes are net consumers of fatty acids (and glycerol and glucose). On a weight-for-weight basis, white adipocytes contain few mitochondria and are metabolically sluggish while brown adipocytes can achieve among the highest known rates of substrate oxidation in their numerous mitochondria (Cannon and Nedergaard 2004).

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 emphasize their similarities more than their contrasts.

8.4.1 Thermogenesis

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). Metabolic depression in temperate-zone frogs hibernating in ice-covered ponds is mediated by changes to ATP synthase and other mitochondrial components in skeletal muscles (Boutilier and St Pierre 2002). Proteins resembling mammalian uncoupling proteins (UCPs) are also expressed in a reptile (the common green lizard, *Lacerta vivipara*) (Rey et al. 2008) and a teleost fish (Jastroch et al. 2005), where their main function is probably removing toxic-free radicals from mitochondria, and thus are particularly important in highly active aerobic muscle.

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 rewarm themselves with a mixture of shivering and non-shivering thermogenesis (Schleucher 2004; Geiser 2008). However, 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 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).

In birds, mitochondria are uncoupled by membrane protein, adenine nucleotide translocase not UCP, increased Na^+/K^+-ATPase activity on the plasma membrane makes a major contribution and the principal thermogenic tissue is muscle, not adipose tissue (Walter and Seebacher 2009). 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 became so important that the contractile components disappeared, though the very small, rapidly mobilizable lipid droplets remained; ATP synthesis was much reduced though mitochondria became numerous, thus forming brown adipose tissue of myogenic origin (Timmons et al. 2007; Mezentseva et al. 2008). 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 specialization 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. A new name, perhaps "thermogenic tissue", would clarify the situation.

8.5 Paracrine Interactions with Adipose Tissue

Functional interpretation of the anatomy of brown adipose tissue has long been well established: it 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). However, interpretation of 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 has lagged far behind.

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 for adipose tissue was not recognized until the late 1990s (Trayhurn and Beattie 2001).

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 (Dixon 2010) vessels.

8.5.1 The Immune System

The involvement of adipose tissue in immune function has become widely recognized during the past decade (Pond and Mattacks 1995; Zhou and Song 2004; Caspar-Bauguil et al. 2009). Other chapters address the exchange of signal molecules, especially adipokines (Fantuzzi and Mazzone 2007; Caspar-Bauguil et al. 2009) and the role of macrophages in inflammation of adipose tissue in obesity (Qatanani et al. 2009; Gustafson 2010). This section concerns functional, non-pathological relationships between adipose tissue and immune structures.

The mammalian immune system is more elaborate than that of reptiles at all levels from gross anatomy to molecular diversity. 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 have only recently recognized the importance of their relationship to adipose tissue (Harvey et al. 2005; Harvey 2008).

Comparative studies show that an association between the immune system and adipose tissue evolved early in mammalian evolution (Pond 2003b). In the echidna (*Tachyglossus*), a primitive protherian 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).

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, a close association between lymphoid and adipose tissues seems to be a fundamental feature of endothermic vertebrates.

8.5.2 Perinodal Adipose Tissue Around Lymph Nodes

Apart from slightly smaller volume and more extracellular 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 adipose tissue nearest to lymph 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). 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. However, the effect can be prolonged, possibly indefinitely, and elicited in adipocytes situated further from the lymph node, by repeated immune stimulation (Pond and Mattacks 2002).

The appearance of more receptors for tumor 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 tumor 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 adipocytes in depots containing lymph nodes, especially perinodal adipocytes, seem to be partially emancipated from supporting energy supplies for 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 popliteal perinodal adipose tissue is most frequently studied only because these depots are easily accessible and the pair (one or a small group of nodes in compact depots in each hind leg) facilitates experimental design. The responses of perinodal adipocytes in different depots 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. 2004b; 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 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).

8.5.3 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 fatty acid compositions of lipids in intercalated dendritic cells closely resemble those of adjacent adipocytes (Mattacks et al. 2004b). 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. 2004b). 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 2003a). 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 synthesized (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. 2004b). However, 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 is indistinguishable from those of immune-stimulated rats eating standard diets and hardly changes 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 correcting the composition imposed by dietary imbalances (Mattacks et al. 2004b). This mechanism may be among the ways that perinodal adipocytes minimize 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.

8.5.4 Adipose Tissue in Normal Immune Function

Figure 8.1 summarizes known properties of perinodal adipose tissue relevant to its paracrine interactions with the immune system. 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

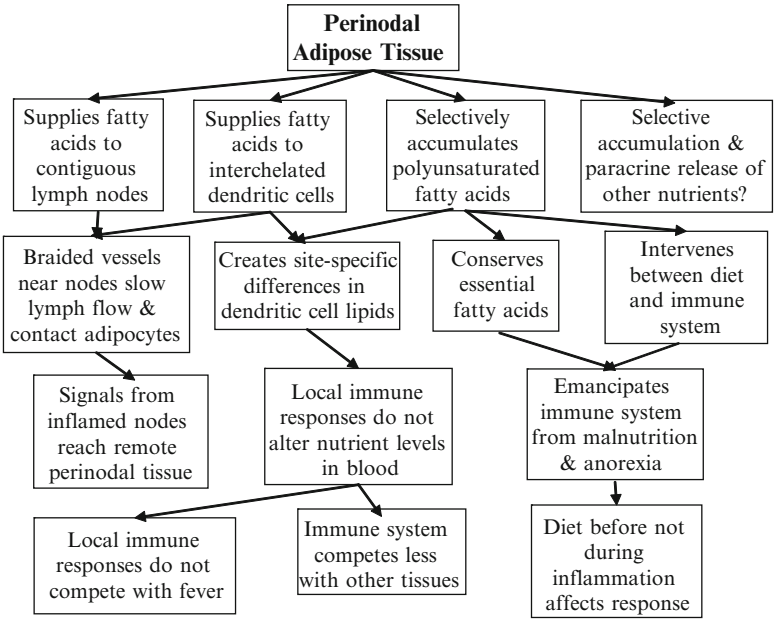

Fig. 8.1 Summary of the structures, properties and functions of mammalian perinodal adipose tissue

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 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 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 defenses 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 specialized adipocytes to the immune system ensures 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 clients 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 metabolism within adipocytes (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 specialized 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 2003a). Ungulates 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 the unusual perinodal adipose tissue may prove 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 criticized 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 co-ordinated 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 tumor cells in culture (Gazi et al. 2007) should prompt further study of paracrine mechanisms.

8.5.5 Human Perinodal Adipose Tissue

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). However, 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 2003a) 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. 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).

"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). 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. 2003; 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 enough of the fatty acids that they need. 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).

Human immunodeficiency virus (HIV)-associated lipodystrophy is another chronic disease in which prolonged inflammation causes site-specific adipocyte hyperplasia and hence permanent enlargement of certain adipose depots, especially those that incorporate infected lymphoid cells. 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). 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. 2004a, 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). Current hypotheses attribute lipodystrophy to a form of premature aging (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.

8.5.6 Paracrine Interactions with Muscle

The history and current understanding of the roles of lipolytic products as fuels for skeletal muscle have recently been well summarized by 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, mobilization 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 metabolized (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).

8.5.7 Cardiac Adipocytes

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) but has recently been studied intensively (Sacks and Fain 2007). Both epicardial and pericardial adipose tissue are found in most lean, healthy wild mammals, especially large species (Marchington et al. 1989). 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).

The dimensions and properties of epicardial and pericardial adipose tissue in humans can be quantified by modern scanners (Iacobellis et al. 2006, 2008a, b; Iacobellis and Sharma 2007) and are intensively studied as indicators of cardiovascular disease (Iacobellis and Sharma 2007; McLean and Stillman 2009). Long-term HIV infection induces hypertrophy of lipids and adipocytes associated with limb muscles (Albu et al. 2007) and the heart (Iacobellis et al. 2007; Lo et al. 2010), as well as the major intra-abdominal depots. Chronic inflammation is unlikely to be an important mechanism in these changes; expression of genes for interleukins and other indicators of inflammation are lower in epicardial adipose tissue than substernal, subcutaneous, or omental depots (Fain et al. 2010).

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). Preliminary studies reveal site-specific properties consistent with the hypothesis that these small depots are specialized 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 specialized 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. Gene expression in epicardial adipose tissue has features in common with that of the omentum (Fain et al. 2008), but since all such tissue samples came from people undergoing bariatric or open-heart surgery, these findings cannot be assumed to represent normality. These studies together are consistent with the hypothesis of paracrine interactions between cardiac muscle and adipose tissue (Chaowalit and Lopez-Jimenez 2008; Iacobellis and Barbaro 2008). Analysis of the huge data set compiled from the Framington heart study concludes that adverse paracrine interactions with cardiac adipose tissue may contribute more than systemic obesity to the risk of cardiovascular disease (Fox et al. 2009). Possible mechanisms of such local exchange of nutrients and

signals have been proposed (Sacks and Fain 2007), but their roles in normal cardiac function remain to be thoroughly elucidated.

Another form of local interaction is thermogenesis in cardiac and other thoracic adipose tissue that warms the heart after hypothermia. Brown adipose tissue is clearly visible in these depots in neonates and hibernators (Nedergaard et al. 1986; Cannon and Nedergaard 2008) and has recently been detected in some adult humans (Cypess et al. 2009). The mRNA for the mitochondrial uncoupling protein (UCP1) is detectable in epicardial adipose tissue of Americans undergoing cardiac bypass surgery and is more abundant in younger subjects (Sacks et al. 2009). It will be interesting to know whether people who are frequently exposed to cold for long periods have increased thermogenic capacity of epicardial adipose tissue. A preliminary study that did not include cardiac depots identified photoperiod as stronger than climate in determining brown adipose tissue activity (Au-Yong et al. 2009).

8.5.8 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 (Rajsheker et al. 2010). These specialized white adipocytes are now known to receive and secrete a wide range of signals (Rajsheker et al. 2010) and to contribute to paracrine control of vascular smooth muscle (Verlohren et al. 2004), immune processes (Wehner and Baldwin 2010) and tissue repair (Takaoka et al. 2010). As with so many physiological functions of adipocytes, these interactions are also implicated in various human pathologies, including atherosclerosis, blood pressure abnormalities and type II diabetes (Henrichot et al. 2005; Lee et al. 2009; Ouwens et al. 2010).

The gene products mediating the relationship between lymph vessels and adjacent adipocytes have recently 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), but the role of such mechanisms in normal mammals remains unclear.

8.6 The Specificity of Fatty Acids

Since leptin was discovered in the early 1990s, the secretion and reception of adipokines have been center stage in adipose tissue research, emphasizing its similarities to other tissues of the immune and endocrine systems (Fantuzzi and Mazzone 2007; Galic et al. 2010). Nonetheless, recent improvements in equipment and techniques for

separating, characterizing and quantifying lipids have greatly advanced understanding of adipose tissue's specialized roles in the sequestration, sorting and selective management of fatty acids and triacylglycerols.

8.6.1 Structural Lipids

All living cells are bounded by fatty membranes and most can oxidize 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 synthesize 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 organization 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 mechanisms involved 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 (Hulbert 2008), and dietary fats correlate with depression and other psychiatric conditions (Horrobin 2001) and 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 (see Fig. 8.1), suggesting that specialized adipocytes supply fatty acids to adjacent immune cells (Pond and Mattacks 2003; Mattacks et al. 2004b; Pond 2009).

8.6.2 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 mobilization 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 maximizes energy density and efficient mobilization 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 mobilized 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.

8.6.3 Fatty Acid Sorting

In mammals including humans, selective deployment and transport of fatty acids begin as dietary lipids are absorbed from the gut (Hodson et al. 2009; Hodson and Fielding 2010). Both brown and white adipose tissue can harbor 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 less 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, as described in Sect. 8.5.

8.6.4 Fatty Acid Sorting in Non-Mammalian Vertebrates

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). However, the process is much more specific and efficient in birds (Speake and Thompson 1999). Avian embryos oxidize 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 12th 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 yolk lipoproteins in 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). 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).

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 recognized, reflects scientific concepts and instrumentation, not biological functions and their evolution.

8.7 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 signaling pathways, and paracrine as well as endocrine interactions. These properties can be related to some of the most fundamental features of mammals: herbivory, variable often high body temperature, lactation, allometric growth and sociality.

8.7.1 Diet

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 as close as it could be to that of the predator. All 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 they are nutritionally almost ideal, a single meal 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 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 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 "futile" metabolic cycles in muscle or liver (Dulloo et al. 2004; Wijers et al. 2009) 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 refined diets fail to recognize the central role of such processes (Kozak 2010).

The presence of brown adipose tissue in superficial depots on the back and neck of adults, including humans (Nedergaard et al. 2007), is also consistent with heat dissipation. The neonatal anatomy, mostly internal depots in the abdomen and thorax, works best for heat retention, as required for rewarming after birth, 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.

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).

8.7.2 Heterothermy

Endothermy has long been regarded as the principal physiological advance of mammals over ancestral mammal-like reptiles. Although core body temperature is maintained very precisely during euthermic periods, heterothermy is highly controlled and many biochemical processes are thermogenic (Silva 2006). Recent accounts of the evolution of endothermy recognize 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.

Mammalian hibernation entails prolonged fasting with the additional physiological challenges of deep hypothermia and rewarming by thermogenesis. Selective gene activation in various tissues and dedicated neural pathways set the minimum body temperature and trigger entry into and emergence from daily torpor and prolonged hibernation (Andrews 2007). During hypothermia, lipids are almost the sole source of metabolic energy (Carey et al. 2003). Metabolism during fasting and thermogenesis closely resembles that of exercise (Newsholme and Leech 2010). At maximum,

thermogenesis can be among the most energy demanding of all biological activities and thus requires rapid mobilization, transport and oxidation of lipids.

Although mammals can oxidize 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 utilizes monounsaturated fatty acids during prolonged hibernation (Falkenstein et al. 2001). Fatty acid sorting ensures that saturates are oxidized 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 described above (Sect. 8.5).

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 protherians (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.

8.7.3 Lactation

Birds are endothermic and most provision their young, mostly by gathering appropriate foods 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 emphasized that lactation is an ancient and fundamental habit of mammals (Pond 1977; Farmer 2000), a conclusion now confirmed by genomics (Lefevre et al. 2010). Lactation enables mammals to breed efficiently on any diet that can support the adults (in contrast, the diets required by hatchling birds and reptiles are usually very different from those of the parents, especially in large species) and to support offspring through periods of food shortage (Pond 1977; Dall and Boyd 2004). 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 8.2 summarizes the causal relationships of these apparently disparate features and habits to brown and white adipose tissues.

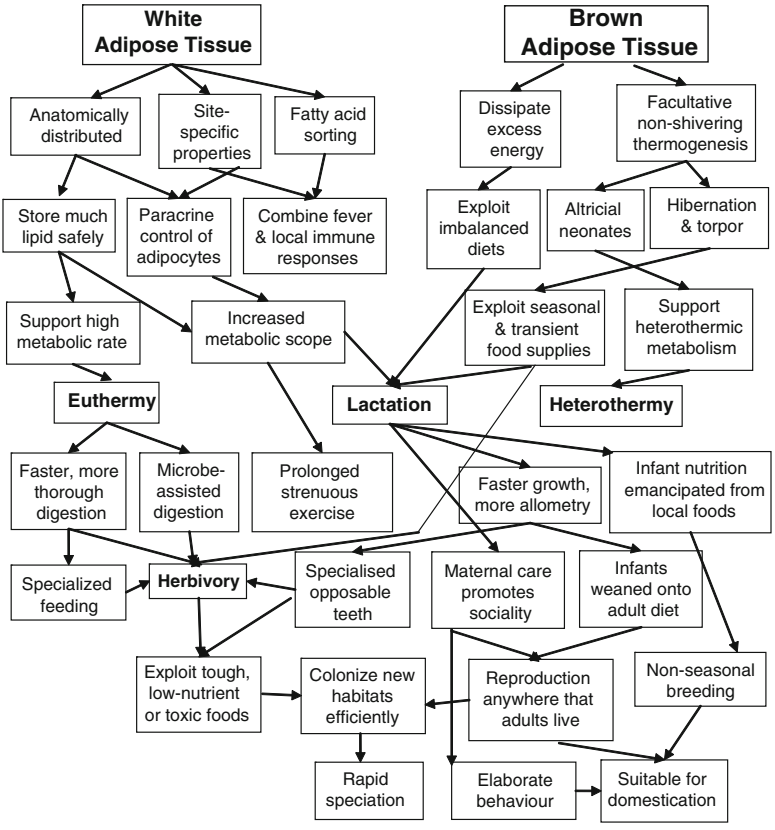

Fig. 8.2 Summary of the contributions of brown and white adipose tissues to the evolution of mammalian structure, habits and reproductive strategy

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 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. Recent reports that the gluteo-femoral depots (always more extensive in women of reproductive age) extract fatty acids from the circulation slightly more slowly than other subcutaneous adipose tissue 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. 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 do 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.

8.7.4 Primates

Despite its obvious relevance to human adipose tissue, surprisingly little research has been done on primates. Sexual dimorphism in the distribution of adipose tissue, such as conspicuous fatty cheeks in mature male orang-utans, contributes to the signaling of age and social status in most apes (Caillaud et al. 2008), though is not more extensive in macaque monkeys and lemurs than would be expected from differences in body size (Pond and Mattacks 1987; Pereira and Pond 1995). The 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).

Humans have more white adipose tissue, especially superficial adipose tissue, 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 1985b). 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 generalized obesity (Harvey 2008). When and why these conditions evolved and their relationship to hairlessness and sexual dimorphism in body shape have been much discussed, but no widely accepted theory has emerged (Pond 1998; Wells 2010).

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). These contrasts are large compared to the small differences between individuals that can be attributed to body conformation and nutrition (Prentice 2005). Using evidence from anthropology, reproductive biology and diet, Wells (2010) concluded that in the great apes, encephalization (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. Hence, the metabolic bases of both these features require efficient digestion and internal distribution of dietary and synthesized lipid.

More than 30 years ago, the greater average fatness of humans and their susceptibility to obesity have been attributed to the belief that until very recently, food supplies were irregular and unpredictable (Wells 2006). The food supplies of other long-lived, slow-maturing apes also have these properties. Measurements made on healthy young adult orang-utans (*Pongo*) 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. Captive specimens are prone to obesity, but there is insufficient information to determine the nature and extent of natural energy reserves.

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). Wrangham has suggested that cooking evolved earlier among the ancestors of modern people than previously believed (Wrangham 2009). The impact of cooking and other forms of manual food processing on dental morphology and digestion are widely accepted. Their contribution to human energetics, including obesity and thermogenic capacity, merits thorough study.

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). Large 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 two to three times larger than those of related lean species (Pond 1998), 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.

8.8 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. The anatomy is closely integrated with paracrine interactions that, by averting competition between tissues, enable adipose tissue to support the specific requirements of many physiological processes simultaneously 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. 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). 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 efficient digestion and utilization of poorer quality diets and rapid colonization of new habitats. Several interspecific comparisons indicate that human obesity is not adaptive.

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Chapter 9
Dietary Determinants of Fat Mass and Body Composition

María A. Zulet, María J. Moreno-Aliaga, and J. Alfredo Martínez

Abstract The stability of body weight and fat composition depends on several components 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 evidence that not all calories 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 fat, 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 in 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.

Keywords Fat mass • Adiposity • Weight loss • Waist circumference • Macronutrient distribution • Fat intake • Protein intake • Glycemic index • Antioxidants • Mediterranean diet

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9.1 Introduction

Obesity is a growing health burden in developed countries and transition countries (McAllister et al. 2009). Moreover, 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.

Several studies have suggested the importance of white adipose tissue (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 (Sethi and Vidal-Puig 2007). 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 (Scherer 2006). Indeed, the development of obesity and accompanying comorbidities is associated with an altered function of the adipocytes, especially concerning the synthesis and secretion of adipokines (Hajer et al. 2008; Galic et al. 2010).

The present chapter reviews the scientific evidence for the effects of several dietary factors on fat mass and body weight regulation (Fig. 9.1), 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 the adipocyte metabolism and secretory functions is also considered.

9.2 Macronutrient Distribution and Energy Density as Determinants of Fat Mass and Body Composition

In many diets designed to reduce body weight and fat mass by restricting the energy content, the macronutrient distribution of energy was commonly set at 15% protein, <30% lipids, and 50–55% carbohydrates. Although this recommendation seemed to be effective for decreasing energy density, lowering and promoting weight and fat loss in the short term, the low achieved levels of satiety are associated with a low dietary adherence over longer periods (Abete et al. 2010). Thus, several nutritional strategies for producing weight loss and reducing the adipose tissue have been investigated (Table 9.1).

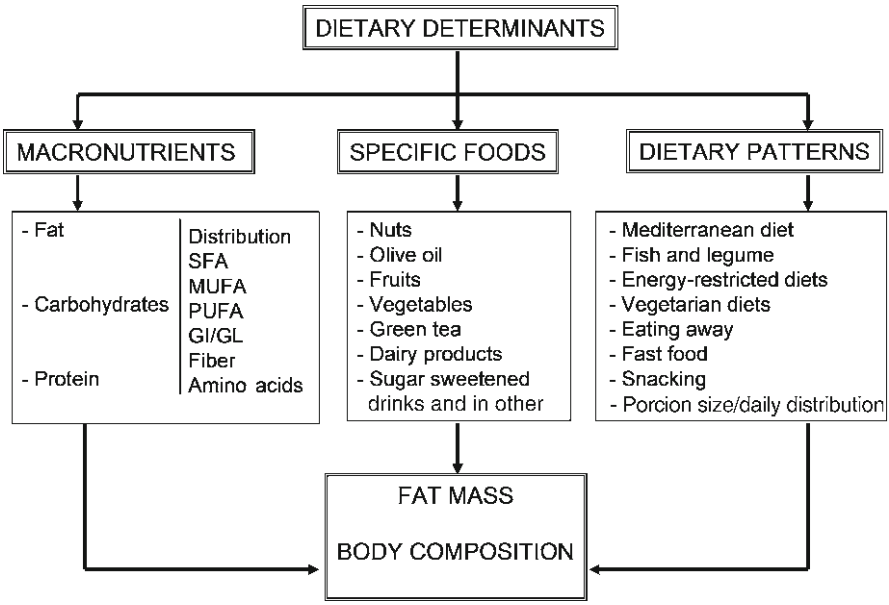

Fig. 9.1 Dietary determinants of fat mass and body composition. *SFA* saturated fatty acids; *MUFA* monounsaturated fatty acids; *PUFA* polyunsaturated fatty acids; *GI* glycemic index; *GL* glycemic load

9.2.1 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 of 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 6 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

Table 9.1 Intervention studies evaluating the role of dietary determinants on fat mass and body composition

Dietary determinant	Subjects/period	Findings	Reference
Macronutrient distribution			
Atkins 30% Protein/65% Lipids/5% CHO Zone 30% Protein/30% Lipids/40% CHO LEARN 15% Protein/30% Lipids/55% CHO Ornish Very-high-CHO	$n=77–79$ 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 Weight loss Atkins, −4.7 kg Zone, −1.6 kg LEARN, −2.6 kg Ornish, −2.2 kg	Gardner et al. (2007)
Conventional 15% Protein/30% Lipids/55% CHO Energy restriction (−500 kcal/day) Moderate Protein 30% Protein/30% Lipids/40% CHO Energy restriction (−500 kcal/day)	$n=130$ 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 At 12 months, protein group had greater improvement in body composition; however, weight loss did not differ between groups	Layman et al. (2009)
Conventional 15% Protein/30% Lipids/55% CHO Energy restriction (−30%) Moderate Protein 30% Protein/30% Lipids/40% CHO Energy restriction (−30%)	$n=19$ 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%) than the control diet	Abete et al. (2009a, b)

Low-fat diet 15% Protein/20–25% Lipids/60–65% CHO Energy restriction (−600 kcal/day) High-fat diet 15% Protein/40–45% Lipids/40–55% CHO Energy restriction (−600 kcal/day)	$n=771$, 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), NUGENOB study
Protein and glycemic index (GI)			
1 of 5 ad libitum diets Low protein/Low GI Low protein/High GI High protein/Low GI High protein/High GI 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	$n=773$ overweight adults from eight European countries, 26 weeks	A modest increase in protein content and a modest reduction in the glycemic index led to an improvement in maintenance of weight loss	Larsen et al. (2010)
	$n=827$ children boys/girls, 5–18 years, 6 months	Neither GI nor protein had an isolated effect on body composition LP/HGI combination increased body fat, whereas the HP/LGI combination was protective against obesity in this sample of children	Papadaki et al. (2010), DIOGENES study
Higher GI 15% Protein/30% Lipids/55% CHO Lower GI 15% Protein/30% Lipids/55% CHO	$n=32$ 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%) 1 Year after the nutritional intervention weight regain was only statistically significant in the higher GI group	Abete et al. (2008a)

(continued)

Table 9.1 (continued)

Dietary determinant	Subjects/period	Findings	Reference
Conjugated linoleic acid (CLA)			
Placebo Olive oil CLA groups 1.7, 3.4, 5.1, and 6.8 g/day 50%c9,t11-50%t10,c12	$n=60$ 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 ($P=0.03$) No additional effect on fat mass was achieved with doses >3.4 g CLA/day No significant differences among the groups were observed in lean body mass and body mass index	Blankson et al. (2000)
Placebo Sunflower oil in chocolate milk CLA groups 3 g/day of 80% CLA 50%c9,t11-50%t10,c12 In chocolate milk	$n=62$ 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 Abdominal and peripheral fat as a percentage of total body weight decreased and differed significantly from that in the placebo group	Racine et al. (2010)
Dairy products			
In addition to their usual diet: Whole-fat dairy supplementation Five servings per day of whole-fat milk/yogurt Low-fat dairy supplementation Five servings per day of low-fat milk/yogurt	$n=45$ men/women, BMI: 23.5 ± 3.6 kg/m^2, Normotensive, Two 8-week periods with a 4-week washout period between both interventions	Weight did not change significantly after the low-fat dairy intervention Whole-fat dairy increased weight significantly compared to low-fat dairy (1.2 kg)	Alonso et al. (2009)

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), The SEAFOODplus YOUNG study
Conventional		The diets did not differ in their effect on weight loss in women	
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 × 150 g portions of cod/week			
Fatty fish			
3 × 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 (four meals per week)			
15% Protein/30% Lipids/55% CHO; Energy-restricted diet (−30% energy expenditure)			

(continued)

Table 9.1 (continued)

Dietary determinant	Subjects/period	Findings	Reference
Fruit			
Low-fruit diet 5% Energy from fructose; 15% Protein/30% Lipids/55% CHO; energy-restricted diet (−600 kcal/day)	$n = 15$ 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)
High-fruit diet 15% Energy from fructose; 15% Protein/30% Lipids/55% CHO; energy-restricted diet (−600 kcal/day)		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%)	
Sugar-sweetened beverages			
All diets were ad libitum and sugar provided 25% of energy Fructose-sweetened beverage Glucose-sweetened beverage	$n = 15$–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 Subcutaneous adipose tissue (SAT) volume was significantly increased in subjects consuming glucose Both total abdominal fat and visceral adipose tissue (VAT) volume were significantly increased in subjects consuming fructose	Stanhope et al. (2009)
Mediterranean diet			
All diets were ad libitum Low-fat diet MD + 1 L/week virgin olive oil MD + 30 g/day mixed nuts	$n = 1{,}224$, subjects at high risk for cardiovascular disease, 1 year	1-Year prevalence of high waist circumference was significantly reduced in the MD + nuts group compared with the control group No significant 1-year changes in body weight	Bes-Rastrollo et al. (2006b), PREDIMED study

BMI Body mass index; *CHO* carbohydrates; *HP* high protein; *HGI* high glycemic index; *LP* low protein; *LGI* low glycemic index; *MD* Mediterranean diet

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) have 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 regard 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. Moreover, in other studies, the usefulness of these results is limited by differences in other macronutrients and high drop-out rates as well as due to the inclusion of additional lifestyle changes (Melanson et al. 2009).

9.2.2 Low-Carbohydrate Diets

Low-carbohydrate diets have been thought as an alternative to a low-fat diet for producing weight loss and fat losses (Krieger et al. 2006). 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 (Yancy et al. 2004; Feinman and Volek 2006; Nordmann et al. 2006). 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). Recently, 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 (1,200–1,800 kcal/day; ≤30% calories from fat), both diets successfully achieved weight loss. Moreover, low-carbohydrate diet was associated with favorable changes in cardiovascular disease risk factors after 2 years (Foster et al. 2010).

However, limitations of these studies include that it could not be determined 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).

9.2.3 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, yet not fully elucidated (Brehm and D'Alessio 2008; Westerterp-Plantenga et al. 2008). Many favorable 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. Also, diets relatively high in protein but with normal carbohydrate content, body weight loss after 2–6 months ranged from 4.9 to 8.9 compared with 3.4 to 6.9 after control diets (Veldhorst et al. 2010). Overall, diets with increased protein and reduced carbohydrates are effective for weight loss, but the long-term effect on maintenance is yet to be investigated. Thus, a study in obese participants consuming moderate-protein (30%) or conventional-protein (15%) energy-restricted diets was carried out to compare changes in body weight after short-term weight loss (4 months) followed by weight maintenance (8 months). The moderate-protein diet was more effective for fat mass loss during initial weight loss, and this group showed greater body composition improvement during long-term maintenance; however, total weight loss did not differ between groups (Layman et al. 2009). Moreover, it has been recently 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. 2010).

Actually, new molecular mechanisms have defined the benefits of protein as a meal threshold for the branched chain amino acid leucine, which has been characterized as a unique signal regulator of muscle protein synthesis (Devkota and Layman 2010). Thus, it has been published that 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). However, current dietary guidelines present protein needs as a percentage of energy in proportion to carbohydrates and fats, but fail to recognize the importance of reaching the leucine threshold at each meal

(Layman 2009). Devkota and Layman (2010) have proposed that humans need to consume at least 25–30 g of protein containing a minimum of 2.5 g leucine/meal to reach an anabolic response that protects metabolic active tissues during weight loss and increases loss of body fat. Moreover, the only way to reverse this catabolic state is to have a high-protein meal as early as possible; thus a balanced daily distribution of protein with increased intake at breakfast and lunch is recommended. Other amino acids could also have a role in energy homeostasis and body composition (Michishita et al. 2010; Galloway et al. 2011).

In summary, there is increased evidence supporting the benefits of higher protein diets. These benefits lie in protein's ability to protect skeletal muscle during caloric restriction. The latest insight into protein research reveals that the bioactive component of dietary protein is leucine. Overall, investigations in humans in relation to these findings are necessary since maintaining healthy muscles during energy restriction is essential for maximizing fat loss and ultimately long-term energy expenditure. Key issues must be resolved regarding the long-term compliance and safety of chronic high-protein intake.

9.2.4 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). 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.

9.3 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). Thus, incidence of obesity was lower in persons who consumed olive oil than those who consumed sunflower oil (Soriguer et al. 2009). 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 18:2t 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). 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 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 metabolic disorders. With respect to adiposity, it has been reported that 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 significant 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. Likewise, current trends lead to decreased consumption of food supplying high *trans* and SFA contents, while foods containing PUFA or MUFA tend to increase.

On the other hand, the consumption of *n*-3 PUFA, eicosapentaenoate (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). 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). Actually, there is also continuing debate as to whether or not *n*-3 PUFA contribute to weight loss and body composition modulation. 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. 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 obese children, the plasma levels of long-chain PUFAs (LC-PUFAs) were associated with the degree of obesity (Scaglioni et al. 2006). 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).

In fact, taking together the 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. Moreover, it has been published that omega-3 fatty acids may have beneficial effects on satiety (Parra et al. 2008).

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).

9.3.1 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). Recently, data has been published showing CLA's efficacy with regard to change in fat and

BMI in children. Thus, CLA supplementation decreased body fatness in children who were overweight or obese. However, long-term investigation of the safety and efficacy of CLA supplementation in children has been recommended by authors (Racine et al. 2010).

On the other hand, 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). With respect to 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), 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. 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). By contrast, other authors 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).

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, DEXA, Infrared), physiopathalogical situations (normal weight, overweight, obese, diabetes, MetS, healthy), in addition to lifestyle (physical exercise or not), among others factors (Table 9.2).

9.4 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 3,734 obese Italian children carried out in the southern Italy, evaluated the association between dietary GI, BMI, and body fat distribution in school children.

Table 9.2 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

The results showed that GI was an independent determinant of both BMI and waist *z*-scores. In particular, GI was the sole nutritional independent determinant of WC marker of abdominal obesity (Barba et al. 2010). 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 8,195 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 1,124 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, the intervention studies in humans focusing on GI or GL also show contradictory results and need more investigation. The comparison of high-carbohydrate (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. 2008a). 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 regard 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 DeltaWC(BMI) (Romaguera et al. 2010a, b). Moreover, a cross-sectional study of 3,931 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).

With regard 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. 2010). 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 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.

9.5 Antioxidants Intake as a Useful Strategy in the Regulation of Body Composition and Fat Depots

In recent years, several studies have hypothesized that obesity might be an inflammatory disorder (Zulet et al. 2007; Tai and Ding 2010). In addition, oxidative stress has been suggested 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 (Valdecantos et al. 2009). 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, 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 recent 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). 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 increasing attention due to identified links with chronic diseases such as obesity. In this sense, antioxidant intake consumption has been suggested to protect against oxidative damage and related inflammatory complications. Thus, consumption of foods containing antioxidants, such as fruits, vegetables, green tea, nuts, olive oil, grapes, and the follow-up of a Mediterranean dietary pattern, etc., 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. (Crujeiras et al. 2006; Barbosa et al. 2008; Romaguera et al. 2009; Razquin et al. 2009) are currently under investigation.

9.6 Specific Foods Consumption in Relation to Fat Mass and Body Composition

9.6.1 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 follow-up 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. 2010). 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).

9.6.2 Fruits and Vegetables

Natural compounds highest in antioxidants 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 beneficial 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 proinflammatory 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 another investigation, two hypocaloric diets with different fruit contents improved antioxidant biomarkers related to lipid peroxidation in obese women, but no differences were observed between diets concerning weight loss and body fat reduction (Crujeiras et al. 2006). In addition, dietary energy density can be reduced by increasing intake of water-rich foods such as vegetables and fruits. Their high-water content allows individuals to eat satisfying portions of food while decreasing energy intake (Rolls 2009).

9.6.3 Green Tea

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 ($n = 1{,}243$ patients) has been recently 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 than 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).

9.6.4 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 (≤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).

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 suggested 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 (1,500 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 1,200–1,300 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).

9.6.5 Sugar-Sweetened Soft Drinks and Water

Sugar-sweetened soft drinks (SSD) are a special target of many obesity-prevention strategies (Malik et al. 2006). However, the inconsistencies of definition, design, statistical treatment, and interpretation make it difficult to draw definitive conclusions as to whether sugar-sweetened beverages are significantly implicated in weight gain. In this context, a systematic review re-examined the evidence from epidemiological studies and interventions, up to July 2008 and identified 44 original studies (23 cross-sectional, 17 prospective, and 4 intervention) in adults and children, as well as 6 reviews (Gibson 2008). Most studies suggested that the effect of SSD is small except in susceptible individuals or at high levels of intake. Of the three long-term (>6 months) interventions, one reported a decrease in obesity prevalence but no change in mean BMI, and two found a significant impact only among children already overweight at baseline. Of the six reviews, two concluded that the evidence was strong, one that an association was probable, while three described it as inconclusive, equivocal, or near zero (Gibson 2008).

However, sweetened beverage intake at age 5 years, but not milk or fruit juice intake, was positively associated with adiposity from age 5 to 15 years. Thus, greater consumption of sweetened beverages at age 5 years (≥2 servings/day) was associated with a higher percentage body fat, WC, and weight status from age 5 to 15 years (Fiorito et al. 2009). In addition, other authors indicate that a greater consumption of SSB is associated with weight gain and obesity and although more research is needed, sufficient evidence exists for public health strategies to discourage consumption of sugary drinks as part of a healthy lifestyle (Fiorito et al. 2009). 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). Newer evidence from clinical and epidemiological studies suggests that there may be risks associated with sugar consumption beyond weight gain, dental caries, and nutritional deficiencies, and that dietary guidelines for sugar consumption need to be reevaluated. In view of these considerations, the American Heart Association recommends reductions in the intake of added sugars. A prudent upper limit of intake is half of the discretionary

calorie allowance, which for most American women is no more than 100 cal/day and for most American men is no more than 150 cal/day from added sugars (Johnson et al. 2009).

With regard to the type of sugar intake, a recent investigation has reported that both subjects consuming glucose-sweetened beverages and those consuming fructose-sweetened beverages exhibited significant increases of body weight and fat mass (Stanhope et al. 2009). Moreover, visceral adipose tissue (VAT) was significantly increased only in subjects consuming fructose, while increased adipose deposition in subjects consuming glucose was mainly distributed in subcutaneous adipose tissue (SAT). In this investigation, the authors concluded that consumption of 25% of energy requirements from fructose for 10 weeks results in increased visceral adiposity and lipids and decreases insulin sensitivity in older, overweight, and obese men and women (Stanhope et al. 2009). Actually, dose–response studies investigating the metabolic effects of prolonged consumption of fructose by itself and in combination with glucose, in both normal weight and overweight/obese subjects, are needed (Stanhope and Havel 2010).

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 vs. water before a single meal. In these comparisons, total energy intakes were 7.8% higher when SSBs were consumed (Daniels and Popkin 2010). With respect to energy intake, consuming 500 mL water prior to each main meal 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).

Taking together, there are promising results for promoting water as a replacement beverage. However, 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., is warranted.

9.7 Dietary Patterns Including Specific Foods and Body Composition

9.7.1 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 (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. 2010).

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 10 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).

In addition, 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. 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 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.

9.7.2 Fish-Based Energy-Restricted Diet

Kunesová et al. (2006) reported significantly greater losses in BMI and hip circumference in obese women following 3 weeks of a very low-calorie diet supplemented

with *n*-3 PUFA vs. placebo. Also, three servings a week of fatty fish included in an energy-restricted diet appears to be a valid strategy for specifically improving insulin sensitivity and leptin levels in obese subjects, which could involve a better body weight regulation after a nutritional intervention period (Abete et al. 2008b).

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), fish oil capsules (1.3 g of omega-3 LC-PUFAs/day), or control (sunflower oil capsules, no seafood). The important finding of the current 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 in BMI 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 energy-restricted diet without marine food, which may be a useful strategy to lose weight and manage adiposity.

9.7.3 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 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.

9.7.4 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). Recently, 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 the 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 (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).

9.7.5 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 than 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).

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. In addition, snacking is also considered an important factor in the development of obesity (Sánchez-Villegas et al. 2002). Thus, 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).

9.8 Regulation of Adipose Tissue Functions by Dietary Factors

Taking together, the studies reported and discussed previously strongly support the proposal that dietary factors are important determinants of adiposity and associated metabolic disorders. 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. 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).

Recently, it has been described that the health effects of food compounds are related mostly to specific interactions on molecular level, such as the regulation of gene expression by modulating the activity of transcription factors. In this context, several studies support that the ability of *n*-3 PUFA supplements in treating hypertriglyceridemia (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 will 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 its anti-obesity effect by inhibiting differentiation to adipocytes (Kim et al. 2006). Lipoic acid, a very important micronutrient with antioxidant and anti-obesity properties (Prieto-Hontoria et al. 2009; Shay et al. 2009), 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).

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).

Stimulation of lipolysis has been suggested to underlie the anti-obesity actions of some dietary components, including CLA and EPA. However, some controversial results have been described. Thus, while some authors observed that *trans*-10,*cis*-12 CLA increases adipocyte lipolysis (Chung et al. 2005), others suggest that the body fat-lowering effect of CLA is not due to this process (Simón et al. 2005). Concerning EPA, 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. 2007).

Adenine monophosphate-activated protein kinase (AMPK) is an important regulator of energy metabolism. In WAT, AMPK activation inhibits fatty acid synthesis and lipolysis, whilst promoting free fatty acid oxidation (Hardie 2008). 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). Also, *n*-3 PUFAs have been shown to upregulate mitochondrial biogenesis and induce 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). CLA also activates AMPK and reduces adiposity in mice adipocytes (Jiang et al. 2009). Moreover, the combination of relatively low doses of lipoic acid and acetyl-L-carnitine improves mitochondrial function in 3T3-L1 murine adipocytes (Shen et al. 2008).

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 dose-dependent manner leptin mRNA expression and leptin 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, and CLA (Pérez-Matute et al. 2003, 2007a, b).

In opposition to leptin, adiponectin concentrations are decreased in obesity and weight loss leads to an increase in adiponectin circulating level (Bruun et al. 2003). Moreover, circulating levels of adiponectin have been positively associated with whole-body insulin sensitivity (Yamauchi et al. 2001). Several assays have suggested that the insulin-sensitizing properties of dietary fish oils could be related to

their ability to increase circulating levels of 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 levels 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).

Visfatin and apelin are two adipokines recently identified (Beltowski 2006). Conflicting results have been described regarding the role played by visfatin in obesity, insulin resistance, and inflammation. 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). Recently, 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 diet-fed) rats (Pérez-Echarri et al. 2009b).

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 rats (Pérez-Matute et al. 2007a, b, c). 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. However, in recent years, *n*-3 PUFA have been demonstrated to serve as substrates for the conversion to a novel series of lipid mediators designated resolvins and protectins, which have been proposed to mediate the protective and beneficial anti-inflammatory

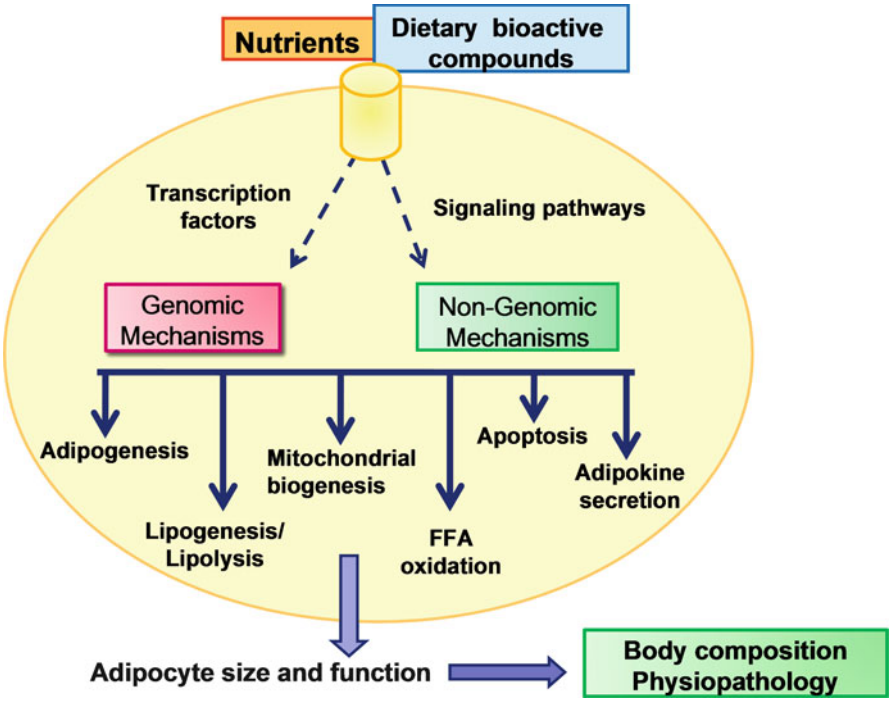

Fig. 9.2 Potential mechanism involved in the regulation of white adipose tissue (WAT) biology and function by nutrients and other dietary bioactive molecules

actions underlying the effects of *n*-3 PUFA (Serhan et al. 2002; González-Périz and Clària 2010). In fact, a recent 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).

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. 9.2).

9.9 Clinical Implications and Future Directions for Research

Body composition determinants are complex with a multifactorial origin, 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 affect

differently 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).

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).
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).
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. However, our present understanding of how diet influences on epigenetic processes remains rudimentary (McAllister et al. 2009).
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).

Finally, the major challenge will be in translating Nutrigenetic/Nutrigenomic/Epigenetics research into dietary guidelines, leading to healthier foods and personalized nutrition (Fig. 9.3). Indeed, personalized nutrition based on nutrigenomics

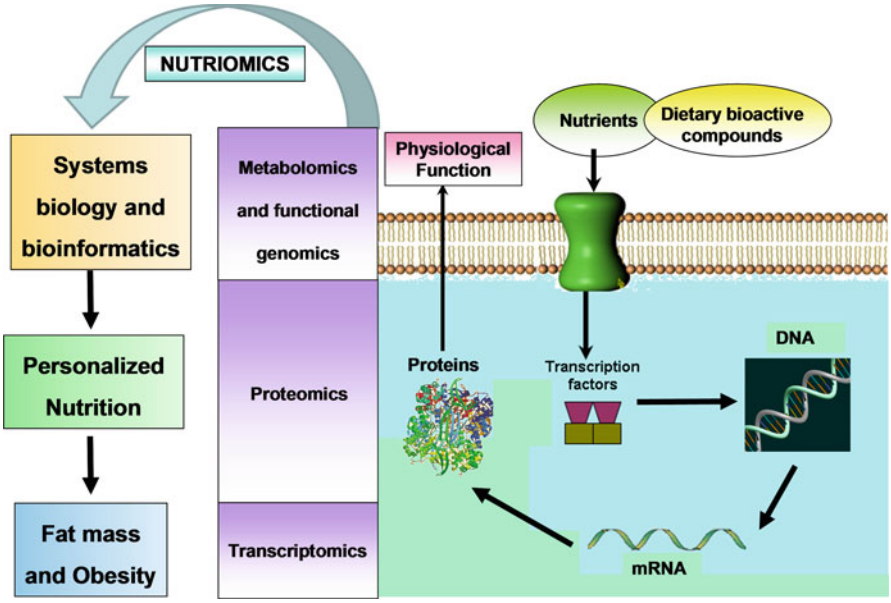

Fig. 9.3 Evolution of "Nutriomics" including epigenomics, transcriptomics, proteomics, and metabolomics will permit better understanding of how dietary factors affect both energy metabolism and fat mass, leading to healthier foods and personalized nutrition

and epigenomics tools 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). The role of the macronutrient content and distribution 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.

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Chapter 10
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 over the past 15 years, candidate gene studies and genome-wide linkage studies were able to identify 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. In less than 4 years time, large-scale genome-wide association studies for body mass index, waist-to-hip ratio and extreme obesity have identified at least 50 obesity-susceptibility loci, most of which had not previously been linked to body weight regulation. Although the combined contribution of these genetic loci to the variation in obesity risk at the population level is small and their predictive value is low, it is anticipated that the recently identified loci will shed new light on the complex physiology that governs 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. This new knowledge could eventually lead to the development of agents 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 personalised implementations.

Keywords Obesity • Genetic epidemiology • Heritability • Candidate gene study • Genome-wide linkage study • Genome-wide association study • Translation • Genetic prediction

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10.1 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 (World Health Organisation 2006). 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 whom nearly half are obese (Flegal et al. 1998, Flegal et al. 2010). Other Western countries have witnessed similar sharp increases and although obesity and overweight prevalences have always been somewhat lower, they follow closely behind those reported for the U.S. population (International Association for the Study of Obesity 2010). Of concern is that obesity is no longer confined to Western societies and a substantial increase in its prevalence has been observed worldwide (Popkin 2008).

It has been well-established that rapid globalisation of the westernised lifestyle is fuelling this growing obesity epidemic. Yet, not everyone in the present-day obesogenic environment becomes obese, and intensive efforts to reduce weight by those who are obese 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 the 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 in particular warrants specific attention. Therefore, the chronological sequence of 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 are being discussed.

10.2 Evidence for a Genetic Contribution to Obesity-Susceptibility

10.2.1 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 standardised 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 body mass index (BMI) data from twin and family studies suggest that the risk of obesity is 1.5–5 times higher for an individual with a family history of obesity compared to the risk in the population at large (Allison et al. 1996; Ziegler et al. 1997; Lee 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 if 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 than U.S.-born individuals upon arrival (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 native-born individuals was the same (Cunningham et al. 2008). These studies suggest that (the American) lifestyle increases the risk of obesity, irrespective of genetic difference between foreign-born and U.S.-born individuals. Yet, 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.

10.2.2 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). However, 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). 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 westernised 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).

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.

10.3 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 that 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).

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 10.1). 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 (Human Genome Sequencing Consortium 2004), The International HapMap (The International HapMap Consortium 2007), The 1000 Genomes (Sudmant et al. 2010), 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. 2010). Here, the contribution of the main gene-discovery approaches to the field of common obesity is being reviewed.

Box 10.1 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,

(continued)

Box 10.1 (continued)

it is expected that the newly indentified 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. Single nucleotide polymorphisms (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 $<5\times10^{-8}$. 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/GWAStudies. 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.

10.4 Candidate Gene Studies

Candidate gene studies are hypothesis driven and rely on the current understanding of the biology that underlies obesity-susceptibility (Box 10.1). 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, in the mid-1990s, genotyping was expensive and tedious, and information on the genetic architecture of the human genome was rather scarce, such that candidate gene studies would examine only one or a few genetic variants in a particular candidate gene. Over time, candidate genes studies became more comprehensive as decreasing genotyping costs, and the availability of catalogues of genetic variation (such as dbSNP and the International HapMap) 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 15 millions SNPs across populations (Sudmant et al. 2010). 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. 10.1).

Despite the large number of candidate gene studies, most of which had valid hypotheses for a given gene to be implicated in obesity-susceptibility, so far, 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 the candidate gene approach are that (1) sample sizes studied are often too small ($n < 1{,}000$) and thus insufficiently powered to identify the small to modest effects that are expected for common obesity, that (2) the genetic variation of the gene of interest was not surveyed comprehensively and that (3) the candidacy was based on limited biological insights.

In recent years, however, an increasing number of candidate gene studies have tested for associations in larger populations ($n > 5{,}000$), and more often, the initiative has been taken to perform meta-analyses of all available published (and unpublished) data. Such large-scale studies have greater statistical power that is needed to confirm or refute associations.

Tables 10.1 and 10.2 summarise the results of large-scale ($n > 5{,}000$) association studies and meta-analyses, respectively. Robust associations have been observed for non-synonymous variants in the melanocortin 4 receptor (*MC4R*), β-adrenergic receptor 3 (ADRB3), prohormone convertase 1/3 (*PCSK1*), brain-derived neurotrophic factor (BDNF), melanotonin receptor type 1 B (*MTNR1B*) genes and for a functional variant near the lactase (*LCT*) gene.

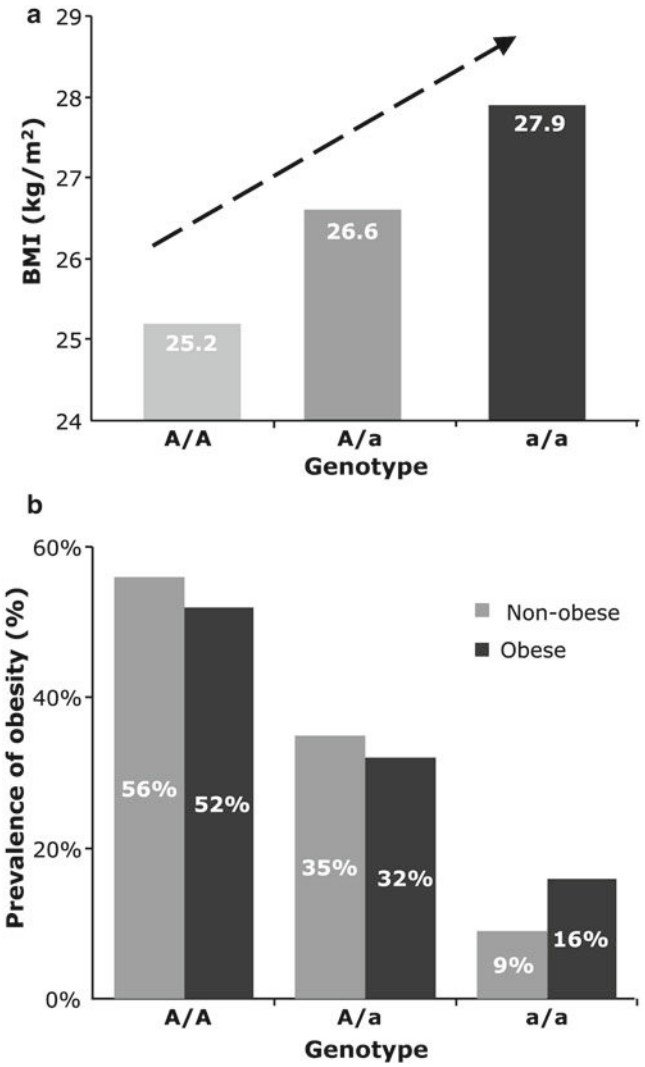

Fig. 10.1 Example of association between a bi-allelic single nucleotide polymorphism (A/a) and body mass index (BMI) (panel a) or obesity risk (panel b), assuming an additive effect of the a-allele. This example shows that each additional a-allele increases BMI and risk of obesity

The 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 (Huszar et al. 1997; Fan et al. 1997). Up to 6% of individuals with severe, early-onset obesity carry pathogenic mutations in *MC4R*, making *MC4R* deficiency the commonest form of monogenic obesity (Farooqi et al. 2003; Vaisse et al. 2000). Patients with *MC4R* deficiency exhibit hyperphagia, increased fat and lean mass, greater bone mineral density and accelerated linear growth (Farooqi et al. 2003).

Table 10.1 Overview of large-scale ($n > 5000$) individual candidate gene studies for obesity-related traits (genes in alphabetical order)

Gene	Design	Variants	IndividualsN	Main outcome	References
ADRB1		Arg389Gly	7,677	No association with obesity or BMI	Gjesing et al. (2007a)
ADRB2		Arg16Gly	7,808	No association with obesity	Gjesing et al. (2007b)
		Gln27Glu		No association with obesity	
ADRB3		Trp64Arg	7,605	No association with obesity	Gjesing et al. (2008)
BDNF		Val66Met	10,109	Val-allele carriers have a higher BMI (+0.76 kg/m^2 [95CI 0.036–1.16]; $P < 0.001$) than Met-allele homozygotes	Shugart et al. (2009)
DIO2		Thr92Ala	7,342	No association with obesity	Grarup et al. (2007b)
ENPP1		Lys121Gln	5,863	No association with BMI or waist	Grarup et al. (2006)
ENPP1		Lys121Gln + 3 marker haplotype	8,676	No association with BMI	Lyon et al. (2006)
ENPP1		Lys121Gln + 3 marker haplotype	8,089	No association with obesity	Weedon et al. (2006)
ENPP1		Lys121Gln + 3 marker haplotype	5,153	No association with BMI, overweight, or obesity. Gln-allele nominally associated with morbid obesity ($P = 0.02$)	Meyre et al. (2007)
ERRalpha		Pro116Pro and ESRRA23	6,365	No association with obesity or BMI	Larsen et al. (2007)
FAAH		Pro129Thr	5,801	No association with obesity or BMI	Jensen et al. (2007)
GHSR	Systematic screen of gene	7 SNPs	15,854	No association with BMI, obesity or overweight	Gjesing et al. (2010)
HTR1C	Systematic screen of gene	6 SNPs	4,973–20,976	No association with obesity, BMI or waist circumference	Vimaleswaran et al. (2010)
IGF2	Systematic screen of gene	3 SNPs	5,000	No association with BMI	Heude et al. (2007)

(continued)

Table 10.1 (continued)

Gene	Design	Variants	IndividualsN	Main outcome	References
INSIG1	Systematic screen of gene	6 SNPs	7,562	No association with obesity or BMI	Szopa et al. (2010)
KLF7	Systematic screen of gene	4 SNPs	14,818	Minor allele of rs7568369 protected against obesity (OR = 0.90 [0.84–0.96], $P = 0.001$), and is associated with lower BMI ($P = 0.002$) and waist circumference ($P = 0.003$)	Zobel et al. (2009a)
LPIN1		3 SNPs	17,538	No association with BMI, waist circumference, or with obesity risk	Burgdorf et al. (2010)
LTA		Thr60Asn	5,630	N-allele carriers have increased waist circumference ($P = 0.009$), but not BMI	Hamid et al. (2005)
MC4R		V103I	7,937	Ile-allele carriers have a reduced risk of obesity (OR 0.69 [95% 0.50–0.96], $P = 0.03$)	Heid et al. (2005)
MTNR1B	Systematic screen of gene for nonsynonymous variants	Six nonsynonymous variants: Gly24Glu, Leu60Arg, Val124Ile, Arg138Cys, Arg231His, Lys243Arg	10,610	The Glu-allele of the Gly24Glu variants associated with increased risk of obesity (OR 1.20; $P = 8.3 \times 10^{-4}$), increased BMI (+0.5 kg/m^2; $P = 1.2 \times 10^{-5}$) and waist circumference (+1.2 cm; $P = 9 \times 10^{-6}$).	Andersson et al. (2010)
NOS1AP		rs7538490	16,913	No association with obesity	Andreasen et al. (2008a)
NPY2R	Systematic screen of gene	3 SNPs	5,971	Minor-allele carriers of rs12649641 have increased risk of obesity ($P = 0.02$)	Torekov et al. (2006)
NPY2R	Systematic screen of gene	5 SNPs	>8,000	No association with BMI	Campbell et al. (2007)

PCSK1	Systematic screen of gene	N221D	13,659	Minor allele is associated with increased risk of obesity (OR 1.34 [95% 1.20–1.49], $P=7.27x10^{-8}$)	Benzinou et al. (2008)
		Q665E-S690T		Minor allele is associated with increased risk of obesity (OR 1.22 [95% 1.15–1.29], $P=2.31x10^{-12}$)	
PCSK1		N221D & S690T	20,249	Neither of the SNPs was associated with obesity, BMI or waist circumference. However, N221D was associated with BMI ($P=0.021$) and obesity ($P=0.022$) in the younger age group (<59 years), but not among the older age group	Kilpelainen et al. (2009)
PKLR		rs3020781	16,801	No association with obesity	Andreasen et al. (2008a)
PPARA		Leu162Val	5,799	No association with obesity	Sparso et al. (2007)
PPARD	Systematic screen of gene	12 SNPs	5,971	No association with BMI	Grarup et al. (2007a)
PPARGC1B	Systematic screen of gene	Ala203Pro	7,790	203Pro allele is associated wit a reduced obesity risk ($P=0.004$)	Andersen et al. (2005)
PYY		Arg72Thr	6,022	Thr-allele carriers have increased risk of overweight ($P=0.02$)	Torekov et al. (2005)
STAMP2	Systematic screen of gene	9 SNPs	5,212	No association with waist circumference	Miot et al. (2010)

Table 10.2 Overview of meta-analyses of candidate gene loci for obesity-related traits (genes in alphabetical order)

Gene	Design	Variants	Individuals*N*	Main outcome	References
ADRB1		Arg389Gly	7,677	No association with obesity or BMI	Gjesing et al. (2007a)
ADRB2		Arg16Gly	7,808	No association with obesity	Gjesing et al. (2007b)
		Gln27Glu		No association with obesity	
ADRB3		Trp64Arg	7,605	No association with obesity	Gjesing et al. (2008)
BDNF		Val66Met	10,109	Val-allele carriers have a higher BMI (+0.76 kg/m^2 [95CI 0.036–1.16]; $P<0.001$) than Met-allele homozygotes	Shugart et al. (2009)
DIO2		Thr92Ala	7,342	No association with obesity	Grarup et al. (2007b)
ENPP1		Lys121Gln	5,863	No association with BMI or waist	Grarup et al. (2006)
ENPP1		Lys121Gln + 3 marker haplotype	8,676	No association with BMI	Lyon et al. (2006)
ENPP1		Lys121Gln + 3 marker haplotype	8,089	No association with obesity	Weedon et al. (2006)
ENPP1		Lys121Gln + 3 marker haplotype	5,153	No association with BMI, overweight, or obesity. Gln-allele nominally associated with morbid obesity ($P=0.02$)	Meyre et al. (2007)
ERRalpha		Pro116Pro and ESRRA23	6,365	No association with obesity or BMI	Larsen et al. (2007)
FAAH		Pro129Thr	5,801	No association with obesity or BMI	Jensen et al. (2007)
GHSR	Systematic screen of gene	7 SNPs	15,854	No association with BMI, obesity or overweight	Gjesing et al. (2010)
HTR1C	Systematic screen of gene	6 SNPs	4,973–20,976	No association with obesity, BMI or waist circumference	Vimaleswaran et al. (2010)
IGF2	Systematic screen of gene	3 SNPs	5,000	No association with BMI	Heude et al. (2007)
INSIG1	Systematic screen of gene	6 SNPs	7,562	No association with obesity or BMI	Szopa et al. (2010)

KLF7	Systematic screen of gene	4 SNPs	14,818	Minor allele of rs7568369 protected against obesity (OR = 0.90 [0.84–0.96], $P = 0.001$), and is associated with lower BMI ($P = 0.002$) and waist circumference ($P = 0.003$)	Zobel et al. (2009a)
LPIN1		3 SNPs	17,538	No association with BMI, waist circumference, or with obesity risk	Burgdorf et al. (2010)
LTA		Thr60Asn	5,630	N-allele carriers have increased waist circumference ($P = 0.009$), but not BMI	Hamid et al. (2005)
MC4R		V103I	7,937	Ile-allele carriers have a reduced risk of obesity (OR 0.69 [95% 0.50–0.96], $P = 0.03$)	Heid et al. (2005)
MTNR1B	Systematic screen of gene for nonsynonymous variants	Six nonsynonymous variants: Gly24Glu, Leu60Arg, Val124Ile, Arg138Cys, Arg231His, Lys243Arg	10,610	The Glu-allele of the Gly24Glu variants associated with increased risk of obesity (OR 1.20; $P = 8.3 \times 10^{-4}$), increased BMI (+0.5 kg/m^2; $P = 1.2 \times 10^{-5}$) and waist circumference (+1.2 cm; $P = 9 \times 10^{-6}$)	Andersson et al. (2010)
NOS1AP		rs7538490	16,913	No association with obesity	Andreasen et al. (2008a)
NPY2R	Systematic screen of gene	3 SNPs	5,971	Minor-allele carriers of rs12649641 have increased risk of obesity ($P = 0.02$)	Torekov et al. (2006)
NPY2R	Systematic screen of gene	5 SNPs	>8,000	No association with BMI	Campbell et al. (2007)

(continued)

Table 10.2 (continued)

Gene	Design	Variants	Individuals*N*	Main outcome	References
PCSK1	Systematic screen of gene	N221D	13,659	Minor allele is associated with increased risk of obesity (OR 1.34 [95% 1.20–1.49], $P=7.27x10^{-8}$)	Benzinou et al. (2008)
		Q665E-S690T		Minor allele is associated with increased risk of obesity (OR 1.22 [95% 1.15–1.29], $P=2.31x10^{-12}$)	
PCSK1		N221D & S690T	20,249	Neither of the SNPs was associated with obesity, BMI or waist circumference. However, N221D was associated with BMI ($P=0.021$) and obesity ($P=0.022$) in the younger age group (<59 years), but not among the older age group	Kilpelainen et al. (2009)
PKLR		rs3020781	16,801	No association with obesity	Andreasen et al. (2008a)
PPARA		Leu162Val	5,799	No association with obesity	Sparso et al. (2007)
PPARD	Systematic screen of gene	12 SNPs	5,971	No association with BMI	Grarup et al. (2007b)
PPARGC1B	Systematic screen of gene	Ala203Pro	7,790	203Pro allele is associated wit a reduced obesity risk ($P=0.004$)	Andersen et al. (2005)
PYY		Arg72Thr	6,022	Thr-allele carriers have increased risk of overweight ($P=0.02$)	Torekov et al. (2005)
STAMP2	Systematic screen of gene	9 SNPs	5,212	No association with waist circumference	Miot et al. (2010)

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. The two most common *MC4R* variants, V103I and I251L, 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) (Table 10.1). 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 V103V homozygotes (Geller et al. 2004; Young et al. 2007; Stutzmann et al. 2007; Wang et al. 2010) (Table 10.2). 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) (Table 10.2). 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.

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) 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 Finnish populations (Widen et al. 1995). Following these first reports in 1995, more than 100 studies have been published on the association between the Arg64Trp variant and obesity-related traits, but results have been inconsistent. Even the results of three consecutive meta-analyses on the association with BMI were inconclusive (Table 10.2). The first meta-analysis combined data from 36 populations published before June 1997 including a total of 7,399 individuals and found no evidence of association between the Arg64Trp and BMI (Allison et al. 1998). The absence of heterogeneity suggested that the results were not affected by ethnicity or diabetes status (Allison et al. 1998). The second meta-analysis included data from an additional eight studies (n_{total} = 9,236) and, in contrast to the first meta-analysis, the Arg64-allele carriers were found to have a significantly higher BMI (+0.30 kg/m^2) compared to Trp64Trp homozygotes. Similar to the first meta-analysis, there was no evidence for effect heterogeneity by ethnicity. The third meta-analysis included data of Japanese only (n = 6,582) and confirmed that carriers of the Arg64-allele had a

significantly higher BMI (+26 kg/m^2) than Trp64Trp homozygotes (Kurokawa et al. 2001). The 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 Arg64Trp 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 Arg64Arg 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%), suggesting that this variant is more important in East Asians. The functional effect of the Trp64Arg polymorphism on the expression and activity of *ADBR3* 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 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 prohormones 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; Farooqi et al. 2007; Jackson et al. 2003). A recent 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, N221D (rs6232) and the Q665E-S690T pair (tagged by rs6235), were consistently associated with obesity in adults and children (Table 10.1). Each additional minor allele (frequency: 4–7%) of the N221D variant increased the risk of obesity by 1.34-fold, while each additional minor allele (frequency: 25–30%) of the Q665E-S690T pair increased the risk by 1.22-fold. However, a subsequent population-based study, including 20,249 individuals of white European origin, could not convincingly confirm the previously observed associations with obesity-related traits (Kilpelainen et al., 2009). In this study, the association between the N221D variant and obesity reached a magnitude (OR $\geq$ 1.24) similar to that observed in the first study, and the association with BMI (+0.25 kg/m^2/allele) reached nominal significance, but only in the younger age group (<59 years) (Kilpelainen et al. 2009). A weak ($P = 0.03$) but directionally consistent association with BMI was also observed for N221D (no data available for Q665E-S690T) in a genome-wide association study of BMI (Willer et al. 2009). The population-based study by Kilpelainen et al. (2009) found no evidence of association between the Q665E-S690T pair and any of the examined obesity-related traits (Kilpelainen et al. 2009). The inconsistency in results reported by Benzinou et al. (2008) and Kilpelainen et al. (2009) may in part be explained by differences in

age of the study participants. Benzinou et al. (2008) included three case–control cohorts of children, whereas Kilpelainen et al. (2009) studied individuals between 40 and 49 years of age. Furthermore, a high number of the obese cases studied by Benzinou et al. (2008) were class III obese (BMI ≥ 40 kg/m^2), whereas only 4% of the obese participants of the study by Kilpelainen et al. (2009) had such a high BMI. This would suggest that the *PCSK1* variants might be associated with a more extreme and earlier onset for of obesity. Functional characterisation of these two variants suggested a modest deleterious effect of the N221D variant, but no functional role for the Q665E-S690T was observed (Benzinou et al., 2008).

Brain-derived neurotrophic factor (BDNF) – *BDNF* is believed to act primarily in the hypothalamus, downstream of the leptin–proopiomelanocortin signalling pathway (Unger et al. 2007; Wang et al. 2007; Xu et al. 2003). *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 report. *BDNF* mutant mice show a reduced expression of the gene in the hypothalamus; they are hyperphagic, obese and hyperactive (Kernie et al. 2000; Fox and Byerly 2004; Rios et al. 2001). While no mutations in humans have been described, a de novo chromosomal inversion at chr11p, a region encompassing *BDNF*, was detected in an 8-year-old girl who was hyperphagic, severely obese and hyperactive (Gray et al., 2006). Furthermore, in patients with the Wilms tumor, aniridia, genitourinary anomalies, and mental retardation 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). The Met-allele has also been associated with 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 recent 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) (Table 10.1). A recent large-scale genome-wide association study reported highly significant associations between variants in the locus that harbours the Val66Met and BMI, confirming *BDNF* a common obesity-susceptibility gene (Speliotes et al. 2010).

Melanotonin receptor type 1 B (MTNR1B) – Genome-wide association studies previously identified common variants in the *MTNR1B* gene to be unequivocally associated with fasting glucose and risk of type 2 diabetes (Bouatia-Naji et al. 2009; Prokopenko et al. 2009), most likely through an effect on beta cells (Lyssenko et al. 2009). Because melatonin is involved in the regulation of circadian rhythms (Claustrat et al. 2005), which contribute to metabolic disorders when disturbed (Scheer et al. 2009), it has been speculated that variation in the *MTNR1B* (which encodes the MT2-receptor) could contribute to obesity-susceptibility (Andersson et al. 2010). While there is no evidence for the previously identified glucose-associated

MTNR1B variants to be associated with BMI (Speliotes et al. 2010), a large-scale candidate gene study examined whether rare non-synonymous variants in *MTNR1B* are associated with risk of diabetes and obesity (Andersson et al. 2010) (Table 10.1). By sequencing *MTNR1B* in 200 individuals, six non-synonymous variants were identified that were subsequently genotyped in a large sample of individuals of European descent. Four variants had a minor allele frequency of <1%, whereas the Lys243Arg (frequency 3.3%) and Gly64Glu (9.2%) were more frequent. While none of the variants contributed to the risk of type 2 diabetes, each additional Glu24-allele of the Gly24Glu polymorphism was associated with a 20% increased risk of obesity, and with increased BMI (+0.50 kg/m^2) and waist circumference (+1.2 cm) in a population-based sample of 10,610 individuals (Andersson et al. 2010). Counterintuitively, the Glu24-allele was also associated with decreased fasting glucose levels. Functional characterisation analyses demonstrated that the Glu24-allele decreased the constitutive signalling activity of MT2-receptor, but not its potency or efficacy (Andersson et al. 2010). The Gly24Glu (rs8192552) variant has not been tested for association in genome-wide association studies, as it was not available on the HapMap release that is used for imputation (see below). Replication of these findings in other large-scale cohorts will be needed to further confirm these observations. No association with obesity-related traits was reported for the Lys243Arg variant.

Lactase (LCT) – LCT is expressed in intestinal epithelial cells and encodes the *LCT* enzyme, which contributes to the digestion of the milk sugar lactose. Its enzymatic activity typically declines during childhood, such that 75% of adults worldwide develop *LCT* non-persistence (or lactose intolerance). However, there are marked regional differences, and the majority of northern and western Europeans, as well as some Middle Eastern, African and southern Asian populations, show *LCT* persistence (lactose tolerance). The down-regulation of *LCT* activity during childhood has been linked to a variant (C/T$_{-13910}$, rs4988235) in a *cis*-regulatory element near *LCT* (Enattah et al. 2002). The T-allele has been associated with a disrupted down-regulation, leading to *LCT* persistence in adulthood. While *LCT* might not seem the most obvious candidate gene in the context of obesity-susceptibility, a recent study hypothesised that because *LCT* non-persistence individuals have a more restricted diet compared to those with *LCT* persistence, this may affect their BMI (Kettunen et al. 2010). In this study, the C/T$_{-13910}$ variant was genotyped in 31,720 individuals from four European populations. The prevalence of T-allele carriers (i.e. those with *LCT* persistence) varied across countries from 80% in Finnish populations to >90% in British and Dutch populations. Overall, the T-allele carriers had a significantly higher BMI than the C/C homozygotes (Table 10.2). However, this association was more pronounced in Finnish populations, than in the other European populations combined (Kettunen et al. 2010). The heterogeneity among populations of European descent, combined with the fact that the association is only observed under a dominant model of inheritance, might explain why recent genome-wide association studies did not report association for the *LCT* variants and BMI (Speliotes et al. 2010).

While such large-scale and often comprehensive candidate gene studies have sufficient power to identify small effects, they are also powered to refute associations. For example, for the Lys121Gln variant in *ENPP1,* four studies with each more

than 5,000 participants and a combined sample size of 27,781 individuals found no association between the Lys121Gln variant and obesity-related traits (Lyon et al. 2006; Weedon et al. 2006; Meyre et al. 2005; Grarup et al. 2006) (Table 10.1). Also for other genes, despite their sometimes strong biological candidacy, there was sufficient data to refute association with obesity-related traits; these include the Ala54Thr variant in *fatty acid binding protein 2* (Zhao et al. 2010b), seven variants in the *grehlin receptor* (Gjesing et al. 2010), the -174 G>C variant near *interleukin 6* (Huth et al. 2009; Qi et al. 2007), four variants in *lipin 1* (Fawcett et al. 2008; Burgdorf et al. 2010), two variants in *liver pyruvate kinase* and *nitric oxide synthase 1 adaptor protein* (Andreasen et al. 2008a), and the -759 C/T variant near the *serotonin 5-HT-2 C receptor* (Vimaleswaran et al. 2010) (Tables 10.1 and 10.2). 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 genetic variation elsewhere in the gene.

For other candidate genes that were examined in large-scale studies or in meta-analyses (Tables 10.1 and 10.2), such as for variants in the *beta-adrenergic receptor 2* (Jalba et al. 2008), *Kruppel-like factor 7* (Zobel et al. 2009a), *lymphotoxin alpha* (Hamid et al. 2005), *peptide YY* (Torekov et al. 2005), *peroxisome proliferator-activated receptor gamma* (Tonjes et al. 2006) and for many of the other proposed candidate genes that have so far only been examined in small studies, the results are more ambiguous, and further follow-up is required to unambiguously prove or refute their role in obesity-susceptibility.

Taken together, despite 15 years of candidate gene efforts, this approach has only recently started to succeed. Large-scale studies and meta-analyses have identified common variants, mostly non-synonymous and/or functional, in at least six candidate genes (*MC4R*, *ADRB3*, *PCSK1*, *BDNF*, *MTNR1B* and *LCT*) to be robustly associated with obesity-related traits. It is reassuring that also genome-wide association studies (see below) show either strong evidence (for *MC4R*, *BDNF*) or suggestive evidence (for *PCSK1*) of association with BMI (Speliotes et al. 2010). Association for variants in the other three genes could not be confirmed by genome-wide association studies as the particular variant (*MTNR1B*), the dominant model (*LCT*) or the specific ethnic group (*ADRB3*) was not examined.

10.5 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 10.1). Genome-wide linkage studies have a rather low resolution and typically identify broad intervals that require follow-up genotyping to pinpoint the genes that underlie the linkage signal.

Since the first genome-wide linkage study on body fat percentage in Pima Indians was published 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, which summarised the literature up to October 2005, 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). However, 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.

10.6 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 10.1).

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, genome-wide association studies have identified more than 900 genetic loci for over 160 diseases and traits, including at least 50 loci for obesity-related traits (Hindorff et al. 2010). 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 genome-wide association studies is robust.

10.6.1 The Genome-Wide Association Study Design

Genome-wide association studies typically consist of a least 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 population-based cohorts to test for association with a continuous trait (e.g. BMI, waist-to-hip ratio (WHR)) 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. 10.1).

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. These consortia use genome-wide meta-analyses to combine summary statistics of a series of individual genome-wide association studies 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 in a study and based on that of a reference panel (e.g. the CEU HapMap population), the genotypes of ~2.5 million untyped HapMap 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. 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 individual genome-wide association studies, are meta-analysed to calculate the overall significance of the associations. The 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 2.5 million SNPs according to their position in the genome (Fig. 10.2).

Given that hundreds of thousands 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 $<5\times10^{-8}$, 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 (de Bakker et al. 2008). Therefore, SNPs for which the 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 $<5\times10^{-8}$ at the replication stage. It should be 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

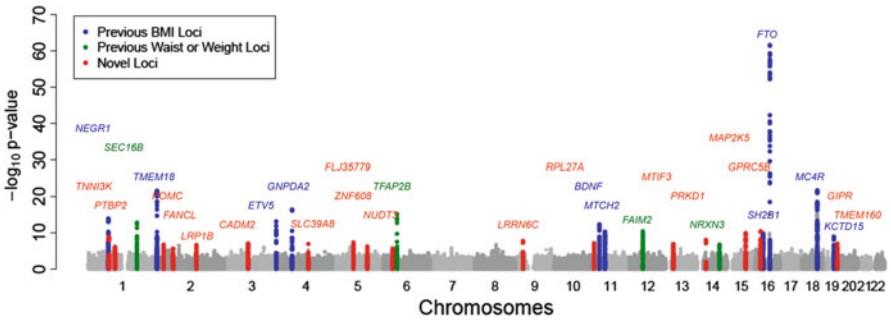

Fig. 10.2 Manhattan plot of the association between genome-wide data and BMI in the meta-analysis of the Genomic Investigation of Anthropometric Traits Consortium. The –log10 *P*-values for the association of each single nucleotide polymorphism with body mass index (BMI) are shown on the *y*-axis. The single nucleotide polymorphisms (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 (BMI) or green (weight and waist circumference). The SNPs that were taken forward from the discovery stage and that were replicated as new BMI hits are shown in red. Adapted from Speliotes et al. (2010), with permission from Nature Publishing Group

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 $<5\times10^{-8}$ 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 $<5.0\times10^{-8}$, 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 often further examined in a third stage to examine their functional implications, to assess their effects on related traits or to fine-map the locus to identify the causal variant or gene.

10.6.2 The Discovery of at Least 50 Obesity-Susceptibility Loci

Since the introduction of the genome-wide association approach in 2005, the obesity genetics field has witness the discovery of at least 50 genetic loci that are unequivocally associated with obesity-related traits (Table 10.3, Fig. 10.3). Large-scale high-density genome-wide association studies and meta-analyses have been performed for BMI, waist circumference, WHR and extreme and early-onset obesity. These have been performed predominantly in adults of white European descent. However, the past few years has seen a growing number of genome-wide association studies in populations of Asian and African origin.

Table 10.3 Summary of three waves of large-scale high-density genome-wide association studies for body mass index and extreme obesity risk (ordered chronologically by trait)

Study	Frayling et al.(2007)	Scuteri et al. (2007)	Loos et al. (2008)	Chambers et al.(2008)	Willer et al. (2009)	Thorleifsson et al.(2009)	Speliotes et al.(2010)	Lindgren et al.(2009)	Heard-Costa et al.(2009)	Heid et al. (2010)	Hinney et al.(2007)	Meyre et al. (2009)	Scherag et al. (2010)
	Body mass index							*Waist circumference and waist-to-hip ratio*			*Extreme and early onset obesity*		
Discovery stage													
Study design	Case-control study	Population-based study	Meta-analysis of 4 population-based studies and 3 disease-case series	Population-based study	Meta-analysis of 10 population-based cohorts and 5 disease-case series	Meta-analysis of 4 population-based cohorts	Meta-analysis of 46 population-based cohorts and disease-case series	Meta-analysis of 16 population-based cohorts and disease-case series	Meta-analysis of 10 population-based cohorts and disease-case series	Meta-analysis of 32 population-based cohorts and disease-case series	Case-control study	Case-control study	Case-control study
Ethnicity	White Europeans from the UK	White Europeans from Sardinia (Italy)	White Europeans from the UK and Switzerland	Indian Asians	Whites from the UK, USA, Sweden, Finland, Italy, Switzerland and Germany	Whites from Iceland, the USA and The Netherlands, and African Americans	Whites from the UK, USA, Sweden, Finland, Italy, Switzerland, Germany, the Netherlands, Iceland, Estonia, and Croatia	Whites from the UK, USA, Sweden, Finland, Italy, Switzerland, Germany, and Iceland	Whites from the USA, Italy, Croatia, and the Netherlands.	Whites from the UK, USA, Sweden, Finland, Italy, Switzerland, Germany, the Netherlands, Iceland, Estonia, and Croatia	White Europeans from Germany	White Europeans from France	White Europeans from France and germany
Traits	Type 2 diabetes*	BMI, hip and weight	BMI	BMI, waist circumfer-ence and waist-hip ratio, weight, and other metabolic traits	BMI	BMI and weight	BMI	Waist circumference and WHR	Waist circum-ference	WHR adjusted for BMI	Early onset extreme obesity	Early-onset and morbid adult obesity	Early onset extreme obesity

(continued)

Table 10.3 (continued)

Study	Frayling et al.(2007)	Scuteri et al. (2007)	Loos et al. (2008)	Chambers et al.(2008)	Willer et al. (2009)	Thorleifsson et al.(2009)	Speliotes et al.(2010)	Lindgren et al.(2009)	Heard-Costa et al.(2009)	Heid et al. (2010)	Hinney et al.(2007)	Meyre et al. (2009)	Scherag et al. (2010)
N	1,924 type 2 diabetes patients; 2,938 population controls	4,741	16,876	2,684	32,387	31,392	123,865	38,580	31,373	77,167	487 young, extremely obese individuals; 442 healthy lean controls	1,380 cases with early-onset and morbid adult obesity; 1,416 age-matched normal-weight controls	1,138 cases with early-onset; 1,120 healthy normal-weight controls
SNPs tested	N/A*	362,129	344,883	318,237	2,399,588	305,846	~2.800,000	2,573,738	~2,500,000	2,850,269	440,794	308,846 SNPs	~2,500,000
Significance level	N/A*	FDR Q value<0.20	$P<10^{-5}$	$P<10^{-5}$	35 SNPs drawn from among the most stronglyassociated loci	$P<10^{-5}$	$P<5x10^{-6}$	$P<10^{-5}$	$P<5x10^{-4}$	$P<1.4x10^{-6}$	15 most significant SNPs (corresponding top<3.6 x 10–6)	FDR Q value<0.20	$P<10^{-5}$
SNPs for replication	N/A*	2	10	23	35	43	42	26	48	32	11	38	44

Replication stage

Study design	Type 2 diabetes case-series, case- series and population-based series. Adults and children.	Family studies. Adults.	Population-based studies,case-series, obesity case-control studies, family study.Adults and children.	Population-based studies. Adults.	Population-based studies, case-series, obesity case-control studies.Adults and children.	GIANT meta-analysis (Willer et al.) and population based study. Adults.	Population-based studies,case-series, obesity case-control studies.Adults and children.	Population-based studies,case-series, obesity case-control studies. Adults.	GIANT meta-analysis (Lindgren et al.). Adults.	Population-based studies,case-series, obesity case-control studies. Adults.	Obesity family study (families with at least one extremely obese child or adolescent). Adults and children.	Four case-control studies and 2 population-based studies. Adults and children.	Two case-control studies and 7 population-based studiesAdults and children.
Ethnicity	White Europeans from the UK, Italy,	European Americans, Hispanic Americans, and African Americans	White Europeans from the UK, Italy, Germany, USA, Sweden and Finland	Indian Asians and white Europeans from the UK	Whites from the UK, USA, Sweden, Finland, Italy, and The Netherlands	Whites from the UK, USA, Sweden, Finland, Italy, The Netherlands and Denmark.	Whites from the UK, USA, Sweden, Finland, Italy, The Netherlands, Australia, Estonia, Germany, France, Norway	Whites from the UK, USA, Sweden, Finland, Germany and The Netherlands	Whites from the UK, USA, Sweden, Finland, Italy, Switzerland, Germany, and Iceland	Whites from the UK, USA, Sweden, Finland, Italy, The Netherlands, Australia, Estonia, Germany, and France	White Europeans from Germany	White Europeans from France, Germany, Finland, and Switzerland	White Europeans from Germany and USA
Traits	BMI and obesity	BMI, hip and weight	BMI and obesity	BMI, waist circumfer-ence and waist-hip ratio, weight, and other metabolic traits	BMI and obesity	BMI and weight	BMI and obesity	waist circum-ference and WHR	waist circum-ference	WHR adjusted for BMI	extreme obesity	obesity and BMI	obesity and BMI
N	38,759	3,205	75,991	11,955	59,082	37,973	125,931	70,689	35,580	113,636	2,309	14,186	31,182
Confirmed loci													
Replicated loci/genes	in FTO	in FTO	in FTO near MC4R	near MC4R	in FTO near MC4R near NEGR1	in FTO near MC4R near NEGR1	in FTO near MC4R near NEGR1	in FTO near MC4R in TFAP2B	in FTO near MC4R in NRXN3	in RSPO3 near VEGFA in TBX15	in FTO	in FTO near MC4R in NPC1	in FTO near MC4R near MSRA

(continued)

Table 10.3 (continued)

Study	Frayling et al.(2007)	Scuteri et al. (2007)	Loos et al. (2008)	Chambers et al.(2008)	Willer et al. (2009)	Thorleifsson et al.(2009)	Speliotes et al.(2010)	Lindgren et al.(2009)	Heard-Costa et al.(2009)	Heid et al. (2010)	Hinney et al.(2007)	Meyre et al. (2009)	Scherag et al. (2010)
					near TMEM18	near TMEM18	near TMEM18	near MSRA		near NFE2L3		near MAF	
					near KCTD15	near KCTD15	near KCTD15	near LYPLAL1 (WHR, women only)		near GRB14		near PTER	
					in SH2B1	in SH2B1	in SH2B1			near LYPLAL1			
					near GNPDA2		near GNPDA2			in DNM3			
					in MTCH2		in MTCH2			near ITPR2 & SSPN			
						in SEC16B	in SEC16B			near LY86			
						near ETV5 & DGKG	near ETV5 & DGKG			near HOXC13			
						in BDNF	in BDNF			near ADAMTS9			
						near BCDIN3D & FAIM2	near BCDIN3D & FAIM2			in ZNRF3			
							in TFAP2B			in NISCH			
							in NRXN3			in CPEB4			

	near RBJ & POMC
	near GPRC5B
	in MAP2K5
	in QPCTL & near GIPR
	in TNNI3K
	in SLC39A8
	near FLJ35779
	in LRRN6C
	near TMEM160
	near FANCL
	in CADM2
	near PRKD1
	near LRP1B
	near PTBP2
	in MTIF3
	near ZNF608
	near RPL27A and TUB
	in NUDT3

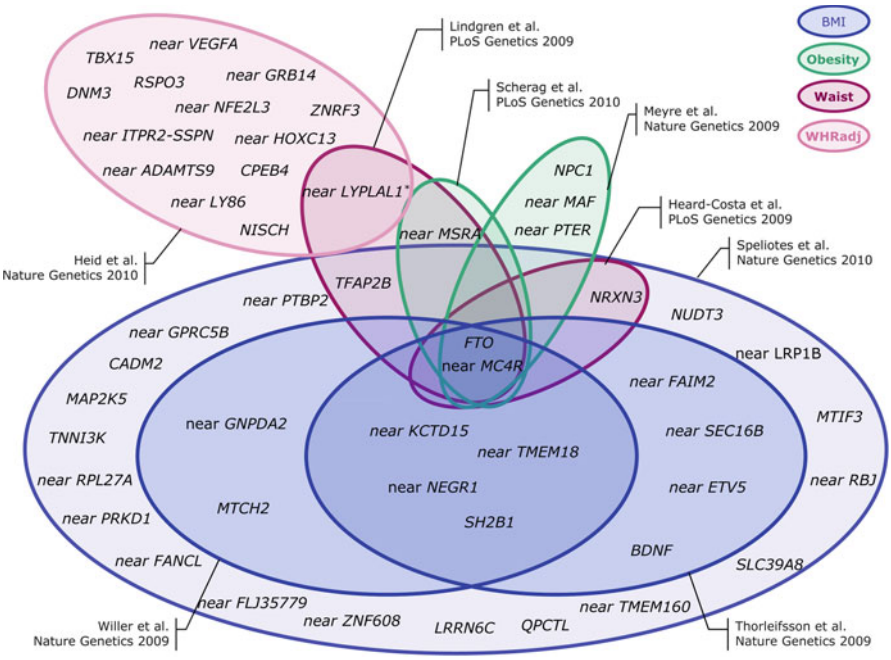

Fig. 10.3 Obesity-susceptibility loci discovered in four waves of genome-wide association studies for body mass index (blue), three waves of genome-wide association studies for waist circumference and waist-to-hip ratio (pink) and two waves of genome-wide association studies for extreme and early onset of obesity (green). Each Venn diagram represents the loci of one paper, except for papers that discovered only one locus, i.e. the fat mass and obesity associated gene (Frayling et al. 2007; Scuteri et al. 2007; Hinney et al. 2007) and the near-MC4R loci (Loos et al. 2008; Chambers et al. 2008), for which no Venn diagram was drawn

10.6.2.1 Genome-Wide Association Studies for Body Mass Index

Most genome-wide association studies for obesity-related traits have been performed for BMI, which is an inexpensive and non-invasive measure of adiposity in adults and which is available in many studies. Four consecutive *waves* of large-scale high-density genome-wide association (meta-)analyses, each characterised by a larger sample size and a growing number of discoveries, have so far identified 32 loci robustly associated with BMI.

First wave of discoveries – The first wave took place in 2007 and comprised two independent genome-wide association studies that each identified *FTO* (fat mass and obesity associated gene) as the first gene of which genetic variation is incontrovertibly associated with common obesity and related adiposity traits (Table 10.3, Fig. 10.3).

The first study, a genome-wide association study for type 2 diabetes, identified a cluster of SNPs in the first intron of *FTO* to be highly significantly associated with type 2 diabetes (Frayling et al. 2007). After adjusting for BMI, the association with

type 2 diabetes was completely abolished, indicating that the *FTO*-type 2 diabetes association was mediated through BMI. The *FTO* rs9939609 variant was taken forward for replication in 38,759 adults and children from 13 cohorts in which its association with BMI, obesity risk and other adiposity-related traits was unequivocally confirmed. Within 6 weeks of the publication of the first report, a second genome-wide association study identified the *FTO* locus to be associated with BMI (Scuteri et al. 2007). In their discovery stage, including 4,741 Sardinians, variants in the *FTO* and *PFKP* (platelet-type phosphofructokinase) genes showed the most significant associations, but only those in *FTO* replicated in 1,496 European Americans and 839 Hispanic Americans.

While the study by Frayling et al. (2007) coincidentally identified the *FTO* locus through a genome-wide association study of type 2 diabetes, the study by Scuteri et al. (2007) is considered to be the first large-scale high-density genome-wide association study with BMI as the primary outcome.

Second wave of discoveries – While the sample sizes of the first genome-wide association studies were relatively small ($N \sim 4{,}800$), they were sufficiently powered to harvest the *FTO* locus as the "low-hanging-fruit". However, scientists soon realised that for *a second wave* of discoveries, collaborative efforts were required to increase the sample size and thus power of the study to identify more common variants with effects smaller than those observed for the *FTO* locus. As such, research groups from across Europe and the USA combined forces and formed the GIANT (Genomic Investigation of Anthropometric Traits) consortium to study the genetics of various anthropometric traits. In their first meta-analysis, data of seven genome-wide association studies for BMI, including 16,876 individuals, were meta-analysed. Ten SNPs representing the ten most significant loci ($P < 10^{-5}$) were taken forward for replication (Loos et al. 2008). Despite quadrupling the discovery stage sample size compared to first wave studies, only *FTO* and one new locus were unequivocally confirmed in the replication stage (Table 10.3, Fig. 10.3). The newly identified locus, represented by the rs17782313 -SNP, maps at ~188 kb downstream of *MC4R* and at ~300 kb upstream of *PMAIP1* (phorbol-12-myristate-13-acetate-induced protein 1).

At the same time, a genome-wide association study in 2,684 Indian Asians identified a locus, represented by the rs129070134-SNP, at ~150 kb downstream of *MC4R*. Association for this locus was confirmed in 11,955 individuals of Indian Asian and European ancestry (Chambers et al. 2008). Although the locus identified by Chambers et al. (2008) maps 38 kb closer to the *MC4R* gene than the locus identified by Loos et al. (2008), both loci are part of the same cluster of genetic variants that are in high linkage disequilibrium ($r^2 > 0.75$ in white Europeans), and they likely present the same association signal. 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.

The identified locus maps in a recombination interval between *MC4R* and *PMAIP1*, of which *MC4R* seems the best biological candidate given its role in the regulation of food intake (Huszar et al. 1997; Fan et al. 1997). As described earlier,

rare coding mutations in *MC4R* result in monogenic forms of obesity (Farooqi et al. 2003) and common variants (V103I or I251L) are associated with a reduced risk of obesity in the general population (Young et al. 2007; Stutzmann et al. 2007; Wang et al. 2010) (Table 10.2).

Third wave of discoveries – For *the third wave* of discoveries, the discovery stage sample size of the GIANT consortium was doubled to 32,387 adults of European ancestry from 15 cohorts (Willer et al. 2009). A total of 35 SNPs, representing the most significant independent loci, were taken forward for follow-up in an independent series of 59,082 individuals. The two previously established loci, *FTO* and *near-MC4R,* were confirmed as well as six new loci; i.e. near the neuronal growth regulator-1 gene (*NEGR1*), near the transmembrane protein 18 gene (*TMEM18)*, in the SH2B adaptor protein-1 gene (*SH2B1)*, near the potassium channel tetramerisation-domain containin-15 gene (*KCTD15)*, near the glucoseamine-6-phosphate deaminase-2 gene (*GNPDA2)* and in the mitochondrial carrier homologue-2 gene (*MTCH2)* (Table 10.3, Fig. 10.3).

At the same time, the Icelandic company, deCODE genetics, performed a meta-analysis of four genome-wide association studies for BMI, including 30,232 individuals of European descent and 1,160 African Americans (Thorleifsson et al. 2009). A total of 43 of the most significant SNPs ($P < 10^{-5}$) in 19 chromosomal regions were taken forward for replication in 5,586 Danish individuals and for further confirmation in the discovery stage data of the GIANT consortium. Besides the *FTO* and near-*MC4R* loci, eight additional loci reached genome-wide significance (Table 10.3, Fig. 10.3). Of these, four loci (near *NEGR1*, near *TMEM18*, in *SH2B1*, near *KCTD15)* had also been identified by the GIANT consortium, whereas four loci were new; i.e. in the SEC16 homologue-B gene, between the ets variant-5 (*ETV5)* and diacylglycerol kinase genes, in the *BDNF*, and between the BCDIN3 domain and the Fas apoptotic inhibitory molecule-2 genes (*FAIM2)*.

At the end of this third wave of discoveries, a total of 12 genetic loci had been confirmed to be convincingly associated with BMI.

Fourth wave of discoveries – In the fourth wave of genome-wide association studies, the GIANT consortium increased its discovery stage further to 123,865 individuals, or almost four times larger than in the third stage (Speliotes et al. 2010). A total of 42 SNPs, representing the 42 most significant ($P < 5 \times 10^{-6}$) loci of the genome-wide meta-analysis (Fig. 10.2), were taken forward for replication in a new series of 125,931 individuals of white European descent. All 12 BMI loci indentified in the previous waves of discoveries were confirmed, two additional (TFAP2B, neurexin 3 (NRXN3)) loci had been identified in earlier genome-wide association studies for waist circumference (see below), whereas 18 loci were novel (Table 10.3, Fig. 10.3).

Taken together, four waves of large-scale high-density genome-wide association (meta) analyses have so far discovered 32 loci unambiguously associated with BMI, at least in adults of white European descent.

10.6.2.2 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, genome-wide association studies have been performed to identify genetic loci for waist circumference and WHR.

First wave of discoveries – The first two genome-wide association studies that focussed on central obesity both examined waist circumference as well as WHR as the main outcomes (Lindgren et al. 2009; Heard-Costa et al. 2009) (Table 10.3, Fig. 10.3). One of the studies was performed by the GIANT consortium and included data from 38,580 individuals at the discovery stage. The 26 most significant SNPs ($P < 10^{-5}$) for either waist or WHR loci were taken forward for replication in 70,689 individuals. While the *FTO* and the near-*MC4R* loci had reached genome-wide significance already at the discovery stage, two new loci (in *TFAP2B*, and near the methionine sulfoxide reductase A gene (*MSRA*)) were identified to be associated with waist circumference, whereas one locus (near the lysophospholipase-like 1 gene (*LYPLAL1*)) was replicated for WHR in women only.

The second genome-wide association for waist circumference was undertaken by the CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) consortium and included 31,373 individuals at the discovery stage (Heard-Costa et al. 2009). A total of 48 SNPs were taken forward to the next stage where the discovery stage data of the GIANT consortium were used as replication. Besides the *FTO* and near-*MC4R* loci, one new locus in the NRXN3 was found to be highly significantly associated with waist circumference (Table 10.3, Fig. 10.3).

Taken together, these two genome-wide association studies confirmed the *FTO* and near-*MC4R* loci as obesity-susceptibility loci and identified three new loci associated with waist circumference and one new locus with WHR in women. It should be pointed out, however, that the three loci for waist circumference (*TFAP2B*, near-*MSRA*, *NRXN3*) were also associated with BMI, suggesting that these loci are most likely involved in overall adiposity and not specific to central fat deposition. Indeed, the loci in *TFAP2B* and *NRXN3* were also identified in the fourth wave of discoveries for BMI (Speliotes et al. 2010), whereas the locus near *MSRA* has been identified to be associated with early-onset obesity (see below, Fig. 10.3) (Scherag et al. 2010). The *LYPLAL1* locus is not associated with BMI, suggesting that this locus more specifically affects fat distribution, at least in women.

Second wave of discoveries – In a second wave of discoveries, the GIANT and CHARGE consortium combined forces and further expanded their discovery stage to include 77,167 individuals (Table 10.3) (Heid et al. 2010). In this study, the main outcome was WHR adjusted for BMI. The adjustment of WHR for BMI allows focussing more specially on fat distribution, rather than on overall obesity. SNPs representing the 32 most significant loci were taken forward for replication in a new

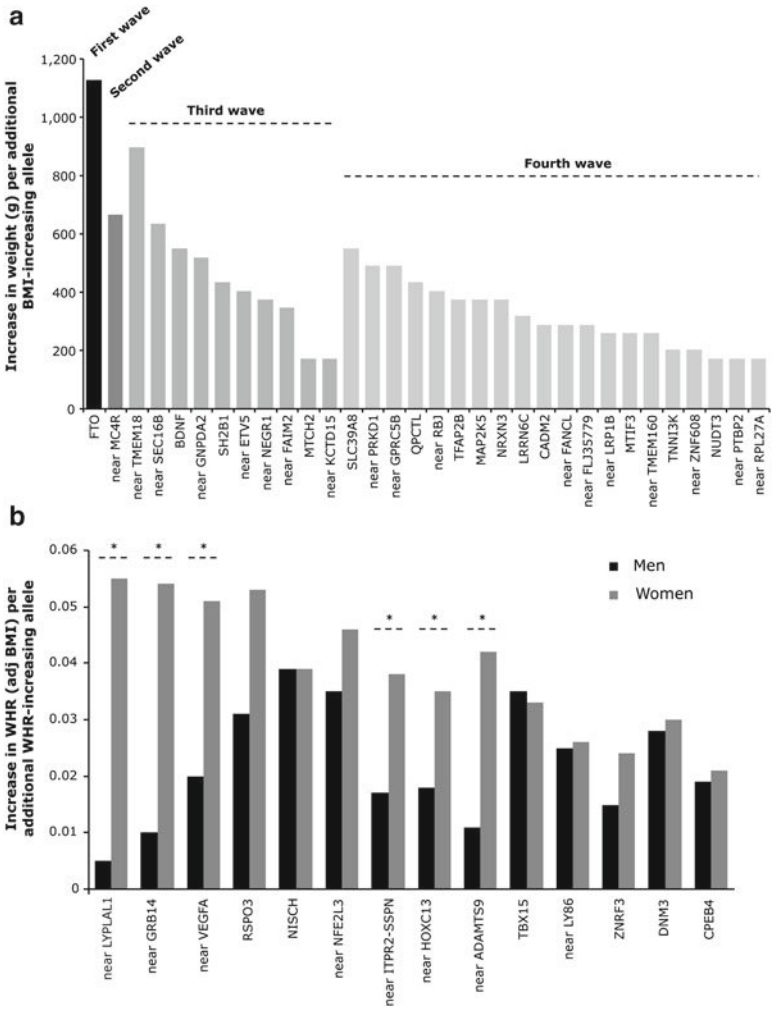

Fig. 10.4 The per-allele effect size for each of the 32 genetic loci for body mass index (BMI) (Speliotes et al. 2010) (panel a) and 14 genetic loci for waist-to-hip ratio (WHR) (Heid et al. 2010) (panel b). The BMI loci are ordered according to effect size, stratified by wave of discovery (panel a). The WHR loci are sorted by overall effect size, but effects are shown for men and women separately (panel b) * Significant difference in per-allele effect between men and women

series of 113,636 individuals. The *LYPLAL1* locus, which had been identified in the first wave of discoveries, was confirmed. Furthermore, 13 new loci were found to show robust association with WHR adjusted for BMI (Fig. 10.3). While these 14 loci reached genome-wide significance in an analysis of men and women combined, secondary analyses showed that the effects of seven loci were significantly more pronounced in women (Fig. 10.4b). The fact that none of these 14 loci have been identified in genome-wide association studies for BMI or early-onset obesity suggests that they specifically affect fat distribution rather than overall adiposity.

Taken together, while the loci identified by genome-wide association analyses for waist circumference turned out to affect general adiposity rather than central obesity loci, the analyses for WHR adjusted for BMI identified 14 loci that specifically influence fat deposition.

10.6.2.3 Genome-Wide Association Studies for Extreme and Early-Onset Obesity

Individuals with early-onset or morbid obesity may be enriched for variants that predispose to obesity in the general population. Therefore, genome-wide association studies for extreme obesity may have more statistical power to identify obesity-susceptibility loci or they may identify loci different from those identified for BMI of waist circumference. So far, there have been three waves of such studies (Table 10.3, Fig. 10.3).

First wave of discoveries – The first genome-wide association study for risk of early-onset extreme obesity was relatively small, including 487 young extremely obese individuals and 442 healthy lean controls (Hinney et al. 2007). Of the 15 most significant SNPs that were taken forward for replication in 644 nuclear families with at least one obese offspring, only the SNPs in *FTO* were confirmed to be associated with extreme early-onset obesity (Table 10.3).

Second wave of discoveries – A second genome-wide association study for early-onset (before age 6 years) and morbid adult obesity (BMI$\geq$40 kg/m^2) compared data of 1,380 cases and 1,416 controls (Meyre et al. 2009). A total of 38 SNPs, representing the most significant independent loci, were taken forward for genotyping in 14,186 adults and children to test for association with BMI and obesity risk. In addition to *FTO* and near-*MC4R*, three new loci were identified; in Niemann-Pick disease type C-1 gene (*NPC1*), near the v-MAF musculoaponeurotic fibrosarcoma oncogene homologue gene (*MAF*) and near the phosphotriesterase related (*PTER*) (Table 10.3, Fig. 10.3).

Third wave of discoveries – The third genome-wide association study focussed on early-onset obesity only and combined the data from the first two studies to include 1,138 cases and 1,120 normal-weight controls (Scherag et al. 2010). A total of 44 SNPs were taken forward for replication with risk of obesity in case–control studies and with BMI in population-based studies. Besides the *FTO* and near-*MC4R* loci, one additional locus was identified near-*MSRA*, which had previously been identified through a genome-wide association study for waist circumference by the GIANT consortium (Table 10.3, Fig. 10.3) (Lindgren et al. 2009).

Taken together, three genome-wide association studies for early-onset and extreme obesity confirm the *FTO* and near-*MC4R* loci, as well as the near-*MSRA* locus, whereas the *NPC1*, *MAF* and *PTER* were not previously established as obesity-susceptibly loci. Although the study by Meyre et al. (2009) and Scherag et al. (2010) confirms that the identified loci are also associated with BMI in population-based studies, they were not identified in the largest genome-wide association study for BMI so far (Speliotes et al. 2010). Furthermore, despite the fact

that the discovery stage of the Meyre et al. (2009) and Scherag et al. (2010) overlapped in part, they were not able to replicate each other's loci. This discrepancy between studies and across traits may be due to heterogeneity of study designs, trait definitions or population-specific characteristics.

10.6.2.4 Genome-Wide Association Studies in Non-White Populations

The large-scale high-density genome-wide association studies described above were performed almost exclusively in populations of white European origin. Even the study by Chambers et al. (2008), of which the discovery stage comprised Indian Asians, used a sample of white Europeans at the replication stage to confirm association of the near-*MC4R* locus. However, a growing number of genome-wide association studies are being performed exclusively in populations of non-white origin. Because of difference in genetic architecture as well as difference in obesity-susceptibility, such genome-wide association studies may identify novel loci that are ethnic specific, but that may also play a role in obesity-susceptibility across different ethnicities.

The largest genome-wide association analysis in non-white individuals so far is the study in a Korean population-based cohort that includes 8,842 individuals in the discovery stage and 7,861 individuals in the replication stage (Cho et al. 2009). Association was tested for eight metabolic and cardiovascular quantitative traits, including BMI and WHR. Of the two SNPs that were taken forward for BMI, only the SNP in *FTO* was confirmed at the replication stage. Two SNPs were also taken forward for WHR, of which a SNP (rs2074356) located in an intron of C12orf51 reached genome-wide significance after replication. The function of the predicted transcript for C12orf51 is currently unknown. Of interest is that WHR-increasing allele of the C12orf51-SNP (rs2074356) has a frequency of 85% in Koreans, which is similar to the frequency seen for Han Chinese and Japanese (based on CHD and JPT HapMap data). However, this SNP is not polymorphic in populations of European or African descent (based on CEU and YRI HapMap data), suggesting that the C12orf51 locus might affect abdominal obesity specifically in East Asians.

A low-density genome-wide association study in Pima Indians identified variation in the ataxin-2 binding protein 1 gene (*A2BP1*) as an ethnic-specific obesity-susceptibility locus (Ma et al. 2010). In the genome-wide association analysis of body fat % in 413 non-diabetic Pima Indians, a SNP (rs10500331) in the *A2BP1* gene showed the most significant association. Replication analyses in a population-based sample of 2,843 Pima Indians confirmed association between the *A2BP1*-SNP and BMI. The association for the *A2BP1* SNP with BMI seems specific to Pima Indians, as no association with BMI or obesity risk was seen in Old Order Amish, in French obese adults and children or in German obese children. Interestingly, other SNPs in the *A2BP1*-locus showed some evidence of association with early-onset obesity in French children, suggesting that the causal variant may be captured by different haplotypes in different ethnic groups or, alternatively, that multiple causal variants are implicated across ethnicities. Deep resequencing of this

locus might provide further insight in how genetic variation in *A2BP1* confers obesity-susceptibility. The *A2BP1* is a promising biological candidate given that it is highly expressed in the hypothalamus, which is known to be involved in the central regulation of food intake. Furthermore, mice deficient of atxn, the protein that binds A2BP1, are obese (Kiehl et al. 2006; Lastres-Becker et al. 2008).

The first genome-wide association study in populations of African ancestry did not identify new, ethnic-specific loci for obesity-related traits (Kang et al. 2010). This may be due to the limited power as the discovery stage included only 1,931 individuals and the replication stage ~3,700 individuals.

Collaborative efforts that combine the ethnic-specific genome-wide association data in large-scale meta-analyses will be needed to continue the discovery of new obesity-susceptibility loci that are specific to certain populations.

10.6.3 Translation of New Discoveries

There is no doubt that the genome-wide association approach has been extremely successful in identifying new obesity-susceptibility loci. In less than 4 years, 50 highly credible genetic loci have been identified that are robustly associated with obesity-related traits. However, there has been little time to celebrate this success as these new discoveries soon raised questions about their clinical relevance for the general population and about their functional mechanisms through which they confer obesity risk. A major challenge is translation of this new knowledge into public health and clinical practice.

10.6.3.1 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 obese 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.

10.6.4 Effect Sizes, Risk and Prediction of Established Loci in Adults of white European Descent

As discovery of new loci is the primary aim, the significance of association has been the main focus for most genome-wide association studies so far. Despite highly significant associations and consistent and repeated replication, however, the effects of the established obesity-susceptibility loci on BMI, WHR and obesity risk are small (Fig. 10.4).

The BMI loci – Of the 32 established loci for BMI, the firstly identified obesity-susceptibility gene, *FTO*, has the largest, yet small, effect on obesity-susceptibility. According to the large-scales genome-wide association studies discussed above (Speliotes et al. 2010), each risk-allele increases BMI by 0.26–0.66 $kg.m^{-2}$, which is equivalent to 750–1,900 kg in body weight for a person of 1.70 m tall. The risk of obesity increases by 1.20–1.32 odds for each additional risk-allele. The *FTO* locus was easily identified by genome-wide association studies 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; Scuteri et al., 2007; Hinney et al., 2007).

The identification of the near-*MC4R* locus in the second wave of discoveries required much larger samples as not only its effect size on BMI was much smaller (0.20–0.32 $kg.m^{-2}$/allele) than that of the *FTO* locus, but also the prevalence of the BMI-increasing allele was substantially lower (27% in white Europeans). Each additional risk-allele increased the BMI by 0.19–0.32 $kg.m^{-2}$ (or 550–925 g in body weight for a 1.70 m-tall person) and the risk of obesity by 1.11–1.15 odds.

The effect sizes of the ten loci identified in the third wave of discoveries range from as low as 0.06 up to 0.31 $kg.m^{-2}$ per risk-allele for BMI (or 170–896 g for a 1.70 m-tall person) and from 1.02 to 1.19 odds for risk of obesity. The frequency of the BMI-increasing allele ranges from 25% to 85% in white Europeans. Despite the fact that the effect size of the near-*TMEM18* locus is larger than that of the near-*MC4R*, it could only be identified in the third wave of discoveries because its minor allele frequency (15%) is much lower than that of the near-*MC4R* locus and therefore a larger sample (and thus more statistical power) was needed for the near-*TMEM18* discovery.

The sample size of the discovery stage of the fourth wave of genome-wide association studies was almost four times larger than that of the third wave. This provided more statistical power to not only identify loci with smaller effects, but also loci with a lower minor allele frequency compared to the third wave of discoveries. The BMI-increasing alleles of the loci identified in the fourth wave of discoveries ranged from 0.06 to 0.19 $kg.m^{-2}$ per risk-allele for BMI (or 170–550 g for a 1.70 m-tall person) and from 1.02 to 1.10 odds for risk of obesity. The frequency of the BMI-increasing allele ranges from 4% to 87% in white Europeans.

Figure 10.4 (panel a) shows the average effect sizes per BMI-increasing allele of the 32 BMI loci, expressed as increase in body weight (for a 1.70 m-tall person), derived from the largest genome-wide association study for BMI, including nearly 250,000 individuals (Speliotes et al. 2010). This study also estimated how much each of the individual loci as well as all 32 loci combined contribute to inter-individual phenotypic variation in BMI. The *FTO* locus, which has the largest effect size of all 32 established BMI loci and which has a rather high minor allele frequency, explained the most the phenotypic variation in BMI, which was a mere 0.34% (Speliotes et al. 2010). The explained variance of the other BMI loci was even less than half that of the *FTO* locus, ranging from 0.1% to 0.15%. Together, the 32 BMI loci explained 1.45% of the phenotypic variation in BMI or 2–4% of genetic variation based on an estimated heritability of 40–70% (Speliotes et al. 2010).

In an analysis of 8,120 individuals of the population-based ARIC-study, the combined effect of the 32 SNPs representing all BMI loci was estimated, by constructing a genetic-susceptibility score, which sums the number of BMI-increasing alleles an individual inherited (Speliotes et al. 2010). Each additional BMI-increasing allele in the genetic-susceptibility score was found to increase BMI by 0.17 $kg.m^{-2}$ (or 490 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 ≥38 BMI-increasing alleles, 1.5 of the population) and those with a low genetic-susceptibility (having ≤21 BMI-increasing alleles, 2.2% of the population) score amounted to 2.73 $kg.m^{-2}$ (or 7.88 kg in body weight for a 1.70 m-tall person). While this difference in body weight due to genetic variation is substantial, it should be noted that it compares only the 2.2% most susceptible to the 1.5% least susceptible of the total population studied. 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 very low predictive value (i.e. the area under the Receiver Operating Characteristic curve was 0.574) (Speliotes et al., 2010). In fact, the answer to a simple question on 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 obesity-susceptibility loci is too low to be used for genetic profiling and personalised management of obesity.

The WHR loci – The average effect sizes of the 14 WHR loci as reported in the latest large-scale genome-wide association study including more than 190,000 individuals are shown in Fig. 10.4(panel b) (Heid et al. 2010). As WHR was adjusted for BMI in the genome-wide association analyses, the 14 identified loci increase WHR but not BMI and can therefore be considered as fat distribution loci rather than general adiposity loci. The standardised effect sizes of the 14 WHR loci are of a similar magnitude as those of the BMI loci. The 14 WHR loci combined explain 1.03% of the inter-individual variation of WHR (Heid et al. 2010). Of interest is that the 14 loci were identified in a genome-wide association analyses of men and women combined, but secondary analyses showed that seven of the 14 loci show sex-specific effects (Fig. 10.4, panel b). More specifically, the effect was always more pronounced in women than in men, such that the explained variance in women only was 1.34%, whereas 0.46% in men only.

The early-onset and extreme obesity loci – The effect sizes of the four early-onset and extreme obesity loci seemed in general more pronounced (OR ~1.20 to ~1.40) than of those discovered through genome-wide association studies of BMI (Meyre et al. 2009; Scherag et al. 2010). This may be due to the fact that the effect sizes were derived from case–control studies and not from population-based studies. Of interest is that the effect of the early-onset and extreme obesity loci on BMI in a population-based study of French adults showed effect sizes similar to those observed for the 32 BMI loci (Meyre et al. 2009).

10.6.5 Impact of Established Loci in Adults of Non-White Origin

With the exception of the near-*MC4R* locus, all 50 obesity-susceptibility loci have been identified by examining populations of predominantly white European descent at the discovery stage. Following the publication of genome-wide association studies, a growing number of studies have examined whether any of these obesity-susceptibility loci also affect individuals of non-white origin. Examining genetic associations across different ethnicities is not only informative towards confirming the role of the respective loci in other populations. They can also contribute to the fine-mapping of the locus to identify the causal variant by taking advantage of the known differences in the genetic architecture between ethnicities.

The *FTO* locus has so far received most attention. Many replication efforts in East Asian populations have provided convincing evidence that genetic variation in *FTO* influences BMI and obesity risk in Chinese, Japanese, Korean and Filipino populations (Hotta et al. 2008; Cha et al. 2008; Chang et al. 2008; Tan et al. 2008; Omori et al. 2008; Ng et al. 2008; Al-Attar et al. 2008; Tabara et al. 2009; Cheung et al. 2010; Liu et al. 2010b, 2010c; Ng et al. 2010; Han et al. 2010; Shi et al. 2010; Croteau-Chonka et al. 2011). The magnitude of the effect on BMI and obesity risk is similar to that observed for European populations. As the frequency of the BMI-increasing allele in East Asians (frequency ~20%) is less than half that of white Europeans (frequency ~45%), the overall contribution of the *FTO* locus to obesity-susceptibility will be smaller. The two studies that so far have examined the association of *FTO* variants in South Asians (frequency ~30%) reported a weak effect on BMI (Yajnik et al. 2009; Sanghera et al. 2008). Interestingly, both reported an effect of *FTO*'s BMI-increasing allele on risk of type 2 diabetes risk, which is independent of its effect on BMI or obesity risk (Yajnik et al. 2009; Sanghera et al. 2008). The evidence of association between the *FTO* locus and BMI or obesity risk in African (Hennig et al. 2009; Adeyemo et al. 2010) or African Americans populations (Scuteri et al. 2007; Grant et al. 2008; Thorleifsson et al. 2009; Bressler et al. 2010; Wing et al. 2010; Bollepalli et al. 2010; Adeyemo et al. 2010; Liu et al. 2010b) has been rather inconsistent, which could be due to the small sample size of some studies or heterogeneity between African-ancestry populations. However, a recent large-scale and comprehensive study that included data of 10,819 individuals of African-ancestry and African American populations provided convincing evidence of association between genetic variation in the *FTO* locus and BMI, with effect sizes similar to or somewhat smaller than those observed in individuals of white European descent (Hassanein et al. 2010). By taking advantage of the population-specific genetic architecture, which tends to be less tight in African-ancestry populations, Hassanein et al. (2010) were also able to fine-map the *FTO* locus and narrow down the number of potentially causal variants.

The locus near *MC4R* was first identified in two genome-wide association studies of which one study included Indian Asians only at the discovery stage (Chambers et al. 2008). A study in Asian Sikhs confirmed association between the near-*MC4R* locus and BMI and waist circumference (Been et al. 2009). The effect sizes reported in this study, which had a type 2 diabetes case–control design, were twofold larger

than those observed in the initial genome-wide association study of Indian Asians (Chambers et al. 2008), which found similar effect sizes to those seen for white Europeans. As the BMI-increasing allele frequency in Indian Asians (36–40%) is higher than in white Europeans (~27%) and as the effect sizes in Indian Asians seem at least as large as in white Europeans, the contribution of the near-*MC4R* locus to obesity-susceptibility is likely larger in Indian Asians. The frequency of the BMI-increasing allele in individuals of Filipino (~12%), Chinese (~19%), Japanese (~24%) and Korean (25%) origin is somewhat lower than in white Europeans. Two genome-wide association analyses of population-based cohorts, including 8,842 Koreans and 1,792 Filipino women respectively, both independently confirmed association between the near-*MC4R* locus and BMI with effect sizes similar to those observed in white Europeans (Cho et al. 2009; Croteau-Chonka et al. 2011). A population-based study of 2,806 middle-aged to elderly Japanese observed a directionally consistent trend but the association did not reach significance ($P = 0.12$) (Tabara et al. 2009). Case–control analyses in Chinese from Shanghai ($n = 5{,}030$) (Shi et al. 2010) and Hong Kong ($n = 1{,}170$) (Cheung et al. 2010) and in Japanese ($n = 2{,}865$) (Hotta et al. 2009) also found significant association between the near-*MC4R* locus and increased risk of obesity. However, a large study of 6,681 Chinese adults from Hong Kong, mainly type 2 diabetes cases (Ng et al. 2010), and a study of 1,228 overweight Japanese individuals (Hotta et al. 2010) found no evidence of association between the near-*MC4R* locus and BMI, which could be due to the case-only study design. Two genome-wide association analyses in populations of African ancestry also found significant association between the variants in the near-*MC4R* locus and BMI (Kang et al. 2010; Thorleifsson et al. 2009). The frequency of the BMI increasing allele (frequency = 16–20%) in the African-ancestry population was lower, while effect sizes tended to be similar or even somewhat larger than in white Europeans.

A number of other obesity-susceptibility loci have been tested for association in Chinese from Shanghai (Shi et al. 2010) and Hong Kong (Cheung et al. 2010; Ng et al. 2010), in Japanese (Hotta et al. 2009; Hotta et al. 2010), in Filipinos (Croteau-Chonka et al. 2011) and in individuals of African-ancestry (Kang et al. 2010; Thorleifsson et al. 2009). These studies have so far focussed analyses on the BMI loci identified by Willer et al. (2009) and Thorleifsson et al. (2009) and on loci for extreme and early-onset obesity identified by Meyre et al. (2009). As these loci were identified in genome-wide association studies of which the discovery stage included more than 30,000 individuals of white European origin and as their effect sizes are typically lower than those of the *FTO* and near-*MC4R* locus (Fig. 10.4), replicating these associations in populations of other ethnicity will likely require large sample sizes to provide sufficient statistical power. It is therefore no surprise that currently the association results observed for each of the loci are inconsistent across the East Asian and African-ancestry population. Larger sample sizes or meta-analyses of available data will be needed to convincingly confirm or refute the role of these loci in obesity-susceptibility in non-white populations.

No data is available on the association of the more recently identified BMI and WHR loci in population of different ethnicity (Speliotes et al. 2010; Heid et al. 2010).

10.6.6 Impact of Established Loci in Childhood and Adolescence

So far, most obesity-susceptibility loci have been identified through genome-wide association studies of adults. Several follow-up studies have examined whether these loci affect BMI and obesity risk already during childhood and adolescence, which may provide insight in the aetiology of obesity through the life course.

The evidence that the *FTO* locus influences obesity-susceptibility early in life is convincing and was already observed by Frayling et al. 2007. Many studies following the initial observation further confirmed association with BMI or risk of obesity during childhood and adolescence (Haworth et al. 2008; Hakanen et al. 2009; Cecil et al. 2008; Zhao et al. 2010a; Liu et al. 2010b; Grant et al. 2008; Cauchi et al. 2009; Rzehak et al. 2010; Hardy et al. 2010; Liem et al. 2010), including in Chinese (Wu et al. 2010; Ng et al. 2010; Fang et al. 2010; Xi et al. 2010), and African American populations (Grant et al. 2008; Liu et al. 2010b; Bollepalli et al. 2010). A meta-analysis that included data of nearly 13,000 children and adolescents found that the effect of *FTO* on BMI was of a similar magnitude as the effect observed in adults (den Hoed et al. 2010). One study found that the *FTO* locus was associated with increased weight and ponderal index already at the age of two weeks (Lopez-Bermejo et al. 2008). Others, however, could not confirm an effect on BMI before the age of 1 year (Jaddoe et al. 2007; Rzehak et al. 2010; Hardy et al. 2010). Studies with longitudinal data found that the influence of the *FTO* locus increases during childhood and adolescence (Cauchi et al. 2009; Rzehak et al. 2010; Frayling et al. 2007), with one life course study showing that it reaches its largest impact at around the age of 20 years, followed by a subsequent weakening of the effect throughout adulthood (Hardy et al. 2010).

Also the near-*MC4R* locus has been found to be associated in children and adolescents of white European (Loos et al. 2008; Cauchi et al. 2009; Liu et al. 2010a; Zhao et al. 2009; Liem et al. 2010; Grant et al. 2009) and Chinese origin (Wu et al. 2010). However, no association was observed in African American children (Grant et al. 2009). In individuals of white European descent, the effect on BMI in childhood and adolescence is similar to that observed in adults (den Hoed et al. 2010). Similar to *FTO*, the effect of the near-*MC4R* locus on BMI seems to increase with age from childhood through adolescence (Cauchi et al. 2009; Loos et al. 2008), reaching its largest influence at age 20, after which it weakens again through adult life (Hardy et al. 2010).

Of the loci identified in the third wave of genome-wide association studies, significant associations with BMI were observed for the near-*TMEM18*, near-*GNPDA2*, near-*NEGR1*, near-*SEC16*, near-*FAIM2*, *BDNF* and near-*KCTD15* loci in a meta-analysis including nearly 13,000 children and adolescents of white European descent (den Hoed et al. 2010). For these loci, effects in childhood and adolescence were largely of the same magnitude to those observed in adulthood (den Hoed et al. 2010). In this meta-analysis, no association was observed for the near-*ETV5*, *MTCH2* and *SH2B1* loci (den Hoed et al. 2010). A study in Chinese children found association with BMI and obesity risk for the near-*FAIM2*, *BDNF* and near-*GNPDA2* loci (Wu et al. 2010). Of interest is that none of the BMI loci discovered in the first

three waves of genome-wide association studies were found to be associated with birth weight (Kilpelainen et al. 2011).

The genome-wide association meta-analysis for BMI that reported on the fourth wave of discoveries reported that all 32 BMI loci showed directionally consistent associations with either BMI or risk of obesity in children and adolescents (Speliotes et al. 2010). Only few associations, however, reached significance as the study in children and adolescents was insufficiently powered. Of the newly discovered loci, the associations for the near-*RBJ*, *MAP2K5*, *TNNI3K*, *SLC39A8*, *CADM2*, near-*PRKD1*, near-*PTBP2*, *MTIF3* and near-*RPL27A* reached nominal significance (Speliotes et al. 2010). Additional large-scale studies will be needed to confirm association for these loci in children and adolescents.

So far, no studies have reported on associations for the 14 loci identified for WHR in children and adolescents.

10.6.7 Gene–Lifestyle Interaction

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 the 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; Bouchard et al. 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 so far focussed on how the effect of the *FTO* locus on BMI is attenuated by physical activity. An increasing number of studies have reported significant interaction between the *FTO* locus and physical activity on BMI (Andreasen et al. 2008b; Rampersaud et al. 2008; Vimaleswaran et al. 2009; Cauchi et al. 2009; Sonestedt et al. 2009; Ahmad et al. 2010). In these studies, the BMI-increasing effect of the *FTO locus* was more pronounced in sedentary individuals compared to physically active individuals, suggesting that the genetic-susceptibility towards obesity induced by *FTO* can be overcome, at least in part, by adopting a physically active lifestyle. One large-scale ($n = 15{,}925$) and thus well-powered population-based study did not observe such an effect attenuation of the *FTO*–BMI association by physical activity (Jonsson et al. 2009). The reasons for the discrepant observation are unclear, but the relatively small main effects of *FTO*

and physical activity on BMI and the higher than average physical activity levels observed in this population may explain the absence of interaction. Gene–environment interaction studies require large sample sizes to provide sufficient power (Luan et al. 2001; Wong et al. 2003), which might explain why the results of studies with small sample sizes have been less consistent.

The other more recently identified obesity-susceptibility loci have been examined for interactions with lifestyle factors in only a few studies. Three reasonably large studies found no evidence for an effect attenuation of the near-*MC4R* locus on BMI by physical activity (Zobel et al. 2009b; Cauchi et al. 2009; Li et al. 2010a).

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 identified in the first three waves of genome-wide association studies for BMI (Li et al. 2010a). Interactions with physical activity were significant for the near-*TMEM18* and *MTCH2* loci and suggestive for the *SH2B1* locus; the BMI-increasing effect for each of these three loci was more pronounced in sedentary individuals than in physically active individuals (Li et al. 2010a). This study also examined the cumulative effect of the 12 loci by calculating a genetic-susceptibility score which summed the BMI-increasing alleles across the 12 loci. Each additional BMI-increasing allele was associated with 0.154 $kg.m^{-2}$ (or 445 g for 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. 10.5) (Li et al. 2010a). Similar interaction effects were observed between the genetic-susceptibility score and physical activity for the risk of obesity. While the main observations were made in cross-sectional analyses, longitudinal analyses confirmed the interaction between the score and physical activity for weight gain. 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). These findings hold an important public health message as they challenge the deterministic view of the genetic predisposition to obesity, showing that even the most genetically predisposed individuals will benefit from adopting a healthy lifestyle.

10.6.7.1 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.

The physiological mechanisms that link the established genetic loci to weight gain and increased obesity risk are not yet well understood. So far, the firstly discovered locus, *FTO*, has been studied most extensively in human and animal studies.

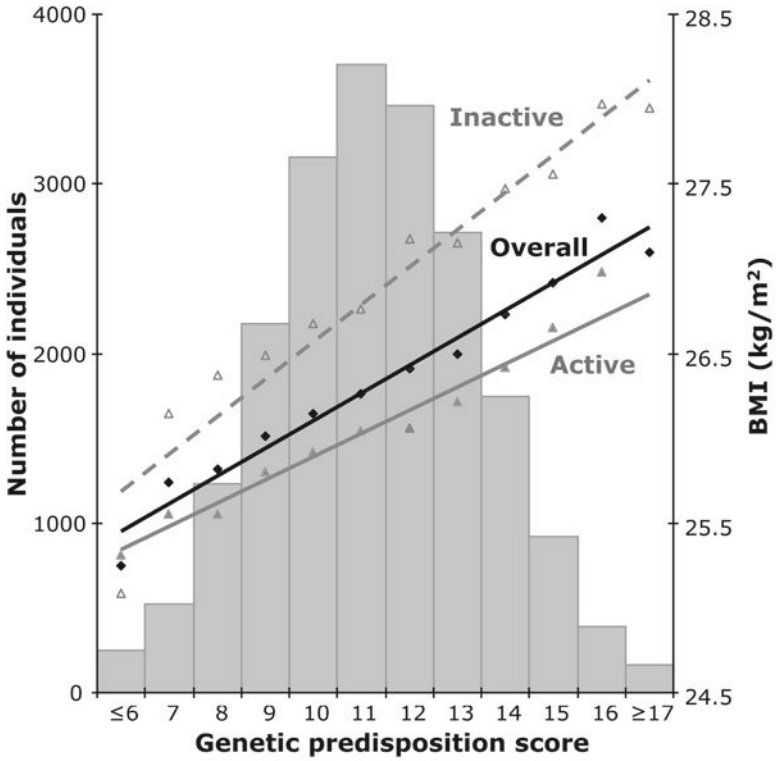

Fig. 10.5 Association between the genetic predisposition score (sum of body mass index (BMI)-increasing alleles from 12 BMI loci) with BMI in all individuals (solid black line), in sedentary individuals (dashed grey line) and in physically active individuals (solid grey line). Adapted from Li et al. (2010a)

The FTO locus – FTO is a member of the non-heme dioxygenase superfamily and was found early on to have demethylase activity (Gerken et al. 2007; Sanchez-Pulido and Andrade-Navarro 2007). This function, however, did not provide immediate insight in the physiological mechanisms by which *FTO* confers increased risk of obesity. Studies in rodents indicated that *FTO* expression is ubiquitous, including in the brain, particularly in the hypothalamic nuclei that are involved in the regulation of energy homeostasis, and its expression is dependent on the energy state (Gerken et al. 2007; Stratigopoulos et al. 2008; Fredriksson et al. 2008). Mice that carry one or two extra copies of the gene display increased *FTO* expression in all tissues, including white adipose tissue, hypothalamus and particularly in muscle (Church et al. 2010). These mice also show increased energy intake and adiposity (Church et al. 2010). Studies in humans have supported a central neuronal role for *FTO* as the BMI-increasing allele was found to be associated with increased appetite and energy intake, and reduced satiety (Timpson et al. 2008; Cecil et al. 2008; Wardle et al. 2008a, 2008b; Haupt et al. 2008; Speakman et al. 2008; Sonestedt et al. 2009).

However, the neuronal hypothesis was challenged as two studies in mice showed that loss of *FTO* function, either by complete gene-knockout (Fischer et al. 2009) or by a single point mutation (Church et al. 2009), results in reduced total weight and adipose tissue. The complete loss of *FTO* in mice led to a significant reduction in adipose tissue and lean body mass, as well as increased energy expenditure and systemic sympathetic activation (Fischer et al. 2009). Interestingly, spontaneous locomotor activity was decreased, while relative food intake was increased. Mice homozygous for a mutation in *FTO* display reduced fat mass, as well as increased energy expenditure without changes in food intake or energy expenditure (Church et al. 2009). These studies suggest that *FTO* may have a peripheral role, influencing body composition through control of energy expenditure (Fischer et al. 2009). A peripheral role for *FTO* was also proposed by a study in healthy women showing that carriers of the risk-allele had reduced lipolytic activity, independent of BMI (Wahlen et al. 2007). Other studies in humans, however, could not confirm association with resting or physical activity energy expenditure (Hakanen et al. 2009; Wardle et al. 2008a; Rampersaud et al. 2008; Berentzen et al. 2008; Cecil et al. 2008; Haupt et al. 2009; Goossens et al. 2009; Do et al. 2008).

A study in mice with systemic *FTO* overexpression suggests that *FTO* implicates both peripheral and central physiology to confer increased obesity risk (Church et al. 2010). Fasted mice that overexpress *FTO* had lower circulating leptin levels than control mice (Church et al. 2010). These observations suggest that systemic overexpression of *FTO* affects leptin expression or secretion from adipose tissue, which in turn affects the central nervous system-mediated control of food intake. However, when *FTO* is only overexpressed in the arcuate nucleus, energy intake was decreased (Tung et al. 2010). Adding to the complexity is the observation that the *rpgrip1l* gene, which lies in opposite orientation to *FTO*, has a similar hypothalamic expression pattern as *FTO* (Stratigopoulos et al. 2008). RPGRIP1L is a component of the basal body of the primary cilium which has been suggested to be involved in syndromic obesity (Baker and Beales 2009). The expression of both genes is co-regulated by CUX1 from within the first intron of *FTO* (Stratigopoulos et al. 2011). Therefore, the function of both genes may be affected in individuals carrying the BMI-increasing allele. It is not completely clear yet which of these two genes (or both) are functionally relevant.

The near-MC4R locus – The near-*MC4R* locus, identified in the second wave of genome-wide association studies for BMI, is located at 188 kb downstream of *MC4R,* which is an obvious candidate gene given its role in monogenic early-onset obesity. While *MC4R* is the gene nearest to the association signal and the phenotypic associations are consistent with effects mediated through *MC4R* function, it has not yet been firmly established whether this locus indeed affects *MC4R* function or whether it affects obesity through other pathways.

Loci identified in the second and third wave of genome-wide association studies for BMI – For most of the loci discovered during the third and fourth wave of genome-wide association studies for BMI, the physiological role in relation to obesity risk is not or poorly understood (Willer et al. 2009; Thorleifsson et al. 2009; Meyre et al. 2009; Speliotes et al. 2010). Many of the loci harbour multiple genes, sometimes

located within a recombination interval with high linkage disequilibrium, which hampers pinpointing the causal variant. For other loci, BMI-associated SNPs are located far away from the nearest gene, suggesting that the locus may contain non-genic regulatory elements that remain to be revealed. Comprehensive resequencing and fine-mapping will be required to unambiguously identify the causal variants before physiologists can start exploring the functional relevance of the locus in relation to the risk of obesity, which will be key for translation into clinical practice.

At least, six loci contain genes with preliminary evidence of a link to obesity risk. For example, one locus harbours *SH2B1* which encodes a protein that is implicated in leptin signalling (Ren et al. 2007). Furthermore, Sh2b1-null mice are obese (Ren et al. 2007). Although the *SH2B1* variant that shows the most significant association is a non-synonymous SNP (Thr484Ala), it is in strong linkage disequilibrium with variants in at least five neighbouring genes. Furthermore, this variant is associated with the expression of SH2B1 and that of three other genes in the locus (Speliotes et al. 2010). Thus, it remains to be confirmed whether the signal is indeed representing SH2B1 function or whether it is caused by any of the neighbouring genes.

Also, the *BDNF* locus has a strong prior candidacy, and one of the SNPs in this locus is the non-synonymous Val66Met SNP that, as discussed above, has previously been examined in candidate gene studies of eating behaviour and BMI (Table 10.1) (Shugart et al. 2009).

Furthermore, the BMI-associated SNPs in the near-*RBJ* locus are associated with the expression of at least two candidate genes, *ADCY3* and *POMC* (Speliotes et al. 2010). The BMI-associated SNPs are in linkage disequilibrium with a non-synonymous variant in *ADCY3*. *ADCY3* is an adenylyl cyclase which expression is increased in lean diabetic rats (Abdel-Halim et al. 1998). Moreover, liver adenylyl cyclase activity is increased in the membranes of male ob/ob mice in comparison to the lean control mice (Begin-Heick 1994). The other candidate in this locus is *POMC* that is post-transcriptionally processed to produce hormones in the hypothalamic–pituitary–adrenal axis, such a-melanocyte-stimulating hormone (MSH), adrenocorticotropic hormone (ACTH) and β-endorphin, which are agonists of *MC4R*. Mutations that inactivate *POMC* have been reported in several children, which are characterised by early-onset morbid obesity, hypocortisolism and alterations of skin and hair pigmentation (Farooqi and O'Rahilly 2008).

Also, the *QPCTL* locus harbours many genes, one of which is *GIPR*, which encodes a receptor of gastric inhibitory polypeptide (GIP) (Speliotes et al. 2010). GIP is an incretin hormone that mediates incremental insulin secretion in response to oral intake of glucose. Interestingly, the BMI-increasing allele of this locus was also associated with increased fasting glucose levels and lower 2-h glucose levels (Saxena et al. 2010). The direction of the effect is opposite to what would be expected based on the correlation between obesity and glucose intolerance but is consistent with the suggested roles of GIPR in glucose and energy metabolism. Mice lacking *Gipr* are protected from diet-induced obesity (Miyawaki et al. 2002). The association of the variant in this locus with BMI suggests that there may be a link between incretins, insulin secretion and body weight regulation in humans as well.

The locus near *FLJ35779* contains a non-synonymous variant and harbours *HMG-CoA reductase*, which encodes the rate-limiting enzyme for cholesterol synthesis. It is furthermore a well-known drug target for cholesterol-lowering drugs like statins. *HMG-CoA reductase* has not previously been linked obesity risk.

TUB is one of the genes located in the *RPL27A* locus and has been implicated in obesity in mice and human before. The *tub* gene is predominantly expressed in the hypothalamus and a loss-of-function mutation in *tub* results in the tubby mouse syndrome, which is characterised by late-onset obesity with insulin resistance, as well as neurosensory defects (Kleyn et al. 1996). Several studies have reported association between genetic variation in *TUB* and obesity-related traits (Shiri-Sverdlov et al. 2006; van Vliet-Ostaptchouk et al. 2008; Snieder et al. 2008).

Of interest is that several of the established BMI loci harbour genes that have previously been implicated in monogenic obesity, such as *MC4R*, *POMC*, *BDNF* and *SH2B1*, and many locate near genes that are highly expressed in the brain and hypothalamus supporting a role for the nervous system in body weight control (Willer et al. 2009; Thorleifsson et al. 2009; Speliotes et al. 2010).

Loci identified in genome-wide association studies of WHR – Several loci identified for WHR contain genes that likely influence body fat distribution and that are involved in adipocyte metabolism (Heid et al. 2010). This is in contrast to the BMI loci of which genes seem predominantly involved in the central regulation of energy homeostasis. One of the interesting candidates for WHR is *GRB14*, of which variants are also associated with triglyceride, high-density lipoprotein-cholesterol and insulin levels (Heid et al. 2010; Ridker et al. 2009). *Grb14*-deficient mice exhibit improved glucose homeostasis despite lower circulating insulin levels, as well as enhanced insulin signalling in liver and skeletal muscle (Cooney et al. 2004). It remains to be examined whether the association with the various metabolic traits are mediated through an effect of *GRB14* on fat distribution or whether they act through independent mechanisms.

The locus near *ADAMTS9* was previously found to be associated with risk of type 2 diabetes (Voight et al. 2010) and with insulin resistance in peripheral tissues (Boesgaard et al. 2009). At the chromosome 6p12 locus, vascular endothelial growth factor A (*VEGFA)* is the most apparent biological candidate given the presumed role of *VEGFA* in adipogenesis (Nishimura et al. 2007) and evidence that serum levels of VEGFA are associated with obesity (Silha et al. 2005; Garcia de la Torre et al. 2008).

Loci identified in genome-wide association studies of extreme and early-onset obesity – Of interest is that apart from the *FTO* and near-*MC4R* loci, none of the loci identified for extreme and early-onset obesity overlap with those identified for BMI (Fig. 10.4), suggesting that extreme obesity and BMI are, at least in part, different phenotypes that are caused by different genes, and thus potentially different physiological pathways.

The locus the harbours *NPC1* represents the best candidate with. More than 200 mutations in *NPC1* have been found to cause Niemann-Pick disease type C1, which is a lipid storage disease (Chang et al. 2005). Although, the *Npc1*-null mice display late-onset weight loss and poor food intake (Xie et al. 1999), mutations in *NPC1* have so far not been found to cause monogenic obesity in humans.

10.7 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 (Fig. 10.6). Various approaches have been proposed to identify more genetic loci and to pinpoint the causal variants.

10.7.1 Continued and Alternative Use of Genome-Wide Association studies

10.7.1.1 Increased Discovery by Efficient Use of Available Genome-Wide Association Data

The statistical power of genome-wide association studies to identify new loci depends largely on the sample size at the discovery stage. The several waves of discoveries

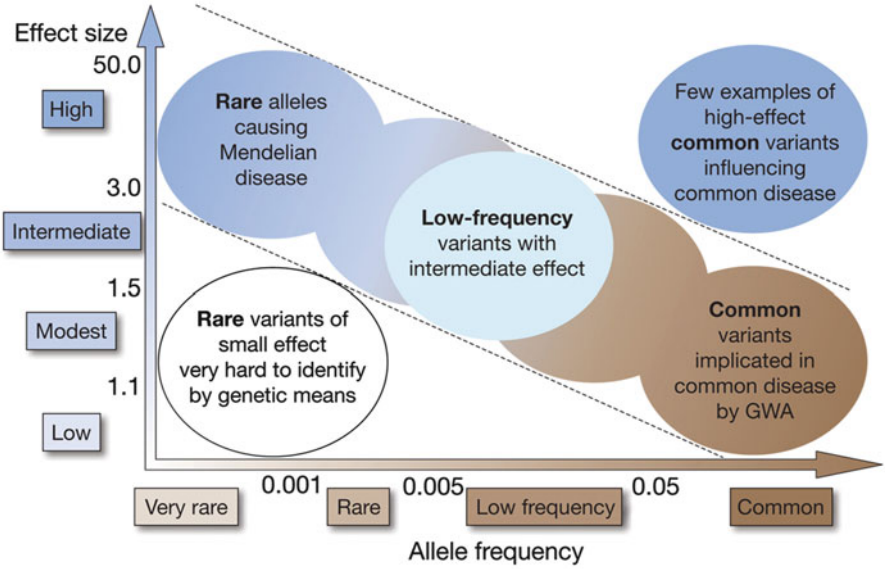

Fig. 10.6 Relationship between effect size (*y*-axis; OR) and allele frequency of the associated genetic variant. Genome-wide association studies are able to identify common loci (allele frequency ≥5%) that typically have small to modest effects (lower right-hand corner). Rare variants (mutations) with large effect are identified for monogenic diseases (upper left-hand corner). There are likely very few complex traits caused by common variants with large effects (upper right-hand corner), and rare variants with small effects are very hard to identify, even with the latest sequencing technology (lower left-hand corner). Current gene-discovery studies aim to identify low-frequency variants with intermediate effect (middle). Adapted from Manolio et al. (2009) and McCarthy et al. (2008)

are an elegant example of how increased sample size results in more loci (Fig. 10.4). At the same time, it should be noted that the additional power not only allows identifying more loci, but also allows identifying loci with smaller effects. While these small-effect loci will not contribute much to explaining the missing heritability, their value lies in the fact that they may point towards new genes of unknown biology.

The most recent genome-wide association studies for BMI and WHR in the discovery stage already include >120,000 and >77,000 individuals, respectively, and they seem to have reached their maximum. However, an alternative approach has been introduced with the Metabochip to make more efficient use of the available data. The Metabochip is a customised array that contains ~200,000 carefully selected SNPs. Half of the SNPs on the Metabochip aim to fine-map the established loci of a variety of metabolic traits, including those of BMI and WHR. The other half of the Metabochip contains SNPs that have reached a respectable significance level in the genome-wide association studies of various metabolic traits but that have not previously been taken forward for replication. These SNPs may need larger sample sizes for replication as they have even smaller effect sizes and/or are of low frequency than those of the previous waves of discoveries.

Taken together, by making use of the available data of the latest and largest genome-wide association studies, the Metabochip aims to fine-map established loci and identify new loci.

10.7.1.2 Studies in Populations of Different Ethnic Backgrounds

The vast majority of GWAS for obesity-related traits have been performed in populations of white European ancestry. As the genetic architecture, including the frequency of genetic variants and the correlation between them, and also the genetic effects may vary across ethnicities, the statistical power to identify new loci may vary across populations. Therefore, the study of ethnicities other than white European may provide new opportunities to discover additional obesity-susceptibility loci. This is elegantly illustrated by the genome-wide association study in Indian Asians, which needed only 2,684 individuals at the discovery stage to identify the near-*MC4R* locus (Chambers et al. 2008), whereas the genome-wide association in white Europeans required 16,876 individuals to find the same locus (Loos et al. 2008). The higher frequency of the risk-allele in Indian Asians (36%) compared to white Europeans (27%) likely provided the former more power to identify this locus with a smaller sample size.

So far, few other genome-wide association studies for obesity-related traits in non-white individuals have been reported (Cho et al. 2009; Ma et al. 2010; Kang et al. 2010). While most confirm the *FTO* and also near-*MC4R* locus, some have also identified a few population-specific loci (Cho et al. 2009; Ma et al. 2010). As discovery stage sample sizes of these studies have been modest, large-scale meta-analyses might increase the power to identify even more obesity-susceptibility loci.

Furthermore, because of the population-specific genetic architecture, comparison of effects in established loci across different ethnicities may contribute in fine-mapping the association signal. As such, the *FTO* locus was narrowed down by examining the effects in the *FTO* locus in an African-ancestry population (Hassanein et al. 2010).

10.7.1.3 Genome-Wide Association Studies in Children and Adolescents

Genome-wide association studies have predominantly been performed in adults. Genetic effects can vary over the life course, and sometimes they may be larger during childhood than adulthood because of limited environmental influence (Hardy et al. 2010). Therefore, performing genome-wide association studies in children and adolescents might be another avenue to reveal new obesity-susceptibility loci.

10.7.1.4 Studies of More Refine Traits of Obesity-Susceptibility

So far, most studies have used BMI and WHR as non-invasive and affordable proxy-measures of adiposity and fat distribution, which are easy to collect in large samples. By using more refined measures of adiposity, such as body fat percentage, the power to detect new loci may increase. However, such measures are often more expensive and harder to obtain, and will be available in fewer cohorts. Whether the gain in power through improved measurement accuracy compensates for the loss in power due to smaller sample size remains to be determined.

10.7.1.5 Studies of Intermediate Traits of Obesity-Susceptibility

Weight gain is the result of a chronic imbalance between energy expenditure and energy intake. Therefore, genome-wide association studies 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 1,644 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.

The main challenge when studying intermediate traits such as physical activity and food intake is the measurement of these traits in an accurate and objective manner. Most often questionnaires are used that differ across studies. This inaccuracy and heterogeneity of measurements will lower the power and hamper pooling of data for meta-analyses. Therefore, harmonisation of measures of lifestyle factors across studies will be essential to achieve a uniform phenotype that can be meta-analysed across cohorts.

10.7.1.6 Genome-Wide Gene–Lifestyle Interaction Analyses

Thus far, gene-lifestyle interaction studies have focussed on the *FTO* locus (Andreasen et al. 2008b; Rampersaud et al. 2008; Vimaleswaran et al. 2009) or a select series of established obesity-susceptibility loci (Li et al. 2010a). 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 genome-wide association studies of intermediate traits, described above, gene–environment interaction studies will require the harmonisation of lifestyle measures before data can be combined in meta-analyses large enough to detect interaction effects.

10.7.2 Identification of Low-Frequency Variation

The risk-allele frequency of the 50 obesity-susceptibility loci ranges from 4% to 94%, and their effects are small. It is unlikely that within this range of allele frequencies, there are new loci to be identified with larger effect sizes than those already observed, as one would expect that at least the most recent genome-wide association studies would have had sufficient power to identify those. Furthermore, it is unlikely that loci will be identified with a lower frequency than those currently observed, because the genome-wide genotyping arrays were design based on information available from the HapMap, which aimed to capture common variants only. To identify rare to low-frequency variants, with potentially larger effects, several new approaches are currently ongoing.

10.7.2.1 Genome-Wide Association of Copy Number Variants

Copy number variants (CNVs) 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 SNPs (Sudmant et al. 2010). Although the extent to which CNVs might contribute to common disease has been debated (Conrad et al. 2010), several lines of evidence suggest that they have a role in obesity-susceptibility.

Two of SNPs of the 32 established BMI loci each tag a CNV; i.e. a 45-kb deletion near *NEGR1* (Willer et al. 2009) and a 21-kb deletion that lies 50 kb upstream of *GPRC5B* (Speliotes et al. 2010).

Furthermore, several systematic genome-wide CNV association studies have further provided evidence for the involvement of small deletion and duplication in the risk of obesity. 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$\geq$40 kg/m^2) (Bochukova et al. 2010; Walters et al. 2010), which was further confirmed in a large-scale population-based study (Bachmann-Gagescu et al. 2010). 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 (Speliotes et al. 2010). These findings highlight the value of using a variety of strategies to increase our insights into the genetic architecture of human obesity.

10.7.2.2 Implementation of the 1000 Genomes Data

The 1000 Genomes Project aims to catalogue all genetic variants, including low frequency variants (~1%) by sequencing populations of various ethnic background (Sudmant et al. 2010). The pilot project already identified more than 15 million SNPs and 22,000 CNVs across the human genome. The data of the 1000 Genomes project can contribute to the identification of low-frequency variants in at least three ways. First, this new detailed information will lead to a new generation of genome-wide genotyping array that more fully capture the genetic variation in humans, which in turn will allow detailed genome-wide association studies. Secondly, cohorts that have been genotyped with the previous generation genome-wide arrays could impute the data of the 1000 Genomes Project and redo their genome-wide association analyses with higher resolution. Thirdly, as the 1000 Genomes Project contains many previously unknown genetic variants, this new information can be used as the basis for fine-mapping of existing loci.

10.7.2.3 Deep-Sequencing Efforts

It is expected that the next-generation sequencing technology will take gene discovery to the next level and will allow identifying the precise variant that contributes to disease. While sequencing is currently still expensive and labour intensive, many exome-sequencing projects as well as several whole-genome sequencing projects are ongoing. One such project that involves the search for genes for obesity is the UK10K project (http://www.uk10k.org/) in which the genomes of 2,000 extremely obese children will be compared to that of a population-based sample of 4,000 individuals. This new approach is still in development and undergoes continuous changes as technologies improve and scientists explore the optimal depth of coverage, the most efficient study design and the statistical tools needed to identify new loci with confidence.

10.7.3 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 deep-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.

10.8 Conclusion

Genome-wide association studies 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 genome-wide association studies in terms of gene discovery has been enormous. In less than 4 years time, at least 50 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 (Contopoulos-Ioannidis et al. 2008), it is too early to evaluate the success of genome-wide association studies 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 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 11
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, such as from the Dutch "Hunger Winter" studies, show that maternal undernutrition 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 foetal adiposity without overall changes in birthweight, obese mothers are more likely to have large gestational age babies, and these offspring are more likely to become 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 preceded by foetal 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 BMI in childhood. Key pathways of foetal 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.

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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.

It can be hypothesised 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. 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.

11.1 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 foetal growth which result from foetal undernutrition between middle to late gestation (Barker et al. 1993). It is recognised that there are critical windows in foetal development when the process is "plastic" i.e. during periods in which the foetus 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 1944–1945) 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 threefold increase in incidence of cardiovascular diseases (Roseboom et al. 2000b).

Hales and Barker have proposed the "thrifty phenotype hypothesis" (Hales and Barker 2001) which postulates that poor foetal 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 foetus will be born. Foetal 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 et al. (2005) defined predictive adaptive responses 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 foetal growth is determined by interaction between foetal environment and foetal 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 (BMI) 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). Furthermore, increased maternal weight and decreased insulin sensitivity are correlated with foetal 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 foetus, leading to foetal 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 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 have 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 foetus 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 foetus and onwards through successive generations (Catalano 2003).

11.2 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 foetal 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 foetuses 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 foetal and early neonatal development can also be better elucidated in the sheep as its diet can be manipulated to coincide with precise periods of foetal organogenesis which are comparable with those during human foetal 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 1 (UCP1) abundance and the hypothalamo-pituitary axis.

Important long-term impacts also result from changes in organ growth rates, foetal 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 foetus and neonate.

11.3 The Programming of Adipose Tissue

Adipose tissue is present from very early in foetal 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). Foetal adipose tissue exhibits characteristics of both brown and white adipose tissue, demonstrating an ontogenic

rise in the BAT specific 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 disappears to undetectable levels in the postnatal period whilst, 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).

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). 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 foetal milieu, adipose tissue is kept firmly regulated (Symonds et al. 2003). However, despite this, foetal adipose tissue is significantly altered by changes in maternal nutrition during foetal 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 foetus (Yuen et al. 1999; Budge et al. 2004; Casteilla et al. 1987). Leptin synthetic capacity of foetal 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 foetal 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 the 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 (PRLRs) and in plasma prolactin are seen along with rise in the metabolically active forms of thyroid hormones in the foetal adipose tissue (Symonds et al. 2003). All these are implicated in upregulation of UCP1 gene expression.

Changes in maternal nutrition during various phases of foetal development can alter foetal adiposity as summarised in Fig. 11.1. These responses may not always manifest as differences in foetal 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 foetus subsequently deposits more adipose tissue with increased 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

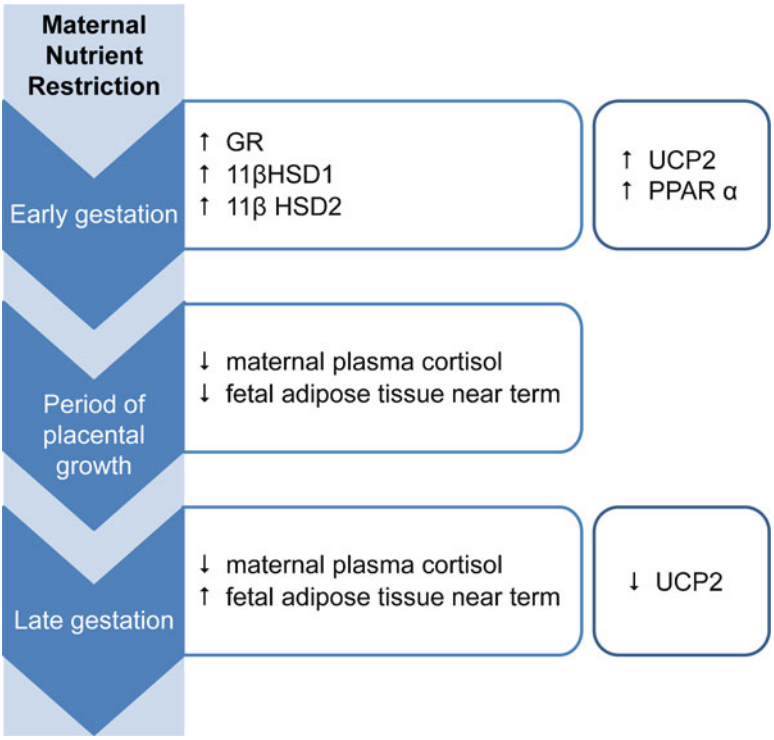

Fig. 11.1 Effects of maternal nutrient restriction on the development of adipose tissue. Nutrient restriction at different phases of development alters the abundances of glucocorticoid receptors (GR), 11β-hydroxy steroid dehydrogenases (11βHSD), uncoupling protein (UCP) 2 and peroxisome proliferator-activated receptor (PPAR) α in foetal adipose tissue deposition

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 foetal adipose tissue in late gestation (Muhlhausler et al. 2003).

11.3.1 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

foetal development and in later life. They are essential for the terminal differentiation of adipocytes as seen by the expression of late markers such as glycerol-3-phosphate dehydrogenase (G3PDH) 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. 2005).

The action of glucocorticoids on adipose tissue is mediated by glucocorticoid receptors (GR) and 11-β-hydroxysteroid 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 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. 2005; 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. 2005). 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. 2005). 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 foetus, 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 foetal 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 glands 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. 2005). Maternal nutrient restriction in sheep during the phase of maximal placental growth results in lower maternal plasma cortisol with an increase in foetal adipose tissue deposition near to term (Bispham et al. 2003), whilst undernutrition in late gestation transiently increases maternal cortisol concentrations when combined with foetal 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. 2005). 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. 2005). These modifications in glucocorticoid sensitivity following maternal nutritional variations could be a pivotal adaptation leading to later obesity, fitting with current theories of foetal 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.

11.3.2 Uncoupling Protein 2 in the Regulation of Obesity

Whilst UCP1 is specific to BAT, 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. 2005), 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. 2005; 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. 2005). 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. 2005; Weisberg et al. 2003).

11.3.3 Peroxisome Proliferator-Activated Receptors in the Programming of Obesity

Peroxisome proliferator-activated receptors (PPAR) 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 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 increases 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 foetal adiposity and its markers. Increased nutrient supply in late gestation results in an increase in the expression of PPAR-γ, LPL, adiponectin and leptin expression in foetal 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).

11.3.4 Role of Prolactin

Prolactin has a role in foetal adipose tissue growth and maturation before birth when there is a rise in PRLR expression during the phase of rapid perirenal BAT deposition in sheep (Symonds et al. 1998). In rats, PRLRs are widely expressed and increase in PRLR expression is seen in late gestation in a number of foetal tissues (Royster et al. 1995). Administration of prolactin to pregnant rats increases UCP1 abundance in both foetus and newborn offspring, accelerating BAT maturation and enhancing its function, suggesting the role of prolactin in development of BAT (Budge et al. 2002).

Both PRLR1 and PRLR2 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 foetal nutrition alone does not affect PRLR expression but hypoxia combined with foetal undernutrition (achieved by removal of endometrial caruncles before mating) downregulates PRLR1 gene expression (Symonds et al. 1998). With an increase in maternal nutrition, foetal 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 foetal plasma prolactin concentrations in late gestation without altering PRLR gene expression in the liver or kidney of the foetus (Phillips et al. 2001).

11.4 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. 11.2). 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

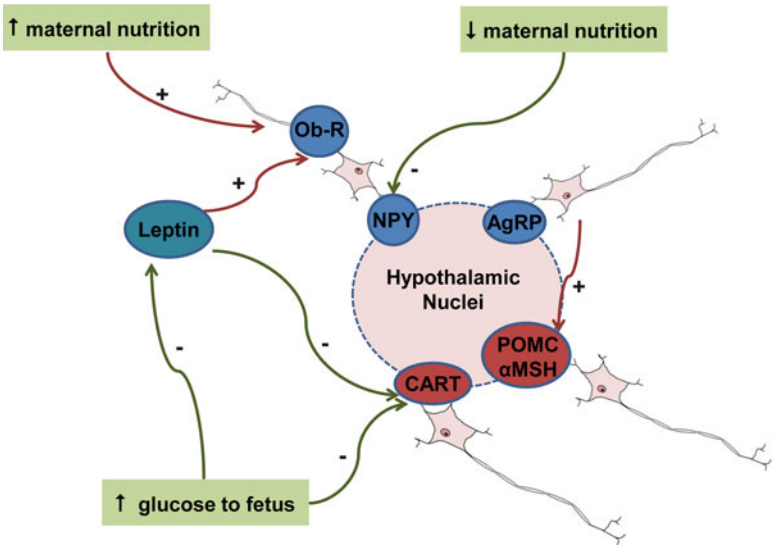

Fig. 11.2 Effects of maternal nutrition on appetite regulation. Decreased maternal nutrition increases the neuropeptide Y (NPY) action on hypothalamic nuclei (HN) whilst leptin acts as an appetite suppressant by inhibiting NPY neurons. Increased maternal nutrition reduces leptin receptors in the HN. Increased glucose administration to the foetus (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)

transcript (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 continue 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 that 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 foetal undernutrition and glucocorticoids increase NPY gene expression in the foetus (Warnes et al. 1998). Glucose administration to foetal 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 foetal 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 foetus 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. 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 foetal 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 have effects on neuronal organisation and expression of orexigenic and anorexigenic neuropeptides 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 foetal 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, leptin receptor (Ob-Rb), signal transducer and activator of transcription 3,

suppressor of cytokine signalling 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 GLUT4 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.

11.5 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) 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.

11.6 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 receive 30% of the ad libitum amount consumed by controls exhibited foetal growth retardation (Vickers et al. 2000). Foetal undernutrition induces inappropriate

hyperphagia in adult life, and postnatal hypercaloric nutrition further amplifies the abnormalities induced by foetal 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 (HPA) 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 (Williams et al. 2007).

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.

11.6.1 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 foetal 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 foetal 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. 2000a) and increased risks of obesity (Ravelli et al. 1999) and metabolic diseases, including a threefold increased incidence of cardiovascular disease (Roseboom et al. 2000b).

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 HPA 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 foetal 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 foetus (Symonds et al. 2007). Enhanced foetal fat stores achieved by promoting nutrient supply to the foetus 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 foetus for excess fat deposition after birth if nutrients are no longer limited (Symonds et al. 2007).

In both sheep and humans, foetal adipose tissue is primarily deposited during the final third of gestation. Over this period, there is an increased abundance of circulating hormones within the foetal circulation which are important in regulating foetal 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 foetuses have an increased abundance of GLUT1 (Dandrea et al. 2001), the enhanced responsiveness to IGF may promote the anabolic effects of glucose on foetal adipose tissue growth. Therefore, maternal nutrient restriction in mid-gestation results in enhanced foetal 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 110 days 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 foetal 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 GLUT4, the major insulin

responsive GLUT, 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 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 foetal 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).

11.7 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 foetus. 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. 11.3.

A study of pregnant women in nine US states showed a 69% increase in pre-pregnancy obesity from 1993 to 2003 (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 has increased to nearly 19 from 9% (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 its huge implications for maternal and foetal outcomes (Catalano and Ehrenberg 2006), maternal obesity is being increasingly recognised as a major public health issue. In addition to the ill-effects of obesity itself, high maternal weight is associated with a substantially higher risk of gestational diabetes mellitus (Chu et al. 2007), exposing the foetus to further risks due to hyperglycaemia 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). Other studies have demonstrated a decrease in birth weight both with increased maternal BMI at conception and with overnutrition during pregnancy (Wallace et al. 2010).

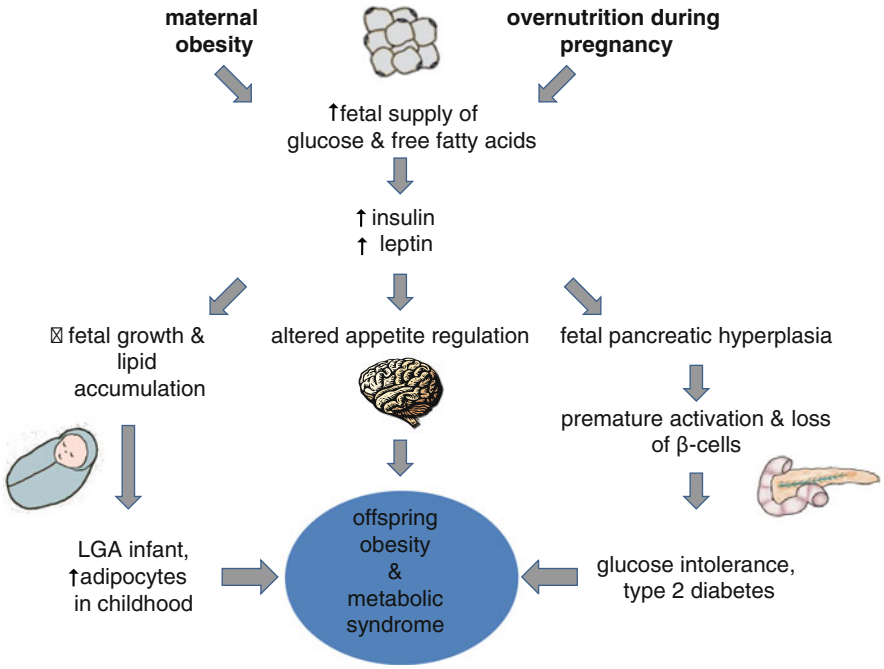

Fig. 11.3 Maternal obesity and overnutrition can programme the foetus to adult obesity and metabolic syndrome

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 foetus (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 emphasise 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 pre-pregnancy 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 increase 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.

11.8 Early Postnatal Growth and Adiposity Rebound

In the Avon longitudinal study in the UK, children who have intrauterine restraint of foetal 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 foetal 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 recognisable 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 IGF-I (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).

11.9 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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