Chapter 3
Adipose Tissue as a Peripheral Clock

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Abstract Adipose tissue is a complex and highly metabolic and endocrine organ, which is capable of expressing and secreting a variety of bioactive peptides, so-called adipokines. These adipokines are involved in coordinating a diversity of biological processes including energy intake and expenditure, insulin resistance, adipocyte differentiation, dyslipidemia, and body fat distribution. One of the most outstanding discoveries in the last year is the presence of an active circadian clock in adipose tissue depots. New data suggest that there is a temporal component in the regulation of all these adipose tissue functions. In fact, studies performed by microarrays have shown that a certain percentage of active genes expressed in adipose tissue in both humans and animal models follow a daily rhythmic pattern. Examples of these genes are clock genes (*PER2, CLOCK, CRY1,* and *BMAL1*), adipokine genes (adiponectin and leptin), and glucocorticoid-related genes among others. Thus, an adequate temporal order in the daily pattern of these genes implicated in adipose tissue metabolism could have important consequences not only in body fat distribution but also in the metabolic alterations associated to obesity. Further investigations of circadian rhythms in adipose tissues will provide insight into the physiology of energy homeostasis and the etiology of metabolic diseases such as obesity.

Abbreviations

LEP	Leptin
LEPR	Leptin receptor
TNFα	Tumour necrosis factor alpha

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ApM1	Adipose most abundant gene transcript 1 or adiponectin
Acrp30	Adipocyte complement-related protein of 30 kDa or adiponectin
GBP28	Gelatin binding protein of 28 kDa or adiponectin
AdipoQ	Adiponectin
ADIPOR1	Adiponectin receptor 1
ADIPOR2	Adiponectin receptor 2
TZDs	Thiazolidinediones
IL-6	Interleukin 6
ASP	Acylation stimulating protein
PAI-1	Plasminogen activator inhibitor-1
TGF-beta	Transforming growth factor-beta
GCs	Glucocorticoids
HPA	Hypothalamic–pituitary–adrenal
GR	Glucocorticoid receptor
11DHC	11-Dehydrocorticosterone
11βHSD1	11β-Hydroxysteroid dehydrogenase 1
11βHSD2	11β-Hydroxysteroid dehydrogenase 2
STAR	Teroidogenic acute regulatory protein
5αR	5-α reductase
PPARγ	Peroxisome proliferator-activated receptor gamma
PCR	Polymerase reaction chain
AT	Adipose tissue
WAT	White adipose tissue
BAT	Brown adipose tissue
SWAT or SAT	Subcutaneous adipose tissue
VWAT or VAT	Visceral adipose tissue
IAAT	Intra-abdominal adipose tissue
FFAs	Free fatty acids
TGs	Triglycerides
MetS	Metabolic syndrome
SCN	Suprachiasmatic nucleus
LPL	Lipoprotein lipase
Pdp1	PAR domain protein 1
RORα	RAR-related orphan receptor alpha
PGC1α	Peroxisome proliferative activated receptor gamma, coactivator 1 alpha
PER2	Period homolog 2 (Drosophila)
BMAL1 or ARNTL or MOP3	Aryl hydrocarbon receptor nuclear translocator-like
CLOCK	Circadian locomotor output cycles kaput
CRY	Cryptochrome
mRNA	Messenger ribonucleic acid
CCG	Clock control genes
ASCs	Adipose-derived stem cells
ACTH	Adrenocorticotropic hormone

Adipose Tissue as Endocrine Organ

Classically, adipose tissue has been regarded as a passive reservoir for energy storage, but this traditional point of view is no longer valid. In 1987, adipose tissue was identified as a major site for metabolism of sex steroids [1]. Nevertheless, the critical change in our perspectives on adipose tissue came in 1994 with the discovery of the cytokine-like factor, *leptin* (from the Greek *leptos*, meaning thin). For the first time in science, it was described that adipose tissue was able to secrete hormones or "adipocytokines" capable of communicating information from periphery to the central nervous system [2]. This outstanding outcome opened a "great window" in the study of obesity. Adipose tissue was not any more a passive reservoir of energy; it was as an endocrine organ and with this discovers a new "époque" started in the obesity research.

Nowadays we know that adipocytes secrete leptin in direct proportion to adipose tissue mass as well as nutritional status, in this way leptin signals the status of energy stores and its secretion can reduce appetite and increase energy expenditure. The capability of leptin to regulate food intake, body weight, and adiposity has been recognized entirely to its actions in the hypothalamus [3]. Nevertheless, it has been reported that leptin is essential in the adipose tissue itself, modulating the adipocytes' metabolic function, up-regulating fat oxidation, and decreasing lipogenesis [4]. These physiological functions are carried out by binding to its receptor (*LEPR*) expressed in adipose tissue [5]. Therefore, changes in leptin or its receptor in adipose tissue can be relevant in the development of obesity and other metabolic disorders [6].

Since the discovery of leptin, numerous research findings show that adipose tissue is a highly active endocrine organ, which is involved in many physiological processes. These metabolic processes are influenced by products of the adipose tissue, so-called adipocytokines or adipokines (Fig. 3.1).

One of the first molecules studied in relation to adipose tissue was *TNFα*. This particular protein was firstly analyzed as an inflammatory molecule in cancer studies.

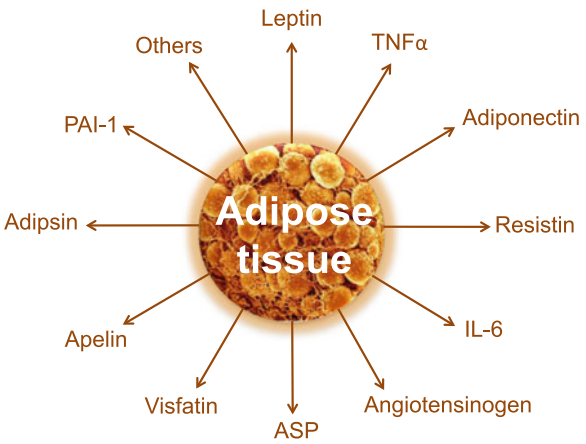

Fig. 3.1 Adipose tissue as an endocrine organ. AT can secrete many adipokines and certain factors involved in the metabolism such leptin, TNFα, adiponectin, visfatin among others

Within adipose tissue, TNFα is expressed by adipocytes and stromovascular cells [7] and nowadays we know that this cytokine is able to repress genes involved in uptake and storage of nonesterified fatty acids and glucose; it also suppresses genes for transcription factors involved in adipogenesis and lipogenesis; it changes the expression of several adipocyte-secreted factors including adiponectin and is capable of impairing insulin signaling [8, 9]. In this context, TNFα production is increased in obesity and it has been implicated in the development of insulin resistance in the adipocyte of the obese by altering insulin signaling through an autocrine or paracrine action.

In relation to insulin resistance, another important cytokine that has attracted much attention in the last years is *Adiponectin* [10]. This cytokine has been described as one of the most protective molecules against the different alterations associated with obesity, such as insulin resistance and inflammation Adiponectin was firstly characterized in 1995, and because it is one of the most expressed genes in adipose tissue it was formerly called apM1 (adipose most abundant gene transcript 1). The identification of adiponectin in the text is not easy and may be confusing, because it has been also named as Acrp30 (adipocyte complement-related protein of 30 kDa), adipoQ, and GBP28 (gelatin binding protein of 28 kDa) [10–13].

Several metabolic effects from adiponectin have been described. On the one hand, its *anti-atherogenic effect* is well known since it is capable of inhibiting expression of adhesion molecules and vascular smooth muscle cells proliferation, and suppresses transformation of macrophages to foam cells. Moreover, it is an *anti-diabetic cytokine* because increases insulin sensitivity and decreases hepatic glucose output and it increases fatty acid oxidation. Besides, adiponectin is decreased in abdominal obesity [14].

The beneficial effects of this hormone are predominantly mediated by binding to two cell membrane receptors, adiponectin receptor 1 (*ADIPOR1*) and adiponectin receptor 2 (*ADIPOR2*) [15]. Both receptors are also present in adipose tissue, suggesting that adiponectin may have biological effects in adipose tissue in an autocrine/paracrine manner [16].

One of the most controversial adipokines described has been *Resistin* (resistance to insulin). This adipokine was identified in 2001 as a novel mRNA induced during adipocyte differentiation but down-regulated by thiazolidinediones (TZDs) in vitro [17]. Initial studies suggested that resistin had significant effects on insulin action, potentially linking obesity with insulin resistance and type II diabetes [18]. Biological activities of resistin include: (a) impairment of glucose tolerance in mice in vivo, (b) antagonism of glucose uptake by cultured 3T3-L1 adipocytes, and inhibition of 3T3-L1 differentiation into adipocytes [19]. Of note, in human studies, the influence of resistin on the development of insulin resistance is controversial. In obese subjects, resistin in adipose tissue is significantly higher compared to normal weight subjects [20, 21]. However, several human studies have failed to demonstrate any impact of obesity and insulin resistance on the concentration of resistin [22, 23].

Interleukin-6 (*IL-6*) is another cytokine associated with obesity and insulin resistance [24]. IL-6 comes from adipose tissue and this amount increases proportionally with increasing body mass [25]. This cytokine decreases the expression of insulin

receptors in peripheral tissues, acts as an inhibitor of adipogenesis and inhibits adiponectin secretion. There is growing evidence that IL-6 and several other pro-inflammatory cytokines are "sleep factors". In addition, it has been shown that these cytokines also influence energy intake by enhancing insulin and leptin sensitivity. The study of this cytokine offers the possibility to connect the showed interactions among energy intake, sleep behaviour, and circadian system with metabolic alterations.

Human studies have indicated that complement proteins, secreted by adipocytes, the so-called *Complement-Related Proteins*, positively correlated with adiposity, insulin resistance, dyslipidemia, and cardiovascular disease [26]. *Adipsin* (complement factor D) is one of several adipose tissue-derived complement components that are required for the enzymatic production of acylation stimulating protein (*ASP*), a complement protein that affects both lipid and glucose metabolism [26, 27].

Every day we have new cytokines in research. In fact, *Visfatin* is a recently discovered adipokine produced and secreted primarily by visceral adipose tissue, which binds to and activates the insulin receptor, exerting insulin-mimetic effects both in vitro and in vivo [28]. There are many other adipokines, such as *Apelin*, which is an adipocytokine whose plasma concentration is increased in obesity, insulin resistance, and hyperinsulinemia [29]. But also other factors that are secreted by adipose tissue include angiotensinogen, plasminogen activator inhibitor-1 (PAI-1), tissue factor and transforming growth factor-beta (TGF-beta), adipophilin, monobutyrin, agouti protein, and factors related to pro-inflammatory and immune processes [30]. Although adipose tissue-derived hormones are identified every day, even those factors that were already characterized, such as leptin, require precise definitions of their physiological effects, as many as 40% are novel genes [31]. The continued identification characterization of these novel genes is likely the endocrine function of adipose between energy homeostasis and systems.

Glucocorticoids: The Circadian Hormone

One of the most outstanding circadian hormones in our body is cortisol. The analyses of its rhythms in plasma are one of the outcomes more frequently described in the classical chronobiology. It has been long established as a catabolic in nature, liberating energy substrates during times of stress to supply the increased metabolic demand of the body. The link between glucocorticoids (GCs) and excess adiposity is clearly demonstrated and well established clinically. However, the effects of GCs on adipose tissue metabolism are still contradictory. In fact, several studies have shown that the patients with elevated GCs, for example, individuals with Cushing syndrome [32] or those on exogenous corticosteroid treatment [33] present increased weight gain and visceral adiposity and are at increased risk for developing type 2 diabetes mellitus [34, 35]. Although elevated GC levels seem to contribute to visceral fat accumulation, most obese individuals do not exhibit elevated peak plasma GC levels [36–38]. Indeed, circulating GCs levels in obese patients might even be lower than in patients with normal weight, and intra-adipose GCs metabolism has

been hypothesized as the reason for this low GCs plasma concentration [39]. On the other hand, subjects with visceral obesity show perturbations of the cortisol diurnal rhythm, characterized by a significant decrease in cortisol variability suggesting that a pathological HPA axis response is associated with abdominal fat distribution [40].

In this context, increased adipose tissue glucocorticoid exposure relies not only on glucocorticoid receptor (*GR*) availability but also on the local enzymatic interconversion of active (cortisol in humans and corticosterone in rodents) and inactive (cortisone in humans and 11-dehydrocorticosterone (11DHC) in rodents) hormones. This interconversion is controlled by two isoenzymes of 11β-hydroxysteroid dehydrogenase (*11βHSD1* and *11βHSD2*). But other enzymes can increase cortisol availability, such as steroidogenic acute regulatory protein (*STAR*), a key factor in steroidogenesis mediating the transfer of cholesterol from the outer to the inner mitochondrial membrane.

By contrast, cortisol can be inactivated by other enzymes, for example steroid 5-α reductase (*5αR*), an A-ring reductase that enhances cortisol clearance in peripheral tissues, mainly the liver [41–43]. In addition, the action of these genes can be regulated upstream by other factors which are crucial for the metabolism of adipose tissue such as *PPARγ*. This gene is an adipocyte-specific nuclear hormone receptor. Agonists of PPAR gamma, such as TZDs, promote adipocyte differentiation and have insulin-sensitizing effects [44]. Moreover, this is well established as *11βHSD1* activity is regulated by *PPARγ*[45], and it is known that some of the beneficial effects of *PPARγ* antidiabetic agents may result, at least in part, from the down-regulation of *11βHSD1* expression in adipose tissue [46]. The study of cortisol, and glucocorticoid receptors in adipose tissue, and their diurnal fluctuations is an important issue in obesity, from a chronobiological point of view, and perhaps could help us to clarify the important associations between stress, chronodisruption, and abdominal obesity.

Brown and White Adipose Tissue: Adipocytes or Stromal-Vascular Cells?

Although when we refer to adipose tissue, we consider it as a "whole", it is important to know that in adult mammals, the major volume of adipose tissue is a loose association of lipid-filled cells called adipocytes, which are held in a structure of collagen fibres. However, apart from adipocytes, adipose tissue contains stromal-vascular cells including fibroblastic connective tissue cells, leukocytes, macrophages, and adipocyte precursor cells, known as pre-adipocytes (not yet filled with lipid). In addition a combination of small blood vessels, connective tissue matrix, nerve tissue, stromovascular cells, and immune cells are also present [25], functioning as an integrated unit. One of the most frequent problems in the study of adipose tissue is the difficulty in assessing from the genes studied which are expressed by adipocytes or by the stromal vascular cells, and technique and histochemical and PCR techniques are helping us to clarify this aspect.

In mammals, mature adipocytes exist as two cytotypes, white and brown adipocytes, which are histologically distinct [47]. Whereas white adipocytes is characterized by

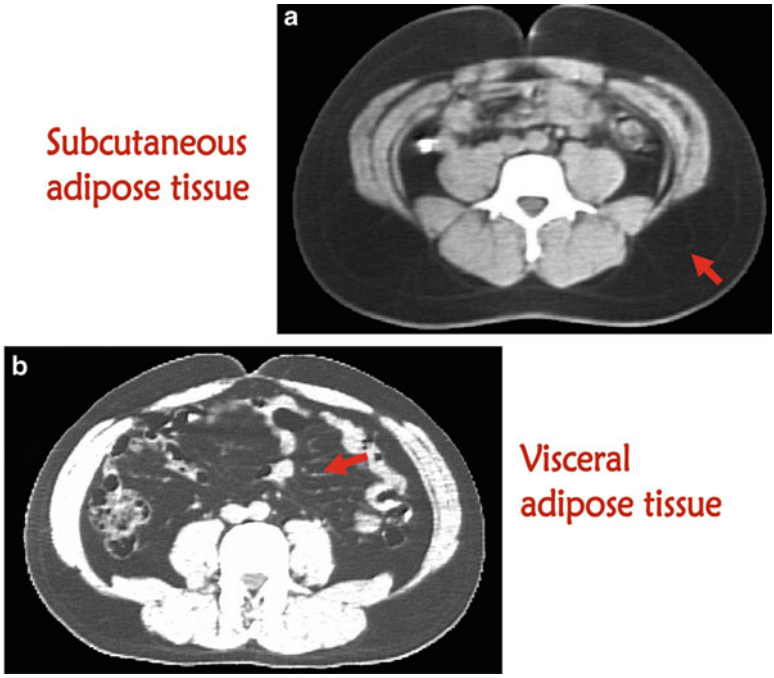

Fig. 3.2 A representation of two computed tomography slides. Subcutaneous fat-type with prominence of subcutaneous AT (**a**). Visceral fat-type: excess fat in the visceral compartment (**b**)

possessing lipids organized within one large and "unilocular" droplet which occupies the majority of intracellular space, compressing the cytoplasm and nucleus into a thin visible rim [48], brown adipocytes are organized into multiple smaller "multilocular" droplets and they are additionally characterized by their high content of large mitochondria packed with cristae within the cytoplasm. Moreover, brown adipocytes are polygonal, have centrally placed nuclei, and are relatively smaller than white adipocytes [49].

In this regard, mammals have two main types of adipose tissue: white adipose tissue (WAT) which contains white adipocytes, and brown adipose tissue (BAT) with brown adipocytes, each of which has different properties. While WAT is specialized in storing energy and is an important endocrine organ involved mainly in the control of weight regulation, the BAT is the main tissue regulating thermogenesis in response to food intake and cold [50]. Particularly, the WAT can be classified, depending on its distribution throughout the body in two major types: subcutaneous adipose tissue (SWAT or SAT) and visceral adipose tissue (VWAT or VAT) also called intra-abdominal adipose tissue (IAAT) (Fig. 3.2). SAT accumulates under the skin (known as peripheral fat mass) whereas VAT is located in the body cavity beneath the abdominal muscles and surrounding the intra-abdominal organs (well known as central fat mass).

Visceral and Subcutaneous, Two Different Adipose Tissues

Several studies have demonstrated that both depots, visceral and subcutaneous, are structural, physiological and metabolically different [51, 52]. Indeed, the type of fat cells or adipocytes, their endocrine function, lipolytic activity and the response to hormones differ between both adipose tissues. From the structural point of view, visceral adipose tissue contains greater number of large adipocytes and higher vascularity and innervation, in contrast to subcutaneous fat that possesses small adipocytes. Moreover, there are regional variations in density, affinity, and signal transduction of several adipose tissue receptors. VAT shows elevated concentrations of glucocorticoid [53] and androgen receptors [54], whereas oestrogen has greater binding capacity in SAT [55]. Respect to adrenergic receptors, visceral fat presents an increased β_3-adrenoreceptor and α_2-adrenergic receptor sensitivity to catecholamine stimulation compared with subcutaneous depot [56]. On the other hand, attending to physiological and metabolic differences, adipocytes from visceral adipose tissue are insulin-resistant, whereas adipocytes from SAT become more insulin-sensitive [57]. In general, visceral fat cells are metabolically more active than subcutaneous adipocytes, being hyperlipolytics and characterized to have higher rate of insulin-stimulated glucose uptake in contrast to SAT adipocytes which are more avid in absorption of circulating free fatty acids (FFAs) and triglycerides (TGs), preventing their deposition in non-adipose tissue [58, 59].

All these functional differences may be related to their anatomical location [60] and various physiological, psychosocial and clinical factors influence the amount and distribution of the adipose tissue throughout the human body, including sex, age, ethnicity, diet physical activity, hormone levels, among others [52]. Indeed, visceral fat accumulation has been associated to cause impaired glucose metabolism, elevated blood pressure, and dislipidemia, and therefore it is considered to be a key player in the metabolic syndrome (MetS) [61]. Moreover, this body composition phenotype is linked to other pathological conditions including several malignancies including prostate and colorectal cancers [62, 63]. On the other hand, peripheral fat mass is negatively correlated with atherogenic risk factors and improvement cardiovascular risk profile [60].

The "When" in the Adipose Tissue: A Peripheral Clock

Introduction

In recent years, numerous studies have provided the molecular mechanisms governing the regulation of circadian rhythms by neurons in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus in the brain, the master circadian pacemaker [64]. In its simplest form, the molecular clock work consists of autoregulatory transcriptional and translational feedback loops that have both positive and negative

elements, the *clock genes* [65]. These genes, orchestrated by the SCN in due time, are necessary for creating and sustaining rhythms of 24 h. In addition, posttranslational mechanisms such as protein phosphorylation, affect stabilization, degradation, and subcellular localization of clock proteins, thus contributing to the molecular clockwork [66, 67]. However, one of the most interesting outcomes in the chronobiological field has been the discovery of different peripheral clocks in many tissues and organs [68]. Peripheral clocks appear to be regulating rhythmicity of at least 10% of the expressed genes within each tissue, and their finding has revolutionized our understanding of how circadian and metabolic networks overlap to regulate physiologic processes in the whole organism [69].

In this context, the current scientific literature is replete with investigations providing a revolution in the study of adipose tissue biology. Many genes in adipose tissue show circadian rhythmicity [70]. Microarrays studies have documented that approximately 25% in humans and 50% in animal models of active genes expressed in adipose tissue follow a daily rhythmic pattern [71, 72] and depending of the tissue, between 10 and 30% of the total genes is under the control of the circadian molecular clock [73]. Thus, in a study, performed in human blood plasma and saliva, it has been demonstrated that approximately 15% of all identified metabolites are under circadian control and this strong effect of the endogenous circadian clock on multiple human metabolic pathways was independent of sleep or feeding [74].

The Importance of "Time" in Metabolism of Adipose Tissue

Food Intake and Lipogenesis and Lipogenetic Processes

All discoveries discussed above are clear evidences that in the metabolism of the adipose tissue it is important not only to understand the "what" and the "how" but also the "when" of the metabolic processes. Moreover, since the antagonism of many of the metabolic processes that occur in adipose tissue, it is expected that not all occur simultaneously. For example, mammals show alternating cycles of *lipogenesis and lipolysis*. Specifically in humans during the night (low activity period), there is a predominance of lipolytic activity, responsible of the body fat utilization, which reduces the frequency of hunger signals and, in consequence, reduces the need for food. In contrast, during the day, l*ipogenesis* predominates, in order to fulfill energy needs during the activity period [75]. Indeed, several studies performed in metabolome and proteosome, point out to the presence of circadian rhythmicity in approximately 75% of the total lipids having their acrophases early in the morning and at 12 h, around the time of food intake [74].

Taking in consideration all these metabolic processes, we can suggest that time is important in adipose tissue and this tissue must be synchronized with different organs and tissues implicated in the food intake processes in order to be able to accumulate fat or mobilize it in the proper time. For that reason we can speculate that in adipose tissue, the genes implicated in these processes display also circadian

rhythmicity in their expression and temporally are very precisely organized. In this sense, Gimble et al. in a study performed in humans have described that if a meal occurs out of phase with lipoprotein lipase (LPL) expression levels, an adipocyte-derived circulating enzyme (responsible for clearing circulating triglycerides), the individual may be prone to store circulating FFA in ectopic tissues, producing lipotoxicity and as a consequence hepatic, muscular or pancreatic comorbidities and MetS [76]. For this reason the circadian deregulation correlates with increased risk of obesity and its comorbidities as cardiovascular disease, diabetes and insulin resistance among other.

Circadian Rhythmicity in Adipokines

Other evidence that suggest a close relationship between circadian rhythms and adipose biology could be the fact that 24 h rhythms have been reported in the plasmatic concentration of leptin and adiponectin in humans [77, 78], being both adipokines. For example, leptin plasma levels during day are variables [77]. Normally, its peak is coinciding with the inactivity phase. Thus, in diurnal animals, such as humans, plasmatic leptin is high during night, when appetite decreases, and low during the day, when hunger increases [79]. However, in nocturnal animals, such rodents, its peak is during the early to mid-light phase [80]. With respect to adiponectin, Gavrila et al. showed that the 24-h variations of serum adiponectin was nearly identical and followed those of cortisol, but were out-of-phase with leptin diurnal rhythms in healthy men [78].

Circadian clocks have been shown to be present in adipose tissue of experimental animals [81] revealing rhythmic expression of clock and adipokines genes, such as resistin, visfatin, and adiponectin [82]. Moreover, diurnal variations in the sensitivity of adipose tissue to adrenaline-induced lipolysis persist ex vivo, suggesting that the intrinsic nature of the adipocyte exhibits a diurnal variation [83]. There are multiple works that show how circadian clock regulates metabolism in the adipose tissue [84].

Adipocytes Differentiation and Adipogenesis

It is well known the implication of clock genes in the adipocytes differentiation as well as in the control of adipogenesis and lipid metabolism [85, 86]. In this sense it has been crucial the availability of genetic models of circadian disruption because they have provided us new opportunities to dissect the interrelationship of circadian and metabolic systems. In fact, experimental studies performed in clock genes knockout mice showed how embryonic fibroblast failed to differentiate into adipocyte, and loss of clock genes led to a significant decrease in adipogenesis and gene expression of some key adipogenic/lipogenic factors [86]. On the contrary, overexpression of clock genes in adipocytes resulted in an increased lipid synthesis activity [86]. Other studies performed in “fat body” of Drosophila showed that *PAR domain*

protein 1 (*Pdp1ε*), equivalent to mammalian *RORα*, modulated a circadian output gene linked to starvation and feeding [87, 88].

Other Key Nutrient Sensors

Additional key nutrient sensors that have been implicated in the cross-talk between circadian rhythms and metabolism are PPARγ and the coactivator PGC1α (PPARγ coactivator). PPARγ is rhythmically expressed and directly regulates *Bmal1* transcription, and mice lacking PPARγ exhibit reduced rhythmicity of clock gene expression.

All these data indicate the importance of circadian rhythms and time in adipose tissue metabolism of experimental animals.

The Adipose Tissue: A Peripheral Clock

One of the most interesting discovers in the last times, related to obesity is the existence of a peripheral clock in human adipose tissue. Thus, it have been recently reported that clock genes are expressed in both human subcutaneous and visceral fat at a certain time of day [89] and this expression was sex dependent [90, 91]. But in addition to the basal expression, it has been showed that both negatives (*PER2* and *CRY1*) and positives (*CLOCK* and *BMAL1*) clock genes, showed circadian rhythmicity in its expression and oscillated independently of the suprachiasmatic nucleus in both adipose tissue explants ex vivo for at least two circadian cycles after surgery in morbidly obese women [92, 93]. These findings show the presence of active circadian clock mechanisms in human adipose tissue, being defined as a peripheral circadian oscillator.

Otway et al. conducted the first study to evaluate human adipose tissue circadian gene oscillations in vivo using serial biopsies, documenting that the oscillatory mRNA profile for core circadian genes was independent of body mass index [94]. These data are in contrast to experimental animals as rodents, in whom obesity attenuates circadian genes amplitude [82].

The Clock Control genes

Clock genes in fat tissue are also capable of modulating other genes, the so-called *Clock Control Genes* (*CCG*), which are not directly involved in the clock machinery but are able to induct the expression of many target genes. In this respect our own research group have published that different genes implicated in adipose tissue metabolism display circadian expression [92]. This is the case for *PPARγ*, a nuclear transcriptor factor which stimulates adipocyte differentiation, or circulating levels of certain hormones and cytokines highly related to adipose tissue, including

adiponectin, leptin, tumour necrosis factor-α, interleukin-6 and plasminogen activator inhibitor-1 (PAI-1). Thus, several studies have demonstrated that some of these factors regulated by *PPARγ*have displayed a strong circadian pattern. Among these, adipokine genes as adiponectin (*ADIPOQ*) which displays a protective role against MetS disturbances, and leptin (*LEP*) closely related to the intake control [97, 98]. Although also their receptors, *ADIPOR1* and *ADIPOR2*, and *LEPR* [97, 98]. Other genes which oscillate with a circadian rhythm in culture of adipose tissue, are the cortisol metabolism-related genes (*GR*, *11βHSD1, 11βHSD2, STAR* and *5αR*), highly implicated in food intake and central accumulation of fat [99].

Techniques in the Assessment of a Peripheral Clock in adipose tissue

The study of circadian rhythms in human adipose tissue has several technical limitations. The quantity of fat needed to assess different points increases, together with the ethical reasons make difficult to assess circadian rhythmicity in adipose tissue in normal weight patients, so most of the studies are made in morbid obese patients. Still, there are varied experimental approaches that have been employed to study adipose tissue from the chronobiological point of view (Table 3.1).

(a) *In vivo*

- *Single time point analyses.*
  The easiest analysis is to take a biopsy of adipose tissue and analyze adipose tissue clock gene expression. This can be performed from subcutaneous fat by kneel aspiration, or during surgery where you can also access to visceral fat.

  This was the method initially used by our group, to assess for the first time the expression of clock genes in human adipose tissue and their correlations with metabolic syndrome characteristic. Although these experiments provide useful preliminary data linking regional adipose clock gene expression with metabolic status, they are nonetheless subject to the disadvantage that a single time point analysis does not permit interpretation of temporal changes in clock genes expression.
- *Serial sampling of biopsies*
  Two studies have now described temporal changes in gene expression using serial subcutaneous adipose biopsies. In the first of these studies, three biopsies were collected in the morning, afternoon and evening from individuals; using array analysis it was estimated that approximately 25% of the human adipose transcriptome undergoes diurnal regulation [71].

  In a subsequent study, diurnal gene expression in individuals who were lean, mildly obese or obese with type 2 diabetes has been analyzed [94]. Biopsies were then collected every 6-h across a 24-h period from the upper buttock region, containing metabolically active adipose tissue [100]. Robust rhythms in all clock genes measured were observed and also in genes that

Table 3.1 Techniques in human adipose tissue circadian rhythmicity

Method	Advantage	Disadvantage
(a) *In vivo techniques*		
Single time point analyses	Study of both subcutaneous and visceral adipose tissue in vivo	Difficult interpretation of temporal changes in clock genes expression
	Provide useful preliminary data linking regional adipose clock with metabolic status	
Serial sampling of biopsies	Analyses of in vivo rhythms	Difficult to check if the clock is independent of the SCN
	Useful as a general marker for human metabolic rhythms	Difficult to determine the circadian pattern because sampling is in general limited to two or three samplings
		Cannot compare subcutaneous and visceral fat
(b) *In vitro techniques*		
Adiopocyte cells	Study of both subcutaneous and visceral adipose tissue	Does not reflect in vivo state of cells
	Identification of endogenous adipose rhythms	
Adipose tissue explants	Study of both subcutaneous and visceral adipose tissue	Limited tissue/sampling resolution
	Identification of endogenous adipose rhythms	Difficult to relate results to in vivo physiology
(c) *Other techniques*		
Bioluminescence imaging of cell culture	Study of both subcutaneous and visceral adipose tissue	Need living cells
	Identification of endogenous adipose rhythms without sampling at different times of day	Can be done in a whole animal but not in human
	Report high temporal resolution from a small amount of tissue	

Table adapted from Johnston JD [115]

have been linked to both circadian and metabolic function. Surprisingly, and in contrast to data from a similar mouse experiment [82], no significant differences in gene expression between our three experimental groups were found.

These studies are rather important particularly to assess potential differences between obese and normal-weight patients. However, these studies cannot answer the question about the existence of a peripheral clock in adipose tissue taking into account that circadian variability could be related to the influence of the SCN, not to the presence of a peripheral clock in the adipose tissue.

Moreover, it is difficult to determine the circadian pattern because sampling is in general limited to two or three samplings.

A third limitation is that we cannot compare subcutaneous and visceral fat.

(b) *In vitro techniques*

In vitro techniques are useful to assess the presence of a peripheral clock, because if rhythmicity persists in vitro, it may indicate the presence of a peripheral clock in the tissue, which is isolated to the SCN influence. These studies can be performed in:

- *Adipocyte cells*
- Authors have studied rhythms in human adipocyte cells differentiated from adipose-derived stem cells (ASCs) [96]. The disadvantage of this procedure is that it does not reflect in vivo state of cells.
- *Adipose tissue explants*
- These experiments also allow the study of both adipose depots and in addition the identification of endogenous adipose rhythms. In our group demonstrated the existence of the peripheral clock in human adipocytes by demonstrating the following statements:

1. Adipose tissue expresses clock genes
2. Clock genes fluctuate during 24 h, even outside the body
3. Clock gene expression is related to the protein expression
4. Clock genes may influence the circadian variability of clock control genes (CCG) and outstanding genes in adipose tissue metabolism.

A single biopsy from both subcutaneous and visceral regions is split into explants which are cultured and then collected for analysis, typically at 6-hourly intervals over a 24 h period. However, the technique does also provide some disadvantages. Firstly, temporal resolution of the analysis is limited by the amount of tissue that can be surgically removed by the current laparoscopic techniques. Our own experience shows that at least 12 g of fat are needed of each depot to define the rhythms of 24 h of any gene with six sampling points. This is heightened when the study is conducted in lean subjects, which normally do not have much fat tissue. Secondly, by moving the tissue into an in vitro environment, it is difficult to relate results to in vivo physiology.

Another important consideration when studying adipose tissue is its heterogeneous nature. Most, if not all, of the adipose cell types contain their own endogenous clock. Furthermore, the relative composition of adipose tissue varies depending upon metabolic state; for instance, obesity is characterized by increased macrophage infiltration into the tissue. However, in a recent study [95] we have performed the histological analysis to assess possible macrophages contamination in human explants of adipose tissue from obese subjects. Our results showed that major changes in gene expression were exclusively a consequence of a differential transcription pattern in adipocytes. Despite these data, in most cases is the uncertainty of which cell type(s) are contributing towards the observed rhythmicity.

(c) *Other techniques*

– *Bioluminescence imaging of cell culture*
This technique has proven to be useful for detecting protein-protein interactions, for tracking cells in vivo, and for monitoring the transcriptional and post-transcriptional regulation of specific genes with a high temporal resolution from a small amount of tissue. Recent applications have included longitudinal monitoring of tumour progression *in vivo*, and monitoring circadian rhythms with single-cell resolution [101]. In this sense, our own research group using this technique is developing the protocol to measure the circadian expression of clock genes in human adipocytes without sampling at different times of day.

A Map of Phases in Adipose Tissue

Energy metabolism and circadian systems have evolved together over millions of years to optimize internal coordination among multiple physiological and molecular processes. The adipose tissue is a metabolically active organ presenting a highly rhythmic behaviour [84]. Each cytokine has to be secreted at the right time and order in order to achieve a concerted function. To date, most published studies have been discussed as if organisms showed only one or few circadian rhythms at a time; however, circadian rhythmicity is exhibited by many variables simultaneously, raising the issue of how do the multiple rhythms relate to each other to generate a precise internal temporal order which is relevant to maintain health. Thus, an adequate temporal order in the daily pattern of genes the different cytokines and proteins implicated in adipose tissue metabolism could have important consequences not only in body fat distribution but also in the metabolic alterations associated to obesity.

Therefore, a recent study performed by our group of research has provided an overall view of the internal temporal order of circadian rhythms in human adipose tissue represented in a phase map (Fig. 3.3) [102]. The data included various genes implicated in metabolic processes such as energy intake and expenditure, insulin resistance, adipocyte differentiation, dyslipidemia, and body fat distribution, and indicated that circadian rhythmicity of the genes studied followed a predictable physiological pattern, particularly for subcutaneous depot.

Timing Interconnections Among Different Adipokines

It is well known that feeding is subject to circadian regulation [84]. Indeed, food intake is a major physiological function in animals and must be entrained to the circadian oscillations in food availability [103]. As expected, leptin, anorexigenic hormone, showed its acrophase (maximum expression) during the night (at 0200 h) in adipose tissue, coinciding with works performed in human plasma and other that demonstrate that leptin and other humoral signals are capable of communicating the nutritional state of the organism to the hypothalamic centres that control hunger and

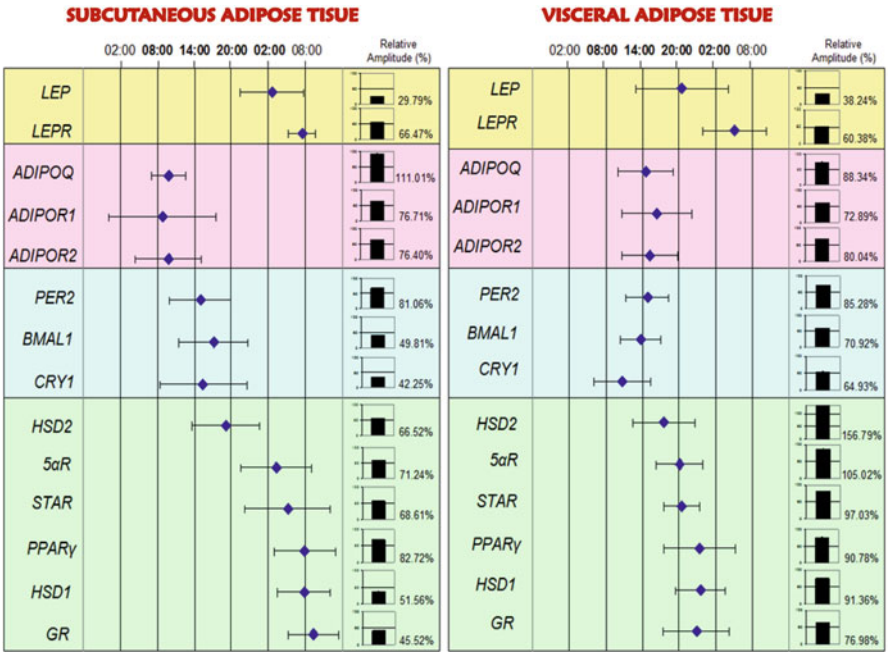

Fig. 3.3 Rhythmic expression of genes studied: (leptin and its receptor (*LEPR*), adiponectin and its receptors (*ADIPOR1* and *ADIPOR2*), clock genes (*PER2, BMAL1,* and *CRY1*) and glucocorticoid metabolism-related genes (*PPARγ, GR, HSD1, HSD2,* and *5αR*)) in human subcutaneous (**a**) and visceral adipose tissue (**b**). Adipose depots were isolated at 6-h intervals over the course of the day from adipose tissue cultures (time at 0, 6, 12, and 18 h). Results are presented relative to the lowest basal relative expression for each gene. Data of relative expression are represented as arbitrary units (AU). Data are reported as means

satiety, in a circadian-dependent manner [79, 104]. The nocturnal increase in leptin levels indicates its role as a satiety hormone, favouring fasting and nocturnal rest.

Adiponectin, the adipose tissue most abundant secreted protein, is highly implicated in glucose metabolism [105]. It has been called the fat-burning molecule because it is able to redirect fatty acids to the muscle for their oxidation. The expression of adiponectin (*ADIPOQ*) achieved its zenith (maximum) during the morning (at 1000 h) which could be implicated in the maximal withdrawal of fatty acids, and the improvement in glucose tolerance and that time [97].

Of note are the relationships between *ADIPOQ, LEPTIN,* and glucocorticoid-related genes circadian profiles and these data in human adipose tissue are consistent with previous findings in serum adiponectin and leptin variations showing out-of-phase 24-h profiles [78]. With respect to cortisol receptor (*GR*), *ADIPOQ* followed similar 24-h rhythmicity. However, although *ADIPOQ* and *GR* reached peak levels around the same time, *ADIPOQ* reached its acrophase 2 h after *GR*. These results are consistent with previous data obtained in plasma from healthy men, and highlight the tightly interactions between AT proteins [78].

PPARγ could be also related to *ADIPOQ* circadian pattern. In fact, the high expression of *PPARγ* during the morning (0800 h), located at the beginning of the of the daily activity, is consistent with results obtained in nocturnal mammals [106] and could be influencing the further increase in *ADIPOQ* expression and the increase in insulin sensitivity during this time of the day.

Other genes studied, *glucocorticoid-related genes such as GR, and the isoenzyme 11β-hydroxysteroid dehydrogenase type 1, (HSD1),* showed their acrophase in the morning (around 0800 h). It has been described that in all species the maximum of corticosteroid rhythms occurs just before or at the onset of activity [107]. In plasma, similarly to what happened in the case performed in adipose tissue, glucocorticoids start to climb from baseline levels about 4–5 h prior to the time of waking, reaching a peak near the time of waking [40]. Over the course of the day they fall, reaching low or undetectable levels an hour or two before bedtime.

It is well known that the corticosteroid rhythm is normally tightly synchronized to the day–night and sleep–wake cycles. The antiphase relationship between leptin and glucocorticoids shown in the current study is reasonable considering that both hormones are strongly interrelated [108] and they exert opposite functions in food intake regulation. While leptin displays an anorexigenic role, glucocorticoids increase appetite (orexigenic function). In fact, in a previous work performed in plasma, leptin ultradian pulses were also inversely correlated with those of ACTH and cortisol [78].

Causation

Regarding the existence of an internal temporal order, an interesting issue concerns causation. Currently, circadian physiologists try to elucidate which genes can be driven by the circadian molecular clock and therefore can be considered as Clock Controlled Genes (CCGs). This is a very difficult question to answer. However, if we observe the clock genes circadian rhythms in the phase map, the advance of phases that suffered *BMAL1* and *CRY1* in visceral with respect to subcutaneous AT, was accompanied with a phase advance in most of the genes studied. Particularly, for *PPARγ* and Glucocorticoid-related genes. Moreover, the phase-relationship of *BMAL1*, and *PPARγ* is maintained in both AT depots. Previously, it has been described that *PPARγ* is a CCG, which is activated by the positive limb, the heterodimmer CLOCK-BMAL1. Moreover, in a study performed in mPer2−/− mice the glucocorticoid rhythms disappeared suggesting that corticosteroids could be considered as CCGs [109].

Differences Between Visceral and Subcutaneous AT

Visceral AT behaved in a different way than subcutaneous for most of the genes studied. Differences were more evident for leptin and glucocorticoids-related genes, both highly related to food intake. The unexpected lower values of leptin and higher

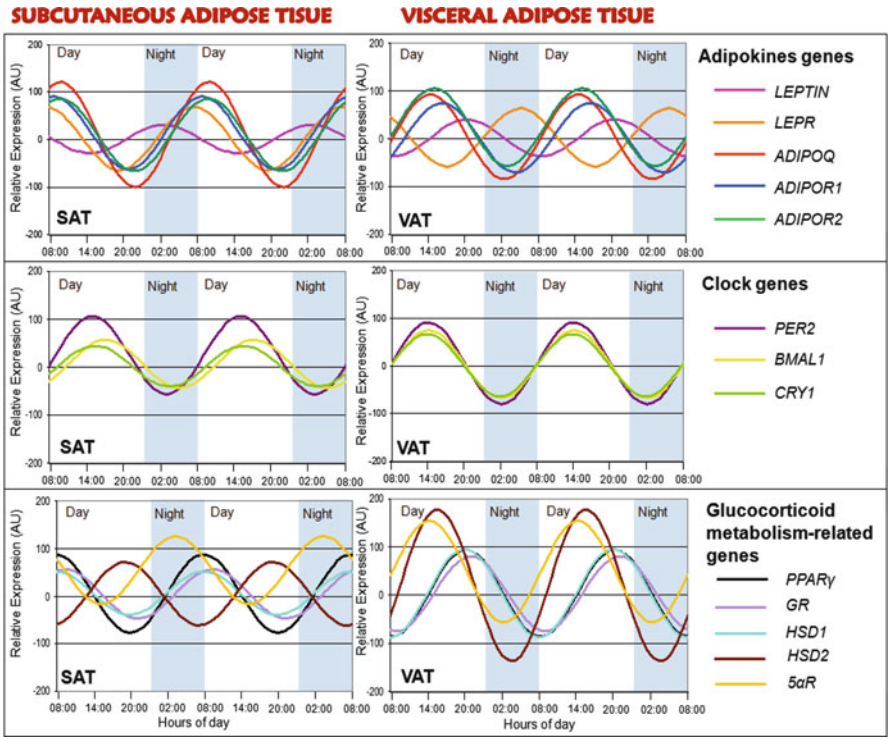

Fig. 3.4 A phase map of circadian rhythms of several genes (leptin and its receptor (*LEPR*), adiponectin and its receptors (*ADIPOR1* and *ADIPOR2*), clock genes (*PER2, BMAL1,* and *CRY1*), and glucocorticoid metabolism-related genes (*PPARγ, GR, HSD1, HSD2, STAR,* and *5αR*)) implicated in human adipose tissue metabolism subcutaneous adipose tissue (**a**), visceral adipose tissue (**b**). This figure shows the acrophase (time of occurrence of the best-fit maximum value) of numerous rhythms. The mean values of acrophases are plotted ± SEM

values of glucocorticoid-related genes during night hours in visceral AT could be accounting for food intake behavioural alterations already described in subjects with a predominance of visceral fat [40]. Night eaters are typically abdominal obese, show anorexia in the morning, hyperphagia in the evening and insomnia at night with frequent awakenings accompanied by food intake [110].

In subcutaneous fat, from the total genes analyzed adiponectin showed the highest circadian rhythmicity, followed by *PPARγ* and *PER2*. For instance, in visceral fat, glucocorticoid-related genes were the genes with the highest amplitude. These data reinforce the particular relevance of adiponectin in subcutaneous and glucocorticoids in visceral fat, already described in previous researches (Fig. 3.4) [105, 111, 112]. In general the relative amplitude of the genes studied was high in this study, as compared previous work carried out in different organs or tissues [113, 114].

Conclusion

For many years, adipose tissue has been considered a single stock of fat tissue. But this idea has completely changed since the last time and today the adipose tissue is defined as a true endocrine organ capable of synthesizing numerous cytokines and other factors that are involved in the metabolism. In addition, the presence of an active circadian clock in adipose tissue depots suggests that there is a temporal component to the regulation of adipose tissue function. Metabolism and maintenance of energy homeostasis require functional coordination among individual adipose depots and other metabolically active tissue sites, to insure proper nutrient/energy flux and substrate use by the organism. Therefore, further investigations of circadian rhythms in adipose tissues will provide insight into the physiology of energy homeostasis and the etiology of metabolic diseases such as obesity.

Summary Points

- Adipose tissue, until recently, considered a single stock of fat tissue, is today defined as a true endocrine organ capable of synthesizing numerous cytokines and other factors that are involved in the metabolism.
- The current scientific literature is replete with investigations providing a revolution in the study of adipose tissue biology. Many genes in adipose tissue show circadian rhythmicity.
- Many of the genes that show circadian rhythmicity in its expression in adipose tissue are involved in important metabolic processes such as energy intake and expenditure, insulin resistance, adipocyte differentiation, dyslipidemia, and body fat distribution.
- An adequate temporal order in the daily pattern of genes the different cytokines and proteins implicated in adipose tissue metabolism could have important consequences not only in body fat distribution but also in the metabolic alterations associated to obesity.

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