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Review

Adipocytes targets and actors in the pathogenesis of HIV-associated lipodystrophy and metabolic alterations

Marie-Lise Gougeon[1], Luc Pénicaud[2], Bernard Fromenty[3], Pascale Leclercq[4], Jean-Paul Viard[5] and Jacqueline Capeau[6]*

[1]Unité d'Immunité Anti-virale, Biothérapie et Vaccins, Département de Medecine Moleculaire, Institut Pasteur, Paris, France
[2]Unite Mixte de Recherche 5018, Centre National de la Recherche Scientifique, University Paul Sabatier, Toulouse, France
[3]INSERM U481, Faculté de Médecine Xavier Bichat, Paris, France
[4]Laboratoire de Bioenergetique Fondamentale et Appliquée, Université Joseph Fourier, Grenoble, France
[5]Service des Maladies Infectieuses et Tropicales, Hôpital Necker Enfants-Malades, Paris, France
[6]INSERM U402, Faculté de Médecine Saint Antoine, UPMC, Paris, France

*Corresponding author: Tel: +33 1 4568 8907; Fax: +33 1 4568 8909; E-mail: mlgougeo@pasteur.fr

The recent clinical use of potent HIV-1 drugs, including nucleoside reverse transcriptase inhibitors (NRTIs) and non-peptidic viral protease inhibitors (PIs), and their combinations, termed highly active antiretroviral therapy (HAART), has dramatically reduced the infection-related mortality of AIDS patients, but it is associated with severe metabolic adverse events such as lipodystrophy syndrome, dyslipidaemia, insulin resistance and diabetes mellitus. The aetiology of this syndrome and metabolic alterations appear to be multifactorial, including HIV drug inhibitory effects on adipocyte differentiation, alteration of mitochondrial functions in adipocytes and altered leptin, adiponectin and cytokine expression in adipose tissue of patients. Adipose tissue may thus be a central regulator in disorganized lipid metabolism and insulin resistance associated with antiretroviral therapy, and we propose in this review to explore how adipose tissue may be a target, but also an actor, in the aetiopathogenesis of the lipodystrophy syndrome.

HIV-associated lipodystrophy syndrome

HIV-associated lipodystrophy, or altered body fat distribution, was originally reported 5 years ago in reports describing wasting of subcutaneous fat in the face and limbs of HIV-infected patients treated with the protease inhibitor (PI) indinavir (IDV), reminiscent of rare congenital and acquired lipodystrophy syndromes [1–4]. Clinical studies have since revealed that long-term use of potent antiretroviral therapy, combining nucleoside reverse transcriptase inhibitor (NRTI) and HIV PI is associated with lipodystrophy (accumulation of visceral fat, 'buffalo hump', breast enlargement, loss of subcutaneous fat in arms, legs and the buttocks) and metabolic alterations, such as elevated serum triglycerides (TG), low-density lipoprotein (LDL)- and very low-density lipoprotein (VLDL)-cholesterol, apolipoprotein B, E and reduced high-density lipoprotein (HDL)-cholesterol, insulin-resistance and diabetes mellitus [4–16]. As underlined in recent reviews [17,18], PI use appears to be more strongly associated with visceral fat accumulation and the metabolic component of the syndrome, characterized by increased levels of TG, TG-rich lipoproteins and insulin resistance, based on the observation that these metabolic changes have been observed after a short-term exposure to PIs in healthy volunteers [19,20], and following introduction of PI therapy in HIV-infected patients [6,13]. NRTIs, on the other hand, while playing a major role in the risk of developing peripheral fat wasting [8,9,21], appear to have relatively less effect on lipid metabolism and insulin sensitivity [6]. HIV infection itself can also play a role in lipid alterations [22]. Glucid and lipid metabolism disturbances are not systematically associated with alterations in fat distribution and are partly independent, although these two aspects are linked in terms of epidemiology and possibly pathophysiology [6]. An important concept that has emerged from clinical trials is that when NRTIs and PIs are combined in highly active antiretroviral therapy (HAART) regimens, their introduction is the most powerful predictor of lipodystrophic body

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composition changes, with a significant increase in relative risk compared with the use of either NRTIs or PIs [15]. Additional factors linked with the development of HIV-associated lipodystrophy have been reported, such as duration of antiretroviral treatment [5,6,15,23], the nadir of CD4 T cells [23], higher age [15,23], elevated C-peptide levels [5], elevated TG [5,6], nutritional status [6] and HCV coinfection [24,25].

Several pathophysiological mechanisms have been proposed to explain the appearance of this syndrome, which are probably associated and could act in synergy to alter adipose tissue. The first hypothetical mechanism, presented by Carr *et al.* [26], suggested that the interference of HIV PIs with lipoprotein receptor-related protein and cytoplasmic retinoid acid binding protein 1, and the cytochrome P450 3A family should induce apoptosis of peripheral adipocytes, leading to increased storage of fat in visceral adipocytes, thereby inducing insulin resistance. However, this hypothesis has not been confirmed, but rather several studies showed that PIs seem to interfere with SREBP-1, a key transcription factor during adipocyte differentiation [27–29]. A second mechanism focused on mitochondrial toxicity of NRTIs on adipocytes and suggested that NRTI-induced inhibition of DNA polymerase-γ leads to depletion of cellular mitochondrial DNA (mtDNA) content through inhibition of mtDNA synthesis [30]. Depletion of mtDNA has been demonstrated in subcutaneous fat samples taken from patients with lipodystrophy [31–34] and some data suggest that NRTIs may alter adipocyte functions independently of their effect on mtDNA. Therefore, even if the extent of peripheral fat loss is not linked to the extent of mtDNA depletion [33,34], a role for NRTI-induced mitochondrial dysfunction in lipoatrophy is highly probable. A third possibility could be the involvement of different cytokines. Indeed, HAART seems to disturb cytokine production in T cells from HIV-infected patients [35], it is associated with altered expression of cytokines in adipose tissue of patients [27,36] and in cultured adipocytes [37]. Since some of these cytokines, such as tumour necrosis factor (TNF)-α, can induce fat loss through apoptosis and insulin resistance, they could play a significant role in lipoatrophy. If both PI and NRTI modify cytokine expression, and possibly mitochondrial function, they could alter fat in synergy. In addition, since TNF-α and interferon (IFN)-α have numerous detrimental effects on lipid homeostasis and insulin sensitivity, and since adiponectin restores insulin sensitivity, HAART-related cytokine over- or under-production may play a significant role in the development of metabolic disorders. Adipose tissue may thus be a central regulator in disorganized lipid metabolism and insulin resistance associated with antiretroviral therapy, and we propose in this review to explore how adipose tissue may be a target, but also an actor, in the aetiopathogenesis of the lipodystrophy syndrome.

Biology of adipose cells and adipose tissues

Differentiation of adipocytes

Until recently, the adipose cell has been considered as having a role of energy storage, but the discovery of adipose tissue secretory capacity has suggested that it may be an important player in numerous physiological functions. Adipocyte develops from a cell of mesodermal origin that can differentiate into chondrocyte, myocyte, osteoblast and preadipocyte [38]. Preadipocyte differentiation has been mainly studied in *in vitro* models of adipogenesis. A summary of the cellular and molecular processes of adipocyte differentiation is depicted in Figure 1 [39]. Proliferation occurs at the stage of adipoblasts or unidentified fibroblastic cells. At growth arrest at the G1/S stage of the cell cycle, commitment to preadipocytes is associated with the emergence of early markers such as type VI collagen (A2COL6) and lipoprotein lipase (LPL). After a phase of clonal expansion (post-confluent mitosis), engagement towards terminal differentiation can be defined by the expression of glycerol-3 phosphate dehydrogenase (GPDH) activity and other late and very late markers, consisting mainly in enzymes necessary for lipogenesis and TG synthesis (glucose transporter Glut4, fatty acid synthase; FAS, acetyl-CoA carboxylase; ACC, aP2), lipolysis (hormone-sensitive lipase; HSL), as well as other proteins that characterize the mature phenotype (adrenergic receptor, adipsin and so on).

Adipocyte differentiation is a highly controlled process, the entire programme being under the dependency of determinant genes through a sequence of transcriptional steps of which the main ones are depicted in Figure 1A [38,40–43]. Within the cascade of transcriptional factors involved, the first to be increased in a transient manner are the CAAT/enhancer binding proteins (C/EBP)-β and -δ. This early event is an important step enabling the distinction between a preadipocyte and a nonadipogenic precursor cell. C/EBP-βδ activity controls the expression of two important factors, peroxisome proliferator activated receptor (PPAR)-γ and C/EBP-α, with PPAR-γ becoming active when heterodimerizing with the retinoic receptor RXR-α. These two factors cross-regulate each other to maintain their gene expression when C/EBP-βδ expression is decreased. PPAR-γ gene expression is also upregulated by SREBP-1c, a member of the sterol regulatory binding proteins (SREBPs) that are known to modulate transcription of genes encoding proteins involved in both cholesterol and fatty acid metabolism. Other factors, such as Id3 and CHOP-10, are inhibitory

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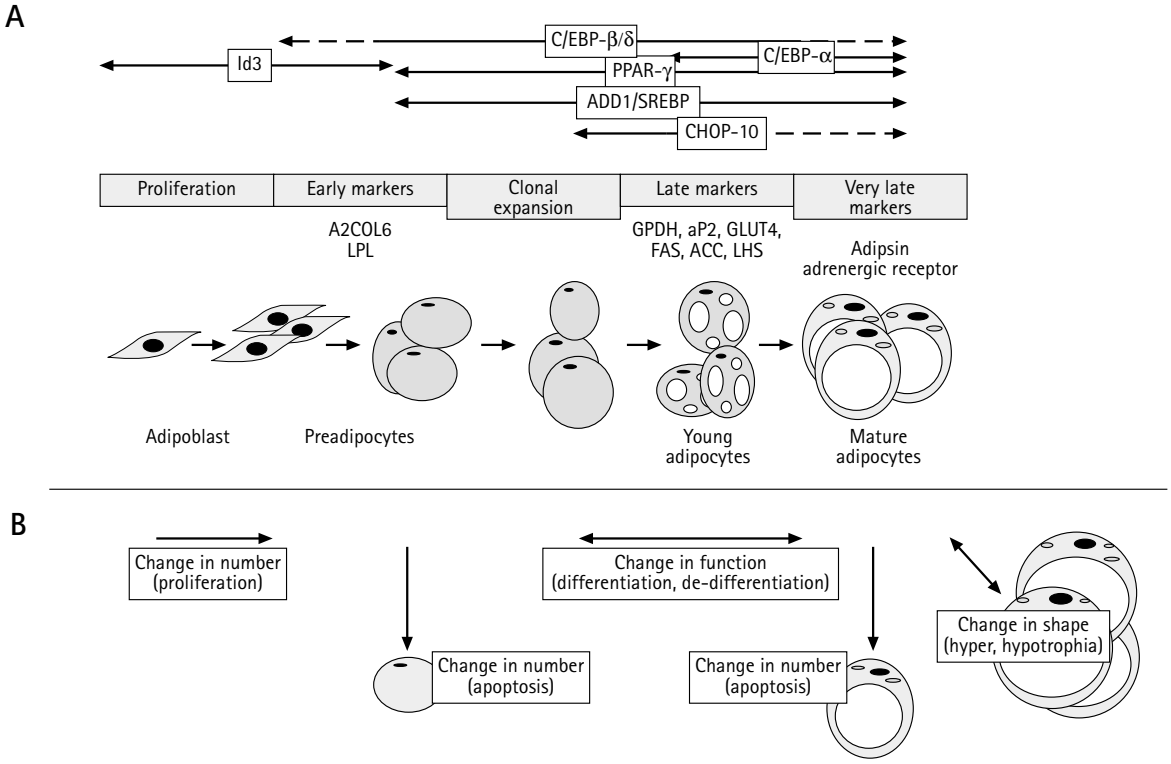
Figure 1. Main events involved in development and plasticity of adipose tissue



(**A**) Adipocyte differentiation mechanism. (**B**) Events in changes in adipocyte number and volume.

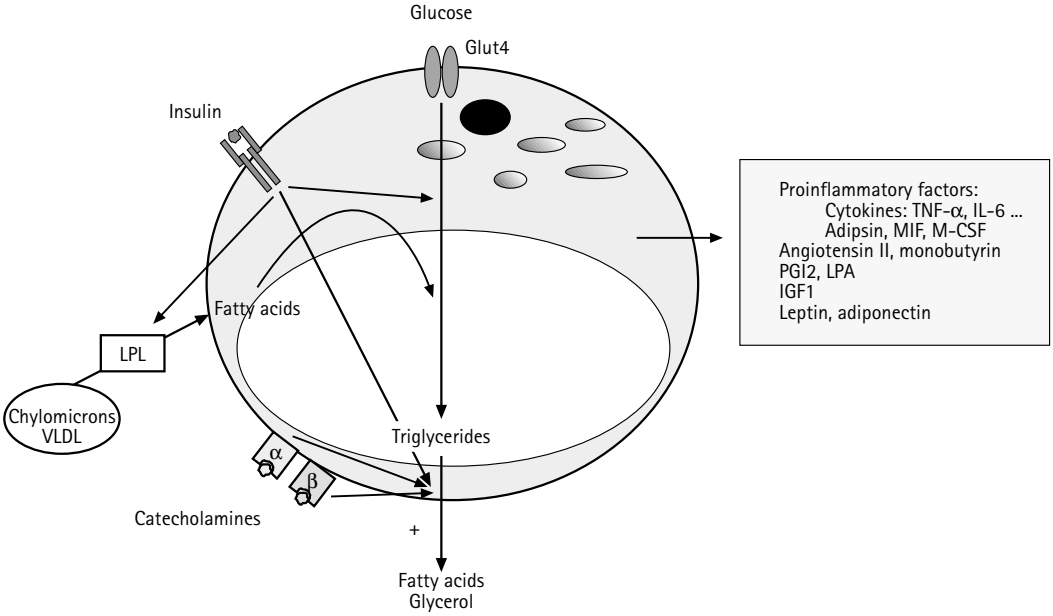
proteins of the differentiation process that maintain the preadipocyte phenotype [44,45]. All of these factors, alone or in combination, control the transcription of many genes encoding proteins involved in the adipocyte phenotype [46].

Functions of adipocytes

Adipocytes are involved in the homeostasis of energy metabolism and they also possess secretory and endocrine functions. The main metabolic pathways of white adipocytes are, on one hand, the synthesis and accumulation of TG and, on the other, their degradation into free fatty acid (FFA) and glycerol (Figure 2) [47]. The increase in lipid stores in adipocytes is achieved by two pathways: 1) the direct uptake of TG associated with lipoproteins coming from the circulation, which are hydrolyzed by LPL into non-esterified (free) fatty acids, the latter being then transported into the adipocyte by a family of fatty acid binding proteins (FABP, FAT, FATP, aP2 and so on); and 2) the lipogenic pathway, that is, the *de novo* synthesis from glucose, which is transported into the adipocyte via Glut4. Glucose allows the synthesis of pyruvate and glycerol-3-phosphate, leading to the synthesis of TG. Pyruvate will be utilized for the formation of acetyl-CoA and its transformation into malonyl-CoA under the control of ACC. The last step is catalyzed by FAS, a multienzyme complex, leading to the formation of long chains of fatty acids. It must be noted that the lipogenic pathway is important in rodents but not in human adipocytes, which mainly use the first pathway. These anabolic pathways are mainly under the control of insulin. The lipolytic pathway is under the control of HSL, which hydrolyzes triacylglycerol into FFA and monoacylglycerol, which is in turn hydrolyzed into glycerol. Catecholamines (noradrenaline and adrenaline) are the main hormones involved in the control of lipolysis [48]. However, catecholamines can exert their effect only if the insulin level is low.

The other functions of adipose tissue are mainly related to the numerous factors that adipocytes synthesize and secrete (Figure 2) [49]. In particular, a strong interplay between cytokines and adipocytes has been suggested by several reports. Indeed, preadipocytes or adipocytes of both peripheral or bone marrow origin synthesize a variety of inflammatory cytokines, among which TNF-α, interleukin (IL)-1 and -6, leukaemia inhibitory factor, macrophage inhibitory factor, IFN-γ, transforming growth factor (TGF)-β, together with adiponectin and leptin [50]. Conversely, some of these factors control adipocyte development and functions. For example, TNF-α and chemokines, such as IL-8, inhibit adipocyte differentiation and enhance leptin secretion. In addition, leptin itself may be directly

Figure 2. Main metabolic and secretory functions of adipocytes



Glut4, glucose transporter 4; LPL, lipoprotein lipase; VLDL, very low-density lipoprotein.

involved in the regulation of immune parameters, since leptin directly activates T cells, enhances the production of oxidative species by neutrophils and induces the secretion of IL-1 receptor antagonist in monocytes. Even more intriguing is the putative role of leptin in the regulation of haematopoiesis. Thus, Laharrague *et al.* [51] demonstrated that bone marrow adipocytes synthesize leptin, which is able to engage haematopoietic stem cells towards the monocytes/macrophages cell lineage. Finally, preadipocytes share numerous characteristics with macrophages [52]. Indeed, both cells are able to phagocyte and kill microorganisms, they express common markers such as MOMA-2 or Mac-1, and deficiency in Mac-1 is associated with murine obesity. Altogether, these data demonstrate a very close relationship between adipose tissues and inflammation, immunity and haematopoiesis.

Adipose tissue heterogeneity and plasticity

An important aspect to consider is the plasticity potential of adipose tissue, that is, its capacity of variations in volume and cell number (Figure 1B). Large alterations in the size of adipocytes (hyper- or hypotrophy) result from changes in metabolic pathways, and reflect anabolic or catabolic conditions, and the extent of these changes might depend on the fat pad localization [53,54]. Variations in the number of adipocytes can be due to the recruitment of new cells, or to the loss of preadipocytes or adipocytes. Proliferation of preadipocytes and differentiation into adipocytes can take place in adult mammals [39], but it decreases with age and depends on the volume of the fat pad, which is under the control of lipogenic factors, such as insulin and glucocorticoids, or lipolytic factors, such as catecholamines, growth hormones and cytokines such as TNF-α. Reduction of adipocyte number can result from preadipocyte and adipocyte apoptosis, as well as adipocyte dedifferentiation [47,55,56]. White adipocytes undergo apoptosis following growth factor deprivation, and *in vitro* treatment with TNF-α increases the cell death process [57], while insulin prevents it [58]. The sensitivity of the adipose cells to these factors, particularly insulin, catecholamines, growth hormone but also TNF-α and IL-6, varies according to the fat depots [59].

Adipocyte as a target of antiretroviral molecules

Due to the complexity of *in vivo* studies and the fact that patients generally receive several classes of antiretroviral drugs, *in vitro* studies have been performed with adipocyte cell lines, generally 3T3L1 or 3T3F442A, in order to analyse the respective influence of individual molecules on adipocyte differentiation and functions. In short-term cultures of adipocytes, a direct inhibitory effect of PIs (such as IDV) on Glut4, when inserted in the plasma membrane, independent of insulin signalling, was reported. This effect was rapid and reversible [60–62]. By contrast, 18 h incubation with nelfinavir (NFV) inhibited insulin signalling in cultured adipocytes [62,63].

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In long-term cultures (several days), different PIs were shown to inhibit adipocyte differentiation in various *in vitro* models (CH3H10T1/2 mesenchymal cells, human adipocytes, murine adipocytes 3T3L1 or 3T3F442A) [64–73]. Their potency differed according to the molecule but also to the presence or not, during the differentiation process, of a strong agonist of PPAR-γ, of the thiazolidinedione (TZD) family. The deleterious effect of some PIs was completely prevented by a TZD, arguing for an impact before the PPAR-γ step while that of others was only partly reversed, arguing for additional alterations after the PPAR-γ step. Interestingly, inhibition of 3T3-L1 adipocyte differentiation and lipogenesis by IDV and ritonavir (RTV) can be amplified by simultaneous treatment with TNF-α [68]. Experiments performed with the 3T3-F442A cell line showed that IDV, NFV and amprenavir (APV) differentially affected adipogenesis with a rank order gradually declining from IDV to NFV and APV [69]. In addition, some PIs induced insulin resistance and adipocyte apoptosis. These deleterious effects were also reversed by a TZD [69].

Interestingly, several studies pointed to SREBP-1 as an altered step in PI-treated adipocytes *in vitro*. Several PIs were found to alter (increase or decrease) the level of the mature form of SREBP-1 in 3T3F442A [69] and 3T3L1 cells [28,29] but in all cases, this resulted in a defective expression of the downstream genes, PPAR-γ, C/EBPa and FAS [28,29,71]. In agreement with these data, IDV was able to inhibit gene activation of two SREBP-1-dependent genes, LPL and FAS in *in vitro* models [72]. An altered cellular location of SREBP-1 induced by some PIs in 3T3F442A adipocytes was revealed with partly impaired nuclear penetration [71,73]. Since SREBP-1 has been shown to interact with the nuclear protein lamin A/C, belonging to lamina [74], the structure and stability of the lamina network was investigated in cultured adipocytes. Some PIs induced an increased nuclear fragility and the presence of dysmorphic nuclei with altered distribution of lamin A/C and lamin B. Those nuclei could not accumulate SREBP-1. PIs also inhibited prelamin A maturation into lamin A. Almost similar nuclear alterations are observed in cells from patients with familial partial lipodystrophy (FPLD), a severe monogenic form of insulin-resistant lipodystrophy due to lamin A/C mutations [75], and in the mouse model of lipodystrophy with defective lamin A/C maturation, the Zmpste24 metalloprotease-deficient mice [76]. Thus, some PIs could impair lamin A/C maturation and induce disruption of nuclear architecture *in vitro* [77]. This could be responsible for SREBP-1 nuclear mislocation and thereby adipocyte dysfunction.

Taken as a whole, different PIs were shown to affect *in vitro* key adipocyte functions, adipogenesis, sensitivity to insulin and apoptosis with different potencies. Some PIs, such as NFV, saquinavir (SQV) and RTV are deleterious even in the presence of a TZD, which suggests that they could act, at least in part, on steps located after PPAR-γ. Conversely, other PIs such as IDV have minimal effects in the presence of a TZD, but are deleterious when adipocytes are differentiated in its absence, suggesting that they are acting mainly at steps prior to PPAR-γ, probably SREBP-1. In addition to its role in adipocyte differentiation and sensitivity to insulin, SREBP-1 plays a major role in hepatic lipogenesis. The effect of RTV was evaluated in liver and white adipose tissue (WAT) in mice, and it appeared to be different [78]. This observation confirmed that the regulation of the expression of SREBP-1 and of its nuclear protein level are different in these tissues [79].

Very few studies evaluated the effects of NRTIs on adipocytes. *in vitro*, Dowell *et al.* observed that stavudine (d4T) alone, or in combination with IDV, RTV or SQV, did not alter lipid accumulation in 3T3-L1 adipocytes [28]. By contrast, using 3T3-F442A adipocytes, Roche *et al.* [70] suggested that NRTIs could interfere with adipocyte differentiation although to a lesser extent than PIs. Interestingly, when PIs and NRTIs were added in different combinations, the resulting effect was different from that elicited by each drug separately. Caron *et al.* [74] observed that chronic exposure of 3T3-F442A adipocytes to d4T or didanosine (ddI) did not alter cell differentiation and insulin sensitivity but moderately impaired lipid accumulation and promoted adipose cell loss. The TZD rosiglitazone almost totally prevented the adverse effects of NRTIs in adipose cell lines, used alone or in association with PIs. This effect has to be confirmed *in vivo*. Therefore, *in vitro*, at clinically relevant concentrations, NRTIs have markedly less adverse effects on adipocyte functions than PIs and have different potencies according to the molecule. This is in line with the NRTI-related clinical lipoatrophy. Moreover, it was observed that the association of PI and NRTI was more deleterious than NRTI alone. This is in agreement with the data observed in HIV-infected patients.

Some studies directly analysed adipose tissue from HIV-infected patients. A higher proportion of small adipocytes was found in subcutaneous abdominal adipose tissue from lipoatrophic patients compared to control fat. Patient fat contained lower mRNA levels of the adipogenic differentiation factors C/EBP-β and -α, PPAR-γ and SREBP-1c. As the differentiation factor SREBP-1 is rapidly targeted by PIs *in vitro*, these observations suggest that decreased SREBP-1c could play a role in peripheral lipoatrophy [27].

Effects of antiretroviral molecules on adipocyte mitochondrial functions

NRTI-induced mtDNA depletion has been shown to be involved in different adverse reactions related to mitochondrial toxicity (such as, myopathy, hepatic steatosis, lactic acidosis) that occur occasionally in HIV-infected patients [30,80,81]. Therefore, it has been hypothesized that, by lowering mtDNA levels in white adipose tissue (WAT), NRTIs play a key role in the pathogenesis of the lipodystrophy [30]. This hypothesis also stemmed from the observation that mtDNA mutations have been detected in patients with inborn mitochondrial diseases presenting abnormal fat distribution [82,83]. There are now several clinical investigations reporting low mtDNA levels and ultrastructural abnormalities of mitochondria in WAT of patients treated with different NRTIs [31–34]. Importantly, one of these studies has found a relationship between mtDNA depletion in subcutaneous fat and lipoatrophy [32], while two others did not [33,34]. The reason for the apparent lack of association between mtDNA depletion and lipoatrophy in these studies is not yet clear but it may be due to their cross-sectional design [33,34]. Alternatively, mtDNA depletion in WAT may not be the sole mechanism whereby NRTIs favour lipoatrophy.

Before considering this hypothesis, it is important to bear in mind that mtDNA depletion does not necessarily lead to the impairment of oxidative phosphorylation (OXPHOS), the process that couples, within the inner mitochondrial membrane, oxidation of substrates and phosphorylation of ADP into ATP. Indeed, electron transport along the respiratory chain and ATP synthesis are impaired whenever mtDNA content falls below a certain threshold for a prolonged period of time [84,85]. In liver (a tissue that greatly relies on mitochondria for ATP synthesis), it is estimated that mtDNA must be severely depleted (to less than 20–30% of residual mtDNA) to impair mitochondrial respiration [86]. In the study reporting a relationship between mtDNA depletion and lipoatrophy in HIV-infected patients, mtDNA levels in subcutaneous fat was decreased by only 36 and 43% when compared to patients without fat wasting and HIV-negative controls, respectively [32]. Clearly, further studies are needed to determine the threshold of WAT mtDNA content below which OXPHOS and other key mitochondrial functions are significantly impaired.

There is evidence suggesting that NRTIs can induce mitochondrial and cellular dysfunction through mechanism(s) not involving inhibition of the mtDNA polymerase-γ, and subsequent mtDNA depletion. For instance, normal mtDNA levels have been found in individuals treated (or exposed) to NRTIs who presented features of mitochondrial dysfunction and diseases, including lactic acidosis and lipodystrophy [87–90]. Besides mtDNA depletion, NRTIs could induce the following mitochondrial abnormalities: 1) oxidative damage to mtDNA including accumulation of the oxidized base 8-hydroxydeoxyguanosine [91,92]; 2) heteroplasmic mtDNA point mutations [93] and multiple mtDNA deletions [88,89]; and 3) direct inhibition of mitochondrial respiration [94], possibly through the inhibition of ADP/ATP translocase, a key OXPHOS enzyme allowing ADP entry within mitochondria [95].

Recently, investigations in mice have brought additional evidence that NRTIs can modify fatty acid and lipid metabolism independently to mtDNA depletion. Indeed, zidovudine (AZT) and d4T (but not ddI, zalcitabine and lamivudine) were found to increase mitochondrial β-oxidation of fatty acids in liver and plasma ketone bodies [86,96,97]. Interestingly, effects of the thymidine analogues (that is, AZT and d4T) were reproduced in mice by the administration of a thymine catabolite, β-aminoisobutyric acid, which is found in significant amount in plasma of d4T-treated primates [86]. Since β-aminoisobutyric acid was also able to reduce body fat mass in mice [96], further investigations are warranted in order to determine if this thymine catabolite plays a role in the syndrome of lipoatrophy occurring in patients treated with AZT and d4T.

Whatever its origin (that is, primary or secondary), mitochondrial dysfunction can trigger some adaptive responses in order to offset OXPHOS deficiency [84]. In patients with inborn mitochondrial diseases, proliferation of mitochondria seems to be a key factor of this metabolic adaptation [98]. In patients with mitochondrial dysfunction related to NRTI therapy this physiological adaptive response seems to vary greatly depending of the affected tissues. Indeed, mitochondrial proliferation was virtually absent in muscle of patients with myopathy [98], whereas it was observed in adipose tissue of patients with lipoatrophy [33,99]. Adipocytes can also respond swiftly to OXPHOS inhibitors by an increased glucose uptake and glycolysis, thus allowing efficient recovery of cellular ATP [100]. These observations suggest that in patients treated by NRTIs, adipocytes could be able to develop some adaptive responses in order to limit ATP shortage and subsequent cellular dysfunction. However, if these compensatory responses become insufficient, cellular dysfunction can ensue. Possible consequences of OXPHOS deficiency in adipocytes could be the reduction of fatty acid synthesis [101], or a decrease in fatty acid oxidation and apoptosis, if one assumes that adipocytes behave like other cells (such as hepatocytes) facing mitochondrial dysfunction [102]. However, data

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regarding this major issue are still lacking and more investigations are required to determine the full repertoire of events occurring when adipose cells are facing severe mitochondrial dysfunction and energy shortage.

Factors other than NRTIs may induce mitochondrial dysfunction in WAT. In patients with HAART-associated lipodystrophy, the following factors may damage mitochondria or impair some key mitochondrial function in the adipose cells: 1) TNF-α, which is able to impair mitochondrial function and induce mtDNA damage. 2) Drugs other than NRTIs such as PIs. RTV was recently shown to induce mtDNA damage in human endothelial cells [103]. It would therefore be important to determine whether PIs can affect mtDNA integrity in adipose tissue. 3) Diabetes that can be associated in different tissues with various mtDNA damages including 8-hydroxydeoxyguanosine, multiple mtDNA deletions and mtDNA depletion [104–106]. It is noteworthy that genetically obese (ob/ob) mice (a model of obesity and type 2 diabetes) have low basal levels of mtDNA in WAT compared either with liver and skeletal muscles, or with WAT from lean (Swiss) mice [97]. Interestingly, d4T administered for 6 weeks to Swiss mice did not deplete mtDNA in WAT but a similar d4T treatment administered to ob/ob mice induced a reduction of mtDNA levels by 45% in epididymal WAT [97]. These observations in ob/ob mice suggest firstly that NRTI-induced mtDNA depletion may occur in fat cells harbouring low mtDNA levels prior to NRTI exposure, and secondly that metabolic factors such as diabetes could favour NRTI-induced mitochondrial dysfunction, at least in some tissues.

Although mtDNA depletion in WAT has been documented in patients treated with different NRTIs including d4T and AZT, *in vitro* studies on cultured adipose cells have given results that are at variance with clinical investigations. Indeed, experiments carried out in 3T3-L1 adipocytes showed that d4T up to 100 μM had no effect on mtDNA content even after 20 days of culture [107]. Higher concentration (200 μM) and prolonged exposure (20 days) of d4T were needed in this model to induce a severe reduction of mtDNA levels. In contrast, studies with other cell lines have shown that 10 μM d4T for 4 days and 36 μM d4T for 25 days reduced by half mtDNA content in CEM cells [108] and HepG2 hepatoma cells [109], respectively. Thus, when compared to other cells, cultured adipocytes seem to be relatively insensitive to the detrimental effect of NRTIs on mtDNA replication. The reasons for this relative insensitiveness is unknown but several hypotheses can be put forward: 1) murine cells (3T3-L1) may differ from human cells (CEM and HepG2) regarding NRTI metabolism, as recently suggested [86,110]. A recent study in 3T3-L1 and 3T3-F442A murine adipocytes showed that d4T and AZT could be phosphorylated in these cells, though AZT and d4T triphosphates were detected in low amounts [111]. Large species difference may exist regarding the ability of adipose cells to activate NRTIs into their triphosphate derivatives and thus to undergo NRTI-induced mtDNA depletion. 2) Cultured murine adipocytes may harbour more mitochondria and/or more mtDNA copies as compared to human adipocytes and sensitiveness of mtDNA to NRTIs could depend on mtDNA levels existing prior to NRTI exposure [97]. Interestingly, 3T3-L1 differentiation is characterized by intense mitochondrial biogenesis with a 20- to 30-fold increase in the concentration of numerous mitochondrial components [112]. 3) Duration of drug exposure should be considered, since several days and weeks of *in vitro* NRTI treatment may not be sufficient to induce significant mtDNA depletion. 4) *In vitro* studies aimed at studying the toxicity of the antiretroviral agents on cultured adipocytes do not evaluate the role of other factors that may also induce (or potentiate) mitochondrial dysfunction *in vivo*.

An interesting hypothesis has been proposed by Kreier *et al.* following the observation that adipose tissue is innervated by sympathetic and parasympathetic nerves resulting, when activated, in respectively decreased and increased fat accumulation. In the hypothalamic area, a strict somatotopy exists for both the sympathetic and parasympathetic nuclei with separate sets of neurons projected towards subcutaneous and intra-abdominal fat [113]. These authors thus speculate that the actual maldistribution of fat seen in HAART-treated patients results from a selective autonomic neuropathy. Interestingly, the regions of the brainstem and hypothalamus, which have been identified to be specifically involved in regulating adipose tissue through the autonomic nervous system, happen to have less of a blood–brain barrier. This not only makes them particularly accessible to natural substances such as hormones derived for instance from adipose tissue, but also to the drugs used for treating HIV. Neurotoxic effects of some of the drugs on discrete sets of neurons could subsequently result in the selective loss of fat in certain parts of the body and fat increase in other parts [114].

Adipocyte and proinflammatory cytokines

Inflammatory cytokines, including TNF-α, IL-1, IL-6 and IFN-α, are inhibitors of preadipocyte differentiation *in vitro* and might play a role in atrophy of adipose tissue and insulin resistance observed in antiretroviral-treated HIV-infected patients. Secreted or membrane-bound TNF-α acts through TNF-α-R1 to inhibit adipogenesis [115], partly through sustained

activation of the ERK pathway. Recently, mapping of early signalling events in TNF-α-mediated lipolysis, performed in human fat cells, showed that it is dependent on the initial activation of members of the MAPK family, mediated by the TNFR1, JNK and p44/42 but not p38 [116]. TNF-α has direct inhibitory effects on insulin receptor signalling by inhibition of autophosphorylation [117]. Exposure of preadipocytes to TNF-α inhibits adipogenesis by blocking induction of PPAR-γ and C/EBP-α [118], and TNF-α treatment of adipocytes downregulates the expression of several genes associated with glucose and lipid metabolism, such as, Glut4, LPL and the atherogenic protein plasminogen activator inhibitor-1 [119].

Elevated expression of TNF-α may participate to mitochondrial dysfunction. Indeed, TNF-α is known to impair the respiratory chain activity and to increase the mitochondrial production of reactive oxygen species (ROS) in different cell lines and tissues [120,121]. These effects are deemed to result from TNF-α binding to its cognate receptor on the cell surface, but a study in cultured human preadipocytes indicated that TNF-α could also be translocated to the mitochondria, where it binds to a 60 kD protein of the inner membrane [122]. Whether the direct interaction of TNF-α with mitochondrial components can lead to mitochondrial dysfunction (including mtDNA damage and depletion) is currently unknown. Interestingly, a recent study in mice overexpressing TNF-α suggested that this cytokine can induce mtDNA damage in heart possibly through an impairment of mtDNA repair capacity and increased ROS generation, but unfortunately other tissues have not been investigated [123].

An altered ability of adipose tissue to secrete adipocytokines has been reported in obese and diabetic patients, and also in lipodystrophic HIV-positive patients and in PI-treated adipocytes. Analyses of fat biopsies taken from lipodystrophic patients under PI and NRTI showed an increased expression of TNF-α and IL-6, while expression of adiponectin and leptin was decreased [27,36,124–126], and IL-6 secretion from abdominal subcutaneous adipose tissue and serum IL-6 were positively associated with visceral fat and negatively associated with the relative amount of lower limb adipose tissue [126]. Otherwise, Jones reported that some PIs and NRTIs were able to increase TNF-α and decrease adiponectin expression in adipocyte cell models [37]. This suggests that ART could play a role in altered expression of adipocytokines observed in patient fat.

An increased level of apoptosis was reported in subcutaneous adipose tissue from patients under HAART including PIs and NRTIs, in favour of a process of adipocyte loss in patient fat [127]. Fat cell apoptosis persisted when patients were switched from PI to nevirapine [128], but when d4T was switched to AZT, apoptosis was reduced although remaining higher than in controls [129]. These data argue for a role of ART in adipocyte apoptosis, which could result from both ART-induced cytokine overexpression and mitochondrial dysfunction. Accordingly, a relation between the extent of apoptosis and the mRNA expression of TNF-α and IL-6 in the adipose tissue of lipodystrophic HIV-infected patients was observed, and also between apoptosis, the expression of the mitochondrial gene COX-2 and the transcription factor PGC1-α [36]. Moreover, a polymorphism G/A at position –238 in the TNF-α gene has been reported to be linked to the prevalence of lipodystrophy in these patients [130], further arguing for a role for TNF-α in the lipodystrophic phenotype. Interestingly, the prevalence of the same TNF-α polymorphism was found to be higher in patients with nonalcoholic fatty liver [131], a condition that can be associated with insulin resistance, type 2 diabetes mellitus and the metabolic syndrome.

A dysregulation in homeostasis of TNF-α T-cell producers has been observed in HIV-positive patients under HAART, and particularly in patients with lipodystrophy. Indeed, we reported that introduction of HAART leads to the progressive accumulation of CD4 and CD8 T cells polarized to TNF-α synthesis, and positive correlations were found between the absolute number of TNF-α CD8 T cell precursors and lipid parameters usually altered in lipodystrophy, including TG, cholesterol and the atherogenic ratio apoB/Apo-A1 [35]. The accumulation of T cells primed for TNF-α is related to their escape from activation-induced apoptosis. Apoptosis plays an important role in the physiological regulation of TNF-α synthesis. The anti-inflammatory function of apoptosis has been reported at the macrophage level [132] and it is exploited by bacterial pathogens to suppress TNF-α production [133]. Combined detection at the single cell level of cytokine synthesis and apoptosis on lymphocytes from untreated patients or healthy donors suggests that apoptosis plays an essential role in the negative regulation of TNF-α synthesis by T cells. Indeed, TNF producers are highly sensitive to apoptosis, and this is partly related to the decreased expression of Bcl-2 molecule [134]. During the natural progression of HIV infection, susceptibility of TNF-α producers to apoptosis progressively increases, and this is correlated to their decreased representation in blood samples from untreated patients [134]. Under HAART, apoptosis in TNF-α producers is highly suppressed and leads to their progressive accumulation in the blood [35]. Suppression of apoptosis under HAART is likely to be a multifactorial process, including decreased *in vivo* immune activation, decreased production of pro-apoptotic HIV proteins,

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increased expression of survival factors, such as Bcl-2, related to restoration of IL-2 synthesis [35,135]. In addition, a direct effect of PIs may be involved since some PIs suppress *in vitro* physiological apoptosis in normal T cells by inhibiting cellular caspase activation [136,137].

Interferons are a class of proteins involved in natural defence, which act by inducing resistance to viral infections and by modulating the immune response [138]. Increased plasma concentrations of IFN-α have been documented in several viral infections including HIV disease [139,140]. IFN-α has major metabolic effects, particularly by regulating lipid metabolism. This is suggested by the following observations: 1) it stimulates hepatic lipogenesis by increasing hepatic *de novo* fatty acids synthesis and VLDL production [141–143]; 2) it decreases adipose tissue LPL activity [144]; 3) it increases lipolysis through a direct effect on fat cells [144] and/or an indirect effect via changes in hormones such as cortisol, noradrenaline and glucagon [145]; and 4) it decreases the clearance of TG [146]. Patients on HAART show increased serum IFN-α concentration compared to controls and lipodystrophy-negative men [147], and longitudinal evolution of patients with lipodystrophy is correlated with modifications in serum IFN-α concentration [148].

Taken as a whole, these data suggest that pathogenesis of peripheral lipoatrophy could result from mitochondrial dysfunction, altered adipocyte differentiation and sensitivity to insulin and altered cytokines expression, most of these alterations possibly resulting from synergistic effects of PIs and NRTIs on adipose tissue.

Adipocyte as an actor of insulin resistance: the role of FFA and adipocytokines

Insulin resistance appears as an important side effect of antiretroviral treatments. It occurs very rapidly with some molecules and it is also present in long-term treatments with HAART including PIs and NRTIs. Several recent studies have clearly revealed that IDV can induce insulin resistance in short-term conditions: in accordance with the direct inhibition of the Glut4 transporter by IDV *in vitro* in a few minutes [61], mice infused with IDV presented an acute and reversible state of insulin resistance at the muscle but not the liver level [149]. Similarly, in control subjects, a single dose of IDV acutely decreased insulin-stimulated glucose disposal, indicating muscle insulin resistance [150]. Moreover, the basal level of FFA and their suppression by insulin were normal, indicating that under these acute conditions there is no resistance at the adipose tissue level.

Interestingly, in patients with HAART-induced lipodystrophy treated for a long time with a combination of PIs and NRTIs, the level of insulin resistance was correlated both with increased abdominal and decreased peripheral fat [151,152]. In all studies, visceral fat accumulation was strongly linked to insulin resistance [153,154]. In addition, several studies have shown that peripheral lipoatrophy was also linked to insulin resistance [151,155,156]. This is in accordance with the dramatic insulin resistance observed in patients with genetic forms of complete lipoatrophy, as observed in the Beradinelli-Seip syndrome [1]. A state of insulin resistance at the adipose tissue level is suggested by an increased lipolysis with decreased suppression of FFA level by insulin [157,158]. The level of fasted FFA was found negatively correlated with subcutaneous fat and positively with visceral fat areas [157]. In these patients, when FFA level was reduced by acipimox, a potent inhibitor of lipolysis, insulin sensitivity was significantly increased [159]. This indicates that lipodystrophic adipose tissue is insulin resistant and plays a role in the insulin resistant state observed in patients treated with both PI and NRTI. A study performed on lipoatrophic adipose tissue from HIV-infected patients showed that the reduced expression of SREBP-1c correlates negatively with glycaemia and insulin resistance, but not with lipid parameters, further suggesting the role played by adipose tissue dysfunction in metabolic alterations [27].

Adipocyte has recently emerged as a major tissue involved in the regulation of whole body insulin sensitivity. Among the adipocytokines, leptin and adiponectin acting through endocrine mechanisms are able to increase insulin sensitivity, while TNF-α and IL-6 through autocrine/paracrine mechanisms are associated with insulin resistance. By the lipolytic process, adipocytes also secrete fatty acids, which are used by different organs in preference to glucose for energy production. At the muscle level, FFA induce insulin resistance by altering proximal steps in the insulin signalling pathway, thereby resulting in decreased Glut4 translocation [160]. At the liver level, FFA oxidation activates gluconeogenesis, leading to increased hepatic glucose production. In this situation, to maintain normoglycaemia, the secretion of insulin is stimulated. This could be pathological when lipolysis is activated, leading to a marked increase in circulating FFA. This is the case for patients with increased abdominal fat, as observed in the insulin resistance or metabolic syndrome, in most cases of type 2 diabetes and in HIV-infected patients with lipodystrophy.

The insulin-resistant state induced by increased circulating FFA and TG levels in muscles is associated with an increased storage of lipids at the intramyocellular level. An increased lipid level in the muscles of HIV-infected patients with lipodystrophy has been reported [161–163], muscle lipid content being correlated with insulin resistance. Similarly, at the liver

level, steatosis with increased lipid content is related to insulin resistance and metabolic alterations in lipodystrophic HIV-infected patients [164,165].

In addition to increased FFA levels, altered levels of adipocytokines could play a major role in muscle and liver insulin resistance, with an emphasis on adiponectin and TNF-α. The role of adiponectin and possibly leptin in peripheral insulin sensitivity has been recently approached in animal models by elegant studies on AMP kinase (AMPK) [166]. This enzyme plays an important role in muscle and liver in lipid and glucose metabolism. Activated by AMP and/or phosphorylation, it can phosphorylate and thereby inactivate the enzyme acetyl-CoA carboxylase, which synthesizes malonyl-CoA from acetyl-CoA. Malonyl-CoA is a strong inhibitor of CPT1, the enzyme required to enter long chain acetyl-CoA into the mitochondrion, a step that is necessary for their oxidation. If malonyl-CoA is present, fatty acid oxidation is impaired. When FFA are increased, the acetyl-CoA derivatives will accumulate inside the cell as intracellular lipids and induce insulin resistance. This deleterious process can be prevented by AMPK. It has recently been shown that AMPK is indeed activated by adiponectin at the muscle and liver level in mice [166]. In addition, AMPK increases glucose uptake inside the muscle cell by recruiting Glut4 transporters to the plasma membrane independently of insulin, and inhibits hepatic glucose production by repressing the expression of gluconeogenic enzymes. A decrease in adiponectin could result in excess lipid content in muscles and liver in mice (as observed in HIV-positive patients), and in insulin resistance. In addition, due to defective AMPK activation, this could result in increased glucose production by the liver and decreased glucose utilization by muscles [167].

A deleterious role for TNF-α in insulin resistance can be inferred from different studies but remains to be definitively demonstrated in humans. Prolonged *in vitro* treatment of adipocytes with TNF-α stimulates lipolysis [168] and thereby upregulates circulating FFA levels, promoting insulin resistance [169]. The involvement of TNF-α in peripheral insulin resistance is suggested by several observations in cell lines, animals and humans: 1) TNF-α regulates the expression of the atherogenic protein plasminogen activator inhibitor-1 [119]; 2) *in vivo* administration of TNF-α-neutralizing antibodies ameliorates insulin resistance in obese rodents [170]; 3) TNF-α gene ablation in mice enhances peripheral insulin sensitivity [171]; and 4) recent studies showed that TZDs inhibit TNF-α-induced lipolysis in murine adipocytes [172], and pretreatment of lean rats with a TZD for 10 days prevents the increase of circulating FFA and ensuing insulin resistance induced by TNF-α infusion [173]. Moreover, a negative relation between TNF-α and adiponectin was shown *in vitro*, TNF-α being able to decrease adiponectin secretion by adipocytes [38,174–176].

The circulating levels of these adipocytokines are altered in patients with lipodystophy [147,148]. Circulating TNFR2 concentrations are increased in HIV lipodystrophic patients and inversely correlated with insulin sensitivity [151], suggesting a role for the TNF-α system in the insulin-resistant state associated with lipodystrophy. In 131 consecutive HIV-infected males under PI-based HAART, insulin sensitivity correlated positively with adiponectin and negatively with leptin and IL-6, and adiponectin, but not leptin, negatively correlated with all metabolic parameters [177]. Insulin resistance, metabolic defects and cardiovascular risk markers were strongly negatively correlated with the adiponectin/leptin ratio (A/L), and positively with sTNFR1. Patients with mixed forms of lipodystrophy presented the most severe metabolic alterations, insulin resistance and A/L decrease [177]. Otherwise, positive correlations were found between the absolute number of TNF-α CD8 T-cell precursors and lipid parameters usually altered in HAART-induced metabolic alterations, including TG, cholesterol and the atherogenic ratio apoB/Apo-A1 [35]. These results suggest that decreased adiponectin and increased activation of the TNF-α system are involved in lipodystrophy, insulin resistance and metabolic alterations in patients under PI-based HAART. Interestingly, IFN-α, the concentration of which is increased in lipodystrophic HIV-infected patients under HAART, could also be involved in metabolic alterations. Indeed, significant correlations were found between serum IFN-α concentrations and increased cholesterol, VLDL-cholesterol and TG, ApoB and atherogenic ApoB/ApoA1 ratio [147,148].

Dyslipidaemia in patients under HAART is thus related to increased production of cytokines involved in lipid metabolism, such as adiponectin, TNF-α and IFN-α, but also to hormonal perturbations, since patients with lipodystrophy show increased serum cortisol levels, decreased levels of DHEA and consequently increased cortisol/DHEA ratio [178]. A positive correlation was found between the cortisol/DHEA ratio and atherogenic lipids [147,148,178]. The rise in cortisol/DHEA ratio may be responsible for the imbalance between lipolysis and lipogenesis in peripheral adipocytes. Excess of cortisol could activate the HSL, inducing increased lipolysis, and leading to the loss of peripheral fat mass. Meanwhile, IFN-α and TNF-α accumulation may enhance hepatic lipid synthesis, particularly FFA, TG and VLDL, and consequently contribute to steatosis and the rise in circulating lipids.

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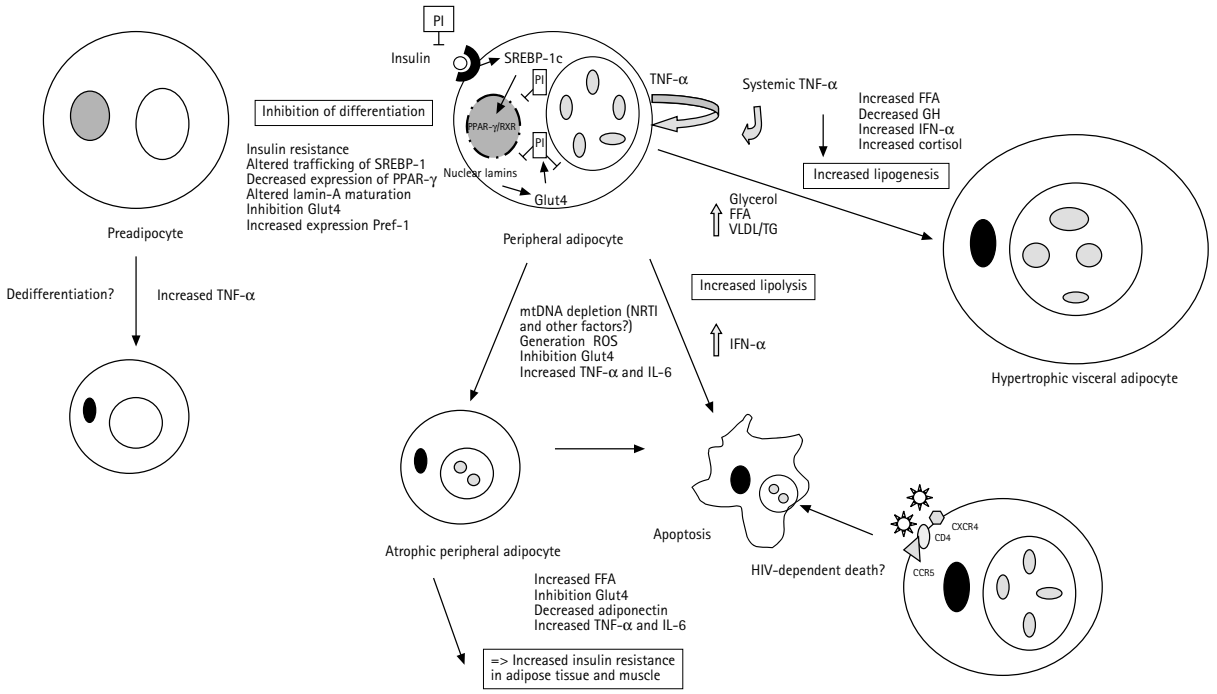
A model of lipodystrophy focused on adipose-specific effects

The more frequent form of lipodystrophy observed in HIV-infected patients is a mixed form with peripheral lipoatrophy and central lipohypertrophy. We have presented different hypotheses, which could explain why adipose tissue could undergo lipoatrophy. NRTIs could induce lipolysis and mitochondrial dysfunction, resulting in apoptosis. NRTIs and PIs could induce secretion of adipocytokines, such as TNF-α, which would increase insulin resistance and lipolysis, and drive adipocytes towards apoptosis. PIs could alter cellular location of SREBP-1 in adipocyte, due to disrupted lamina architecture, resulting in altered phenotype and insulin sensitivity. These alterations would result in a decrease in adipocyte size and number giving rise to a lipoatrophic phenotype (Figure 3). In addition, if peripheral fat is atrophic, there is no possibility of lipid storage at that level. The increased lipolysis will thereby increase the level of FFA, which will be preferentially stored in visceral adipocytes leading to hypertrophy. Moreover, regarding the central adipocytes, hormone dysregulation could also explain adipocyte hypertrophy. These adipocytes have been found to express high levels of the enzyme 11β-hydroxysteroid dehydrogenase, which is able to convert inactive cortisone in active cortisol inside the adipocytes [179]. Cortisol has been found to increase differentiation of adipocytes, this effect being more striking for visceral adipocytes, which express more cortisol receptors. When overexpressed in WAT in transgenic mice, a syndrome of visceral obesity with metabolic alterations was observed, close to the human metabolic syndrome [180]. Interestingly, the expression of the enzyme is lower in subcutaneous fat explaining the central and not peripheral adiposity of the animal model. An increased ratio of cortisol to DHEA has been observed in HIV-positive patients with lipodystrophy, which was correlated with the evolution of lipodystrophy [178]. Moreover, the enzyme 11β-hydroxysteroid dehydrogenase is activated by TNF-α [181], for which levels are increased in HIV-infected lipodystrophic patients. This could explain visceral hypertrophy. In addition, growth hormone (GH) could also play a role in this phenotype. GH has been shown to decrease visceral fat and it is able to inhibit 11β-hydroxysteroid dehydrogenase [182]. Decreased GH levels have been reported in HIV patients with lipodystrophy [183]. Conversely, a treatment with GH of these patients was able to decrease the amount of visceral fat [184]. Therefore, adipose tissue hypertrophy could result from: 1) increased disposal of FFA, which can be stored in visceral adipocytes; and 2) hormonal alterations with an increased synthesis of cortisol at the local level stimulated by TNF-α, and a decreased level of GH. The lipodystrophic adipose tissue, either atrophic at the periphery or hypertrophic at the central level probably participates to the insulin-resistant state through increased secretion of FFA and altered secretion of cytokines (increased TNF-α and decreased adiponectin), resulting in insulin resistance, in particular at the muscle and liver level and in dyslipidaemia.

Figure 3. A model of lipodystrophy focused on adipose tissue



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