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Review

Antiretoviral therapy and the lipodystrophy syndrome, part 2: concepts in aetiopathogenesis

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Clinical research has indicated that the use of nucleoside reverse transcriptase inhibitor (NRTI) and HIV protease inhibitor (PI) therapy is associated with a risk of long-term toxicity syndromes, and that the aetiopathogenesis of these adverse effects is independent of the antiretroviral effects of these drugs. In relation to the lipodystrophy syndrome, it appears that the most powerful determinant of subcutaneous fat wasting is an interaction between these two drug classes. In this review, possible mechanisms underlying the contributions of both PI and NRTI drugs are reviewed, with an emphasis on their effects on adipose tissue. On this basis, an 'adipocentric', or minimal model of the syndrome is developed, in which divergent effects at the adipocyte of NRTIs (mitochondrial toxicity) and PIs (insulin resistance and impaired adipocyte maturation) interact to produce a phenotype that is consistent with clinical observations.

Introduction

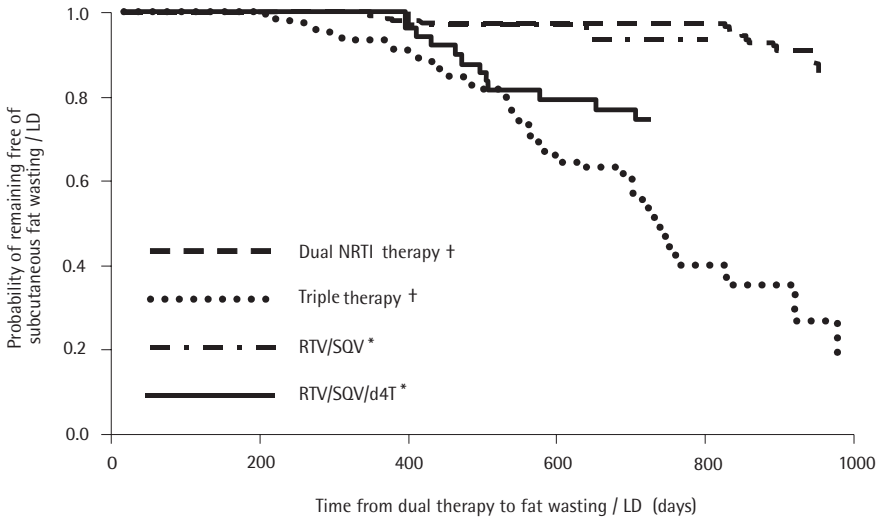
Clinical studies, whether based on observational cohorts or clinical trials, have contributed significantly to an increased understanding of the lipodystrophy syndrome that accompanies long-term use of antiretroviral therapy [1]. Taken together, they provide a conceptual framework that can inform basic science research, with the ultimate aim of gaining an understanding of the underlying aetiopathogenesis of the syndrome at the molecular level. The elucidation of these mechanisms should then be able to guide the selection of antiretroviral therapy combinations less likely to induce long-term complications, as well as provide a rational basis for the clinical management of individuals with established lipodystrophy.

An analysis of the available clinical data was the subject of a previous review [1], and will be briefly reiterated. This review also addressed methodological issues relating to the clinical evaluation and definition of the lipodystrophy syndrome. Of particular importance is the recognition that component clinical features of the syndrome (body composition changes, such as subcutaneous fat wasting and/or visceral or localized fat accumulation; and metabolic abnormalities such as insulin resistance and dyslipidaemia) need to be analysed separately, so that their relation to each other, as well as to interventions, such as introduction or cessation of antiretroviral drugs, can be objectively assessed.

Initial aetiopathogenic models of the lipodystrophy syndrome focused primarily on the role of HIV protease inhibitors (PIs), as the introduction of this class of drug into clinical practice was temporally associated with the first descriptions of the syndrome. It has since become apparent that subcutaneous fat wasting, in particular, can also occur in patients receiving only nucleoside reverse transcriptase inhibitors (NRTIs) [2,3], and that NRTIs contribute to lipodystrophic changes in body composition when both drug classes are used in combination [4]. PI use appears to be more strongly associated with the 'metabolic' component of the syndrome, characterized by increased levels of triglyceride, triglyceride-rich lipoproteins and insulin resistance, based on the observation that these metabolic changes have been observed after short-term exposure to PIs in healthy volunteers [5,6], and following introduction of PI therapy in HIV-infected patients in the absence of changes in body composition [7,8]. NRTIs, on the other hand, while contributing to the risk of developing peripheral fat wasting [2–4], appear to have relatively less effect on lipid metabolism and insulin sensitivity [1].

Assessing the effects of specific drugs within these two drug classes, there is no current evidence that choice of PI significantly affects the incidence or severity of morphological [9] or metabolic [10] compo-

Figure 1. Nucleoside reverse transcriptase inhibitors and protease inhibitors interact to cause fat wasting in highly active antiretroviral therapy-associated lipodystrophy



Combining the results of the Prometheus study* and the Western Australian HIV Cohort Study† demonstrates the interactive effect on fat wasting of combining nucleoside reverse transcriptase inhibitors and protease inhibitor therapy, compared with dual nucleoside reverse transcriptase inhibitors or dual protease inhibitor regimens. NRTI, nucleoside reverse transcriptase inhibitor; RTV, ritonavir; SQV, saquinavir; d4T, stavudine; LD, lipodystrophy.

nents of the lipodystrophy syndrome, with the notable exception that ritonavir therapy is associated with higher triglyceride levels [10,11]. Within the NRTI class there is evidence from observational studies, as well as randomized clinical trials, that stavudine therapy is associated with an increased relative risk (approximately twofold) of peripheral fat wasting compared with zidovudine [2–4,9,12–14].

An important concept that has recently emerged is that when NRTIs and PIs are combined in highly active antiretroviral therapy (HAART) regimens, it is the interaction of these drug classes that is the most powerful predictor of lipodystrophic body composition changes, with a significant (>5-fold) increase in relative risk compared with the use of one drug class. This observation has now been made in clinical trials, in which NRTI therapy is introduced to pre-existing dual PI regimens [15–18] as well as in the more familiar comparison of dual NRTI regimens versus PI-containing HAART [4] (Figure 1). The magnitude of this interaction effect in clinical studies is far greater than observed differences in immunological or virological response to dual therapy regimens (either PI- or NRTI-based) [4,15–18] compared with combination HAART, indicating that the interaction between these drug classes is not mediated by a non-specific effect on immune restoration. This is probably best exemplified in the Prometheus study, in which the addition of stavudine to stable long-term ritonavir/saquinavir therapy had no significant effect on virological or immunological response [16], while the relative risk of developing clinically apparent changes in body composition increased ~fivefold [15]. This is also true for differences within the NRTI class, where equivalent anti-HIV efficacy but divergent effects on lipodystrophy have been shown in comparisons of zidovudine and stavudine therapy [2–4,12].

In this review we propose to further examine the effects of PI and NRTI therapy that may be relevant to the aetiopathogenesis of the lipodystrophy syndrome, and to explore how these effects may interact at the molecular/cellular level to produce the lipodystrophy phenotype. We propose a 'minimal model' of lipodystrophy pathogenesis that focuses on adipose-specific effects, and accounts for the clinical observations, in an attempt to provide a theoretical framework for further research. Undoubtedly this model will require modification in light of the results of future studies, as knowledge of the underlying mechanisms is refined.

The contribution of NRTIs to lipodystrophy

The lipodystrophy phenotype that is observed in PI-naive NRTI-treated individuals differs in some respects to that seen in association with PI treatment. Subcutaneous fat wasting appears to be a dominant manifestation [19], and although mild increases in visceral adiposity have been observed in clinical studies [2,9], the magnitude of this effect is far less than that observed in PI-treated patients [9,20], and does not appear to be influenced by choice of NRTI [9,12].

In terms of the metabolic component of the syndrome, studies by Saint-Marc [2] and Galli *et al.* [21] have demonstrated mildly elevated levels of triglycerides and decreased HDL cholesterol in stavudine-treated patients. Risk of developing these metabolic alterations was not found to be associated with body composition changes [21], suggesting that NRTIs may have an independent effect on some aspects of lipid metabolism. However, the addition of stavudine to dual PI regimens produced no incremental rise in triglyceride or cholesterol levels, suggesting that these lipid changes have minor clinical significance in the presence of PI therapy [22]. Similarly, NRTI-associated lipodystrophy, and peripheral fat wasting in the absence of visceral fat accumulation in general ('pure lipoatrophy'), is not associated with significant hyperinsulinaemia, insulin resistance, or the dyslipidaemic profile that accompanies insulin resistance (that is, elevated apolipoprotein B and VLDL cholesterol in addition to elevated triglyceride and decreased HDL-cholesterol levels) [2,9,23].

A study of *in vivo* lipid metabolism by Ware *et al.* has demonstrated an increased retention time of dietary lipid within the circulation as free fatty acid in PI-naive patients with lipodystrophy [24]. The authors suggested the existence of an NRTI-induced defect in cellular uptake of fatty acids, and proposed a mechanism involving the inhibition of fatty acid-binding proteins (FABPs) that are responsible for fatty acid uptake and intracellular trafficking [24]. Consistent with this hypothesis, FABP mRNA levels were found to be decreased in stavudine-treated mice [25]. Given the postulated association between NRTIs and mitochondrial toxicity, it is interesting to note that FABPs are derived from a mitochondrial precursor (mitochondrial aspartate aminotransferase), which requires intramitochondrial activation before being secreted into the cytosol [26,27].

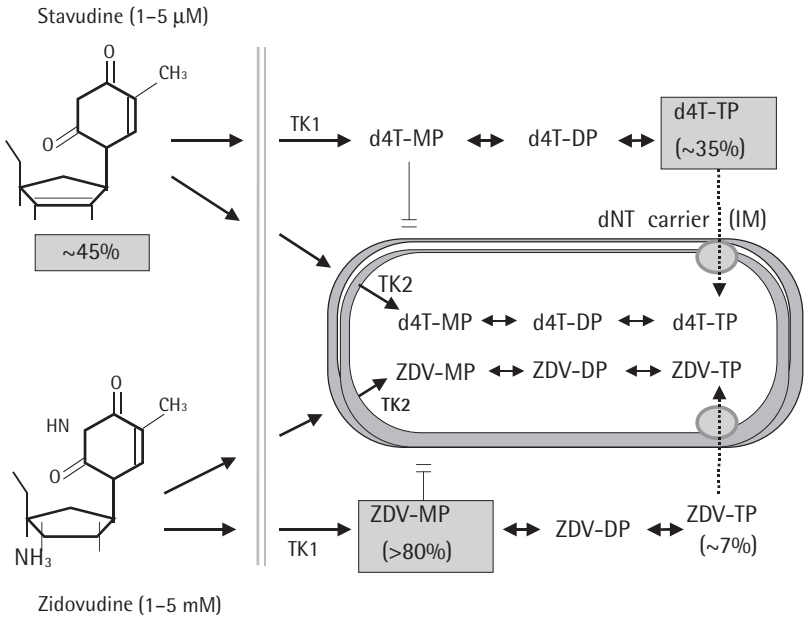
Mechanisms of NRTI-mediated toxicity: mitochondrial toxicity and the 'pol-γ' hypothesis

NRTIs act as false substrates for the polymerase activity of HIV reverse transcriptase, and are characterized by the lack of a hydroxyl group in the 3′ position. Thus, when the activated triphosphate form of these compounds are used by a viral (or host) polymerase and added to a nascent DNA chain, there is no site of attachment for the next nucleotide, terminating DNA synthesis at that position. While the major polymerase involved in nuclear DNA synthesis (polymerase-α) is able to discriminate effectively against these nucleoside analogues, this ability is not shared by the sole polymerase present in mitochondria, polymerase-γ [28]. This polymerase performs a number of critical functions, as mitochondria within a cell (of which there may be hundreds) contain multiple copies of their own (extrachromosomal) mitochondrial genome. The maintenance of bioenergetic function in all metabolically active cells therefore requires ongoing polymerase-γ-mediated mitochondrial (mt) DNA synthesis as well as repair (even in post-mitotic cells, in which nuclear DNA synthesis is negligible), creating an ongoing requirement for nucleoside uptake and use by mitochondria. It is perhaps not surprising, therefore, that chronic toxicities induced by NRTI compounds appear to be the consequences of mitochondrial dysfunction.

The possible link between polymerase-γ inhibition and lipodystrophy was first presented by Brinkman in 1999 [29], and this area has since been reviewed by Kakuda [30] and White [31], in which the syndrome is considered in the context of other NRTI-induced toxicities. The basic premise of the 'pol-γ' hypothesis is that NRTI-induced inhibition of polymerase-γ leads to depletion of cellular mtDNA content through inhibition of mtDNA synthesis. Toxicity at the cellular and tissue level is the consequence of loss of mitochondrial bioenergetic function, once mtDNA levels have fallen beyond a critical level where the production of mtDNA-encoded protein subunits of the mitochondrial respiratory chain [13], and RNAs (22 tRNAs and 2 rRNAs) is insufficient to meet the cell's energy requirements.

There is an increasing understanding of the importance of mitochondrial partitioning of these drugs (that is, their ability to enter the mitochondrial compartment within the cell cytosol) in determining toxicity [32], particularly following the important discovery of a mitochondrial inner membrane transporter that is able to facilitate the entry of NRTI triphosphate derivatives into mitochondria [33]. Based on these considerations, a modified version of the 'pol-γ' hypothesis is proposed (modifications in italics): (1) the NRTI has the pharmacodynamic capability to enter the target cells, *and subsequently to enter the mitochondrion in either its free-drug or phosphorylated form*; (2) the target cell possesses *activated* cellular nucleoside kinases to mono-, di- and subsequently triphosphorylate the NRTI, *so that the active triphosphate form of the drug is present within the mitochondrion*; (3) the triphosphorylated NRTI can inhibit DNA polymerase-γ either by serving as a competitive (ineffective) alternative substrate or by chain termination of the nascent mtDNA strand (non-competitive); (4) the target tissue has a metabolic reliance on *the maintenance of mitochondrial function* (rather than 'oxidative phosphorylation').

Figure 2. Mitochondrial partitioning of zidovudine and stavudine



ZDV, zidovudine; d4T, stavudine; MP, monophosphate; DP, diphosphate; TP, triphosphate; IM, inner membrane; dNT, deoxynucleotide; TK, thymidine kinase.

The examples of zidovudine and stavudine (both thymidine analogues) may be considered in this light, as NRTIs of interest in relation to the lipodystrophy syndrome (Figure 2). Structurally, the two drugs are similar, with the important difference being desaturation of the 2′ and 3′ positions of the sugar in stavudine, and the presence of an azide group in zidovudine. Both compounds are hydrophilic, with relatively short plasma half-lives (1–1.5 h) [34], and thus have no redistribution phase to adipose tissue. At the cellular level, zidovudine and stavudine enter the cell by passive diffusion and are then sequentially phosporylated to mono-, di-, and finally to the active triphosphate derivative. This process involves, in turn, thymidine kinase (TK), thymidylate kinase and pyrimidine diphosphate kinase [35]. At the subcellular level, however, there is evidence that these compounds differ in their ability to enter the mitochondrial compartment, and subsequently to inhibit mtDNA synthesis (Figure 2).

In the thymidine activation pathway, partitioning into cytosolic and mitochondrial compartments occurs at two stages. TK has a cytosolic form (TK1), which is active only in S phase of mitosis, and a mitochondrial form (TK2), which is expressed in proportion to the mitochondrial content of the cell and is not cell-cycle regulated [36]. It is likely that thymidine as well as the free-drug forms of stavudine and zidovudine are able to cross the mitochondrial double membrane without requiring a specific carrier [37]. The rate limiting step for activation of zidovudine is the conversion from zidovudine-monophosphate (MP) to zidovudine-diphosphate (DP) by thymidylate kinase (decreased V_{max}) [38]. Subsequently, low levels of zidovudine-triphosphate (TP) limits access to the mitochondrion via a specific deoxynucleotide carrier [33]. Zidovudine is a relatively weak inhibitor of polymerase-γ (K_i 1–10 μM), and since steady-state whole-cell concentration of zidovudine-TP is <0.5 μM, this suggests that zidovudine at pharmacological doses fulfils the 'pol-γ' hypothesis criteria only mildly.

Stavudine may be able to enter the mitochondrion in its free-drug form, or in its TP form via the deoxynucleotide carrier. It has a relatively high affinity for polymerase-γ (<0.1 μM), and on this basis it could be predicted that pharmacological doses of stavudine have the potential to lead to inhibit polymerase-γ, particularly in post-mitotic cells in which TK1 is inactive.

At present it is difficult to reconcile the available data regarding the important issue of intracellular (and intramitochondrial) activation of stavudine. As shown in Figure 2, it is clear that thymidine kinase-mediated phosphorylation of free stavudine is the rate-limiting step of stavudine phosphorylation, and that affinity of this drug for TK1 is low [39]. In relation to affinity for TK2, activity in the presence of stavudine was shown

Table 1. Evidence of mitochondrial DNA depletion in subcutaneous fat samples from individuals with highly active antiretroviral therapy-associated lipodystrophy

Researchers	Methods	Findings
Walker *et al.* (Germany) [44]	32 samples: 11 with LD, 12 HIV positive, ART positive with no LD, 8 HIV negative controls, 1 HIV positive, ART naive	No difference in mtDNA content between HIV-negative controls and HIV positive patients treated with NNRTI+PI (n=4)
	Southern blot analysis	NRTI treatment associated with ↓mtDNA (P=0.009)
		Among ART-treated, 38% average ↓mtDNA content in patients with LD compared with LD-negative (P=0.04)
Shikuma *et al.* (Hawaii) [45]	69 samples from 24 individuals (8 with LD)	14/23 (61%) with LD had reduced/absent mtDNA, compared with 6/20 (30%) of non-LD controls (P=0.04), and 3/20 (15%) of HIV-negative controls (P=0.008)
	Semiquantitative assay with size fractionation of PCR product on agarose gel	No large mtDNA mutations
Mallal *et al.* (Australia) [46]	17 samples: 5 with LD, 5 HIV positive, ART positive with no LD, 3 HIV positive ART-naive, 4 HIV negative controls	No difference in mtDNA content between HIV-negative and HIV positive controls
	Real-time PCR-based quantitative assay	MtDNA depletion associated with NRTI treat ment (P <0.001). Within ARTpositive group, depletion associated with LD (P=0.05, versus non-LD)
mtDNA		
	Biorad microplate assay of mt protein content	Average mtDNA content compared with controls: LD-positive 15%, LD-negative 58% (adjusted for mt protein mass)

LD, lipodystrophy; mt, mitochondrial; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non- nucleoside reverse transcriptase inhibitors; PI, protease inhibitor; ART, antiretroviral treatment.

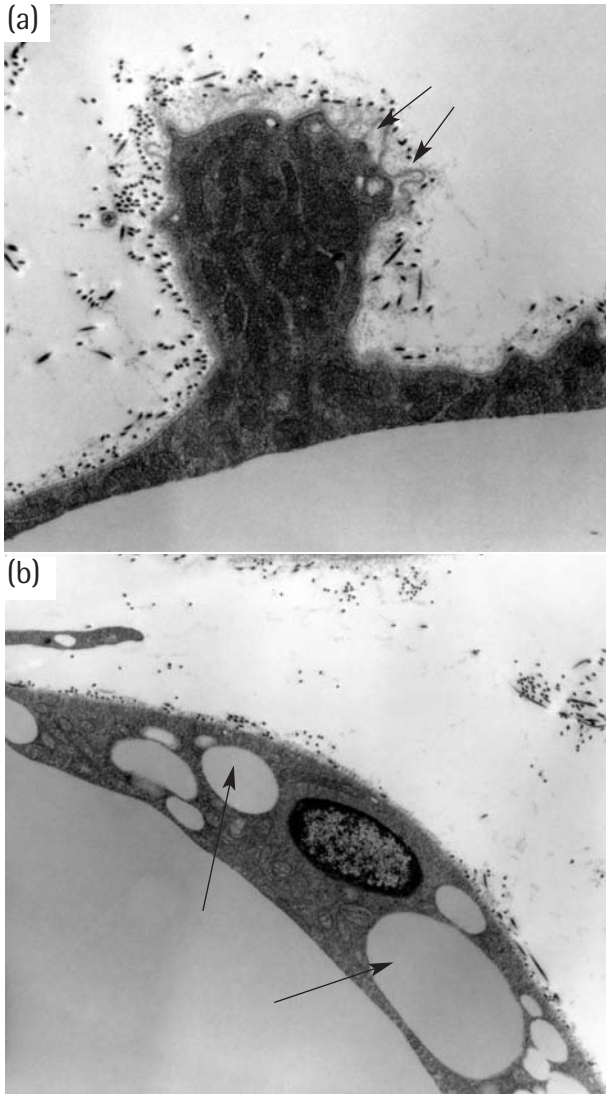
to be 0.005% of that observed with an equivalent concentration of thymidine (20 μM) by Munch-Peteresen *et al*., and this has been confirmed more recently with recombinant TK2 [36,40]. This result is surprising, given that stavudine retains anti-HIV activity within the pharmacological dose range in TK1-deficient cells (IC_{50} HIV 0.27 μM with TK, 2.5μM in TK-deficient CEM cells) [41], and has shown efficient phosphorylation to the active stavudine-TP form within isolated mitochondria [42].

This discordance may relate to the fact that the TK2 gene shows multiple transcripts, and several forms of TK2 mRNA may also be derived from alternative splicing or by the addition of a 3′ polyadenylation signal [36]. It is possible, therefore, that experiments to date have not used the TK2 specific for stavudine. Another explanation is that stavudine may act as substrate for another mitochondrial kinase, but this appears less likely. The other human deoxynucleoside kinases, deoxycytidine kinase (dCK) and deoxyguanosine kinase (dGK), show extensive sequence identity to TK2 and are closely related and separate from TK1 [40]. However, dCK is cytosol-specific, while mitochondrial dGK is unable to recognize thymine as a base, because of the presence of a phenylalanine residue (Phe-156) at a critical site for thymine recognition, where thymidine kinases use a tyrosine (Tyr-172) [43].

NRTIs and lipodystrophy

Depletion of mtDNA has now been demonstrated in subcutaneous fat samples taken from individuals with lipodystrophy by three independent groups [44–46] (summarized in Table 1), providing important *in vivo* data of the effects of NRTIs in the putative target tissue. Analysis of the ultrastructure of adipocytes has also demonstrated abnormal mitochondrial forms (whorled cristae, elongated and branched forms) as well as a striking increase in mitochondrial organellar size and number [44,47,48] (Figure 3). A correlation been mitochondrial DNA depletion and increased mitochondrial mass has been shown in one study [46], consistent with genetically-determined mtDNA depletion syndromes, in which proliferation of mitochondria appears to represent a compensatory response directed by the nuclear genome [49]. This may lead to a ‘vicious cycle’ in which increasing levels

Figure 3. Ultrastructural changes in subcutaneous fat affected by highly active antiretroviral therapy-associated lipodystrophy



(a) Mitochondrial proliferation with elongated mitochondrial forms within an expanded cytoplasm is demonstrated. Redundant folds of basal lamina (arrows) indicate loss of cellular volume, consistent with a reduction in the size of the major intracellular triglyceride droplet. (b) Intracytoplasmic lipid droplets (arrows) are frequent, suggestive of mobilization of triglyceride stores for lipolysis and/or oxidation. These changes are also seen in 'converted adipocytes' in β_3-adrenoceptor-agonist treated rats. Mitochondrial proliferation is also evident.

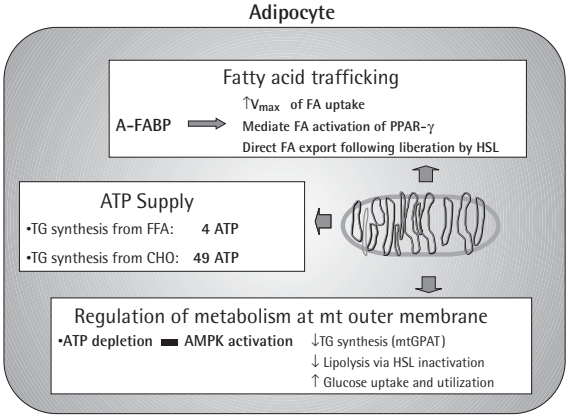
of TK2 (expressed in proportion to mitochondrial mass) allow the further accumulation of intramitochondrial NRTI derivatives. This 'proliferative' response may also exacerbate the pathological effects of mtDNA depletion, by further decreasing mtDNA content relative to the mitochondrial mass and activity that this genome must support. In this context, a study in which cellular mtDNA content was adjusted for mitochondrial mass (measured by mitochondrial protein content) has shown ~85% average depletion of mitochondrial DNA content in samples from individuals with lipodystrophy, compared with controls [46]; a level of depletion that would be considered sufficient to cause mitochondrial dysfunction. It is, however, difficult to ascribe a 'cut-off point' at which the magnitude of mtDNA depletion is considered pathological, particularly because assays of mitochondrial depletion provide an 'average' measure of mtDNA in a tissue sample. Hence, the extent of mtDNA depletion in individual cells may have been underestimated, given that cells within a tissue are heterogeneous in terms of mtDNA content and bioenergetic status. This concept has been referred to as the 'bioenergetic mosaic' theory [50].

This underlines the importance of assessing the *functional consequences* of mtDNA depletion (for example, affects on mitochondrial respiratory activity) *in vivo*, so that proximal effects of polymerase-γ inhibition on the mitochondrial genome (decreased mtDNA synthesis and repair) can be related to cellular pathophysiology.

The consequences of mtDNA depletion and associated mitochondrial dysfunction have traditionally been considered in terms of the affect on oxidative phosphorylation [29]. Adipose tissue, however, has a low oxidative capacity in keeping with its role as an energy substrate store [51]. The role of mitochondria in adipocytes appears to be more complex and is directed towards providing energy for triglyceride synthesis and storage (Figure 4). Experimentally-induced mitochondrial dysfunction in adipocytes increases basal glucose uptake, without influencing insulin-stimulated glucose uptake [52]. While glycolysis is increased, however, loss of mitochondrial membrane potential in adipocytes correlates with a diversion from energy storage (triglyceride synthesis and *de novo* fatty acid synthesis) to energy use associated with increased lactate production [53]. This is consistent with a body of work suggesting that there are a number of cellular mechanisms that are used to 'sense' the energy and fuel status of the cell (and hence of the mitochondria) [54–57]. In lipogenic tissues, such as adipose and liver, a decrease in the cytosolic ATP/ADP ratio activates a protein kinase system (AMP-activated kinase) that diverts resources away from energy-consuming biosynthetic reactions, such as triglyceride synthesis (thus providing a fuel store that can be called on by *other* cells), towards oxidative reactions that provide energy for the ATP-depleted cell. In adipocytes, activation of this response decreases both lipogenesis and lipolysis [56,57].

Returning to the potential role of FABPs, there is an adipose-specific form (A-FABP, also known as aP2 or ALBP) whose activation is dependent on the maintenance of the mitochondrial membrane potential [58]. It

Figure 4. Role of mitochondria in adipose tissue



Triglyceride (TG) synthesis and storage (the major functions of adipose tissue) are energy-requiring processes. Depletion of ATP activates the AMP-activated protein kinase pathway, which inhibits triglyceride synthesis and *de novo* fatty acid synthesis from carbohydrate precursors. AMPK activation also inhibits lipolysis (fatty acid release from the adipocyte) by a direct effect on hormone-sensitive lipase. Hence, mitochondrial dysfunction in adipocytes limits lipogenesis and lipolysis, and directs metabolism towards oxidation while increasing basal glucose uptake. Mt, mitochondrial; FA, fatty acid; FFA, free fatty acid; CHO. cholesterol; HSL, hormone-sensitive lipase; AMPK, AMP-activated kinase; mtGPAT, mitochondrial glycerol phophate acyltransferase.

plays a critical role in intracellular fatty acid trafficking, increasing the V_{max} of free fatty acid uptake by adipocytes [59] and directing fatty acids to nuclear transcription factors involved in regulating lipogenesis [60]. Through an interaction with hormone-sensitive lipase, A-FABP also appears to direct fatty acid export from the cell, so that liberated fatty acid may be released rather than be made available for re-esterification within the adipocyte [61]. While other FABPs are likely to be involved in fatty acid uptake it may be that there is less redundancy in relation to fatty acid export, as A-FABP-null mice exhibit reduced lipolysis and accumulate fatty acid intracellularly [62]. Decreased mitochondrial activation of A-FABP, therefore, would be predicted to reduce the efficiency of fatty acid uptake as well as export (in keeping with the other cellular responses to ATP depletion).

Chronic sub-lethal mitochondrial dysfunction induced by NRTIs in adipose tissue may therefore lead to increased glucose uptake, glycolysis and lactate production, as well as to the accumulation of intracellular fatty acids, which are directed towards oxidative rather than biosynthetic pathways. Severe mitochondrial dysfunction would be predicted to produce cell death through apoptosis following loss of bioenergetic viability [28], while milder mitochondrial injury may produce an adipocyte phenotype with reduced capacity for triglyceride synthesis or fatty acid export, thus 'isolating' it from the metabolic demands of the body as a whole. This would be consistent with the clinical phenotype of NRTI-associated lipodystrophy, in which loss of subcutaneous adipose mass is not accompanied by insulin resistance or elevated apolipoprotein-B or VLDL-cholesterol (both markers of increased fatty acid delivery to the liver), or with the induction of increased visceral adiposity. A reduction in V_{max} of adipocyte fatty acid uptake may contribute to slower extraction of fatty acid form the circulation, and therefore to increased opportunity for lipid exchange between triglyceride and HDL-cholesterol resulting in elevated triglyceride, and reduced HDL-cholesterol, levels.

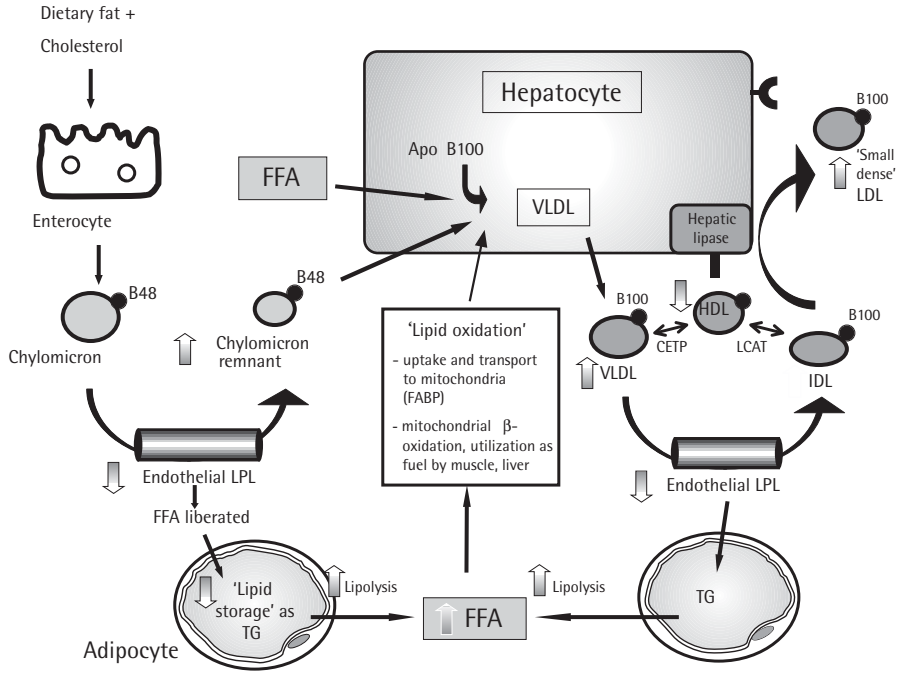
In this context, it is interesting to note that two recent large studies have shown that in patients receiving PI-containing HAART, those who develop peripheral fat wasting without increased visceral fat accumulation ('pure lipoatrophy'), triglyceride levels are higher and insulin sensitivity is preserved compared with those who develop a mixed syndrome in which visceral fat accumulation is a feature [9,23]. This will be discussed further, when the combined effects of NRTIs and PIs are considered.

The contribution of PIs to lipodystrophy

As mentioned previously, the use of PI therapy is strongly associated with the 'metabolic syndrome' component of the lipodystrophy syndrome. In keeping with the observation that the introduction of PI therapy is associated with changes in lipid and glucose/insulin metabolism [5–8], cessation of PI therapy and replacement by NRTI (abacavir) or NNRTI therapy in 'switching studies' has been shown to improve metabolic parameters [reviewed in 1]. In relation to body composition changes, the interaction of NRTIs and PIs is the dominant predictor of subcutaneous fat wasting as well as increased visceral adiposity in PI-containing HAART [9]. Unfortunately, the metabolic changes that accompany long-term dual PI therapy are not well characterized at this time.

The 'metabolic syndrome' associated with PI therapy (Figure 5) appears to be characterized by increased levels of triglyceride and triglyceride-rich lipoproteins (chylomicrons and VLDL-cholesterol). These lipid fractions are normally elevated in the post-prandial phase, and are destined for delipidation in the peripheral circulation by the action of endothelial lipoprotein lipase so that fatty acids may be removed to adipose tissue and stored (after conversion to triglyceride). Elevated plasma triglyceride levels are accompanied by increased total, VLDL- and IDL-cholesterol, apolipoproteins B and CIII as well as E, and small-dense LDL-cholesterol as well as increased HDL_3 [23,63–67]. Although total LDL-cholesterol is often not elevated, or elevations are mild, this pheno-

Figure 5. Insulin resistance and lipid metabolism



Insulin resistance decreases delipidation of chylomicrons (derived from dietary fat) and VLDL-cholesterol (from hepatic processing of fatty acids) at the level of lipoprotein lipase, which is produced and secreted by adipocytes in response to insulin (Figure 6). Increased lipolysis from adipocytes, as well as increased chylomicron remnants, increase hepatic processing to produce VLDL-cholesterol. VLDL metabolism normally produces LDL, which is then readily cleared by LDL receptors. 'Altered', triglyceride-enriched VLDL, however, is diverted towards the production of IDL from HDL, as well as small, dense LDL that is less effectively cleared via the LDL receptor and is more susceptible to oxidative modification.

type is now known to confer excess cardiovascular risk – an issue addressed in a previous review [1]. A case report demonstrating the 'unmasking' of ApoE2/E2-related dysbetalipoproteinaemia by PI therapy (ritonavir and indinavir) highlights a possible role for defective clearance of apolipoprotein E-containing lipids [68].

These lipid changes are commonly accompanied by elevated insulin levels, and increased levels of free fatty acid have also been observed [9,64]. Dysregulated fatty acid metabolism was also found in a study by Sekhar *et al.*, [69] who used stable isotope tracer techniques to provide a comprehensive assessment of *in vivo* lipid metabolism dynamics in patients receiving PI-based HAART. Turnover of fatty acids was found to be dramatically increased, with elevated fat oxidation and re-esterification in adipose and liver, increased lipolysis, and markedly decreased clearance of triglyceride-enriched VLDL and chylomicrons (Figure 4).

This metabolic phenotype is typical of defective post-prandial lipid metabolism, which in turn is intimately associated with insulin resistance. A critical role for insulin is the production of a coordinated metabolic response to the post-prandial (or 'fed') state, so that carbohydrates are used as the primary source of fuel for oxidative reactions, while lipids are directed into adipose stores for use when metabolism must rely more heavily on fatty acids as a fuel source (in the post-absorptive or 'fasted' state). Hence, increasing levels of insulin following food intake increase fatty acid uptake and triglyceride synthesis within adipocytes, and inhibit lipolysis (free fatty acid release from triglyceride) from adipose stores. A primary defect in insulin signalling will, therefore, inevitably produce abnormal post-prandial lipid metabolism. An alternative scenario also exists, in which defective post-prandial lipid metabolism may induce insulin resistance (as would be predicted if lipoprotein-receptor-related protein (LRP) [70], or the acylation-stimulating protein pathway [71] were involved in pathogenesis).

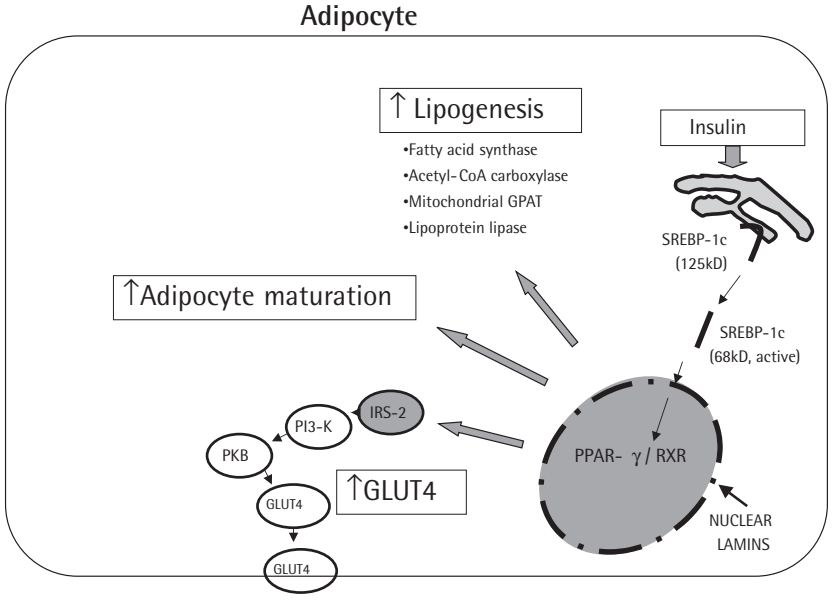
The possibility that adipose tissue may be a target of PI-mediated effects is supported by a number of *in vitro* studies indicating that pharmacological doses of PI drugs may directly induce insulin resistance in adipocytes [72–74], as well as a defect in the late stages of adipocyte maturation. This block in the adipocyte differentiation programme occurs after the adoption of adipocyte morphology, and is characterized by decreased expression of nuclear transcription factors C/EBP-α and PPAR-γ [72,75–77]. These studies have also identified sterol regulatory element-binding protein (SREBP) as a candidate factor that may mediate these effects [72,76,78]. This endoplasmic reticulum-derived transcription factor, common to

adipose tissue and the liver, appears to play a critical role in inducing the signature adipocyte response to post-prandial insulin release. In adipocytes it is involved (either directly or via downstream stimulation of PPAR-γ) in increasing fatty acid uptake (lipoprotein lipase) and fatty acid synthesis from carbohydrate precursors (fatty acid synthase), inhibiting lipolysis (hormone sensitive lipase), and increasing adipocyte differentiation, as well as increasing uptake and utilization of glucose as energy substrate (GLUT-4, glucokinase) (Figure 6) [79–81]. Caron *et al.* [72] have provided some clues to the site of a PI-induced defect in SREBP activity, demonstrating that PI therapy inhibited the translocation of active SREBP-1c from endoplasmic reticulum and nuclear membranes to the nucleus. They also detected altered electrophoretic mobility of activated SREBP-1, suggesting abnormal processing or phosphorylation of active SREBP after proteolytic activation from its 125 kDa precursor form. Interestingly, defective SREBP processing may also be central to the pathogenesis of Dunnigan-type familial partial lipodystrophy, a severe monogenic form of insulin resistant lipodystrophy that resembles HAART-associated lipodystrophy phenotypically [82]. The underlying genetic defect in this condition involves mutations in the *LMNA* gene that encodes nuclear lamin A, a protein involved in the organisation of the nuclear membrane and the regulation of trafficking of transcription factors into the nucleus. It has been proposed that mutated *LMNA* products may interact abnormally with SREBP, so that activation of nuclear transcription factors is impaired [82] (Figure 6).

Could adipose-selective insulin resistance be central to the PI-associated 'metabolic syndrome', and to the interaction between NRTIs and PIs in the development of subcutaneous fat wasting? Certainly, interest in the possibility that adipose tissue, rather than muscle, may be the central regulator in insulin resistance has been revived by a number of recent observations [83]. Mice with an adipose-specific reduction of GLUT4 (the insulin-responsive glucose transporter) also developed insulin resistance in muscle and liver [84], and the subsequent isolation of resistin, an adipose-derived protein, has provided a plausible mechanism whereby defective adipose insulin signalling determines more generalized insulin resistance [85].

A recognition of depot-specific differences between subcutaneous and visceral fat is important when considering these effects, particularly in the context of HIV lipodystrophy in which subcutaneous fat wasting may be accompanied by visceral fat accumulation. Subcutaneous fat acts as a more efficient and stable fat storage reservoir, with greater triglyceride synthetic capacity [71] and responsiveness to insulin [86], indi-

Figure 6. Sterol regulatory element-binding protein and peroxisome proliferator activated receptor γ insulin signalling pathways in adipocytes



Sterol regulatory element-binding protein is activated by insulin, and acts to promote lipogenesis and adipocyte differentiation via multiple pathways, acting both directly and via intranuclear activation of peroxisome proliferator activated receptor γ / retinoid-X receptor nuclear transcription factors. SREBP, sterol regulatory element-binding protein; IRS-2, insulin receptor substrate-2; PI3-kinase, phosphotidylinositol 3-kinase; PKB, protein kinase B; PPAR-γ, peroxisome proliferator activated receptor γ; RXR, retinoid-X receptor.

cating a bias towards lipogenesis. This is also supported by the fact that subcutaneous preadipocytes differentiate more readily to mature adipocytes in response to PPAR-γ agonists [87]. Visceral adipose tissue, on the other hand, is a more labile fat store with increased lipid turnover and greater expression of specific receptors for hormones that promote lipid accumulation (cortisol) as well as lipolysis (androgens, growth hormone, catecholamines) [88]. Lipoprotein lipase (LPL) activity is also higher in visceral adipose tissue in males and females [89]. Significantly, increased fatty acid levels promote insulin resistance in subcutaneous fat and muscle, while visceral adipose tissue responds by increasing glucose use and GLUT4 expression (as well as PPAR-γ and CD36/fatty acid translocase), thus channelling fatty acids towards the visceral fat depot, presumably in an attempt to limit lipotoxic damage to other organs [90]. Visceral fat accumulation, then, may represent an adaptive response to insulin resistance and dysregulated fatty acid metabolism [91], even in the context of adipose-specific insulin resistance. Given the role of visceral fat as a hormonally-responsive adipose depot, the hormonal changes that have been seen to evolve with the development of lipodystrophy (for example, increasing cortisol/DHEA ratios [63], changes in growth hormone dynamics [92], and hyperandrogenaemia in women [93]) may be primarily determined by visceral adiposity *per se*, rather than its underlying cause.

Adipocyte insulin resistance and impaired maturation has been demonstrated *in vitro* in the presence of indinavir, nelfinavir, ritonavir, saquinavir and amprenavir in the studies previously mentioned [72–77], suggesting that this is a class effect of PIs. As previously mentioned, ritonavir use appears to be associated with relatively greater increases in triglyceride levels compared with other PI drugs [10,11]. Ritonavir therapy in HIV-negative volunteers also induced marked elevations of triglyceride-enriched lipoproteins over a 2-week period (increased plasma triglyceride, VLDL cholesterol, IDL cholesterol and apolipoprotein B compared with controls and to baseline levels, $P<0.01$ for all values) [5]. Unlike other HIV-1 protease inhibitors, ritonavir appears to have activity as a proteasome inhibitor [94], and shows specificity for a proteasomal pathway involved in the degradation of SREBP-1 [95]. Accordingly, ritonavir appears to act mainly to increase hepatic triglyceride synthesis via increased stabilization of nuclear SREBP-1c [96], suggesting that its hypertriglyceridaemic activity may be due to the additive effect of hepatic and adipose targeting of ritonavir action. Similarly, it is possible that the rapid induction of insulin resistance in HIV-negative volunteers after 4 weeks' exposure to standard-dose indinavir therapy [6] may relate to the ability of this drug to impair insulin signalling in multiple insulin-responsive tissues, including liver [97] and skeletal muscle [98] as well as adipose tissue. The effects of other PI drugs are yet to be studied in these tissues.

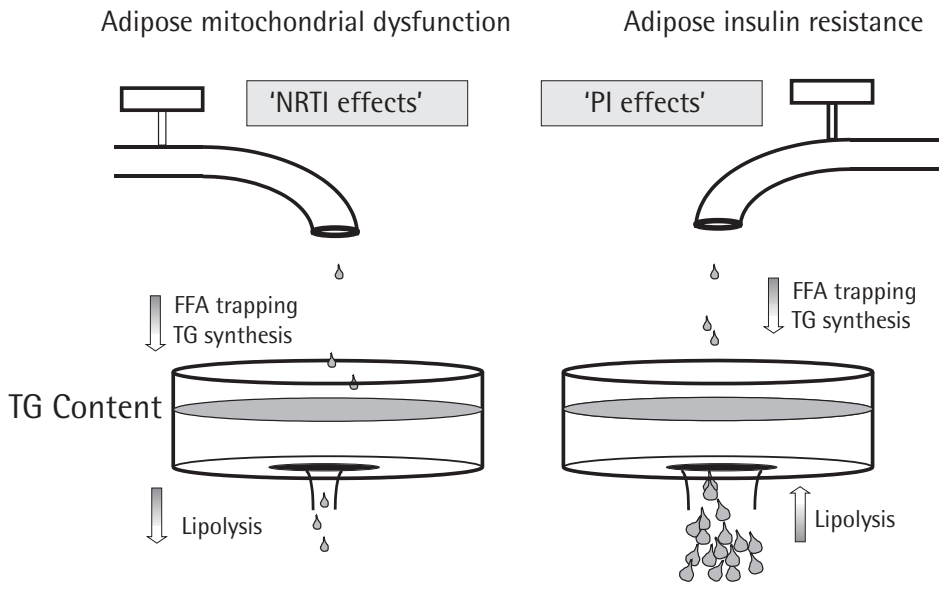
These *in vitro* studies have provided some critical insights into possible mechanisms underlying PI-induced adverse effects. It is hoped that *in vivo* studies that are informed by these results will be undertaken, in which involved tissue (for example, adipose tissue) is sampled and assessed. In this way, the clinical relevance of these findings (and the relative effects of select PIs within this class of drugs) may be determined.

Interactions of NRTIs and PIs – an 'adipocentric' model of lipodystrophy

Taken together, the proposed effects of NRTI-induced mitochondrial dysfunction and PI-induced insulin resistance/impaired maturation on adipocytes each contribute to a disorganized lipid metabolism that appears consistent with *in vivo* dynamic metabolism studies [66] (Figure 7). Loss of the lipogenic and triglyceride-storing capacity of adipose tissue is central to this effect, so that lipid metabolism is unable to respond appropriately to the 'fed' state. Uncontrolled fatty acid flux represents a response to loss of insulin signalling, producing an ongoing 'fasting' metabolic response in which fatty acid is liberated from adipose tissue, and fat oxidation is stimulated. While a diversion from triglyceride and fatty acid biosynthesis to oxidative pathways within the adipocyte appears to be common to the proposed NRTI and PI effects, it could be predicted that NRTI-induced mitochondrial dysfunction would lead to decreased lipolysis, while loss of insulin sensitivity would increase it. It is possible that the balance between these two effects may determine if the phenotype is predominantly 'lipoatrophic', where NRTI effects are prominent (with less evidence of insulin resistance clinically, although triglyceride levels may be higher [9,23]), or 'mixed', in which the presence of visceral fat accumulation and insulin resistance indicate that lipolysis is dysregulated and fatty acid flux is increased ('PI effect') [9,23].

Does this model provide an explanation for the fact that combining NRTI and PI therapy greatly increases the risk of developing subcutaneous fat wasting compared with the use of either drug class alone? One clue may be provided by the adipose tissue morphology that is seen in HAART-associated lipodystrophy (Figure 3), in which increased mitochondrial mass is accompanied by evidence of decreased cellular volume (redundant basal lamina) and loss of the

Figure 7. Dynamic regulation of intracellular triglyceride content



Intracellular triglyceride stores in adipocytes are determined by the dynamic equilibrium between triglyceride synthesis (in turn dependent on fatty acid uptake or re-esterification, and/or fatty acid synthesis from carbohydrate precursors, as well as assembly of fatty acids and glycerol to form triglyceride); and triglyceride lipolysis (by hormone sensitive lipase) and export of liberated fatty acids from the cell. In the presence of mitochondrial dysfunction ('NRTI effect'), both processes are inhibited while energy is conserved so that the adipocyte can meet its own bioenergetic requirements. This 'isolates' the cell from the demands of whole-body metabolism, and causes a shift from energy storage (as triglyceride) to energy use, including fat oxidation. Adipose insulin resistance ('PI effect') would be predicted to cause loss of the post-prandial insulin response in adipocytes, resulting in reduced triglyceride storage and increased fatty acid oxidation, decreased adipocyte differentiation, and inappropriate activation of lipolysis in the post-prandial phase.

central lipid store in favour of multiloculated intracytoplasmic deposits [47,48]. This phenotype is also observed in 'converted adipocytes', described by Himms-Hagen *et al.* [99], in which diversion of adipocyte metabolism towards fatty acid oxidation (accomplished by β_3-adrenoceptor agonists in this experimental setting) produces morphological changes in mature adipocytes consistent with those seen in lipodystrophy. These adipocytes are also characterized by increased expression of uncoupling protein-3 (UCP-3), a mitochondrial protein induced by increased cellular fatty acid levels [100]. These data suggest that increasing adipocyte oxidative metabolism, as is proposed for both NRTI and PI effects may: (1) increase adipocyte mitochondrial mass independent of the effects of mtDNA depletion or toxicity; (2) increase intracellular fatty acid levels; and thus (3) invoke mechanisms designed to limit cytotoxicity associated with excessive fatty acid concentrations. These responses may each contribute to enhancing tissue mitochondrial dysfunction and cellular toxicity. In particular, there is a well characterized mechanism whereby increased cellular fatty acid levels induce partial 'uncoupling' of mitochondrial respiration, involving UCP-3 as well as other members of a closely related family of mitochondrial inner membrane anion transporters [101]. 'Uncoupling' refers to an imperfect correlation between substrate use (glucose and fatty acids) and subsequent ATP production within mitochondria, so that excessive substrates can be 'burned' to produce heat rather than ATP. This regulated response minimizes the effects of excess fatty acids (for example, increased reactive oxidative stress and direct toxicity within mitochondria) while also avoiding excessive ATP production (which would inhibit multiple biochemical pathways within mitochondria) [101].

It may be proposed that the appropriate physiological responses that are evoked by impaired mitochondrial function induced by NRTIs (mediated by AMP-activated kinase), and by insulin resistance and increased fatty acid flux (mediated by fatty acid induced uncoupled respiration) are incompatible, and lead to a pathophysiological state in which the cell is unable to achieve energy homoeostasis. In this scenario, a 'vicious metabolic cycle' is created, in which restoration of the ADP/ATP ratio in response to mitochondrial dysfunction ('energy depletion') is subverted by an ongoing demand for decreased efficiency of mitochondrial respiration by increased fatty acids ('fuel excess').

A possible clinical correlate for this hypothesis has been provided by Kosmiski *et al.* [102], who demonstrated increased resting energy expenditure (REE) in patients receiving PI-based HAART, consistent with data in other insulin resistant lipodystrophy syndromes.

Elevated metabolic rate in HAART recipients had been noted in a previous report, in which no association between immunological or virological parameters and REE was found [103]. However, Kosmiski *et al.* have demonstrated that metabolic, rather than antiviral, effects of PI therapy appear to determine increased REE, which was found to be ~25% higher than predicted in patients receiving PI-based HAART with clinical evidence of lipodystrophy and ~6% higher in those without clinically apparent lipodystrophy [102]. This effect was not abrogated by adjustment for the expected influence of fat free mass. A strong negative correlation between accurately measured insulin sensitivity and REE was found (correlation coefficient =–0.68, $P<0.005$), and subsequent logistic regression analysis suggested that insulin resistance was the major determinant of REE independent of any effects of therapy on body composition (measured by DEXA scanning and lumbar computed tomography scans).

These findings may provide *in vivo* evidence of fatty acid-induced uncoupling, as increased metabolism in the resting state is likely to represent the consequences of uncoupled mitochondrial respiration, while accurately measured insulin resistance would be predicted to provide a marker of increased tissue fatty acid flux.

The role of inner mitochondrial membrane transporters such as the UCPs is also interesting from the point of view of NRTI effects. As stated previously, the characterization of a mitochondrial deoxynucleotide carrier with a high affinity for activated (triphosphorylated) NRTIs represents a major advance in this area of research [33]. This transmembrane protein has significant homology with other members of the mitochondrial membrane transporter family (including uncoupling proteins as well as transporters critical to mitochondrial respiratory chain function such as the ADP/ATP translocator), so it is conceivable that interactions with other transporters by NRTIs could influence fatty acid handling and mitochondrial function through direct non-'pol-γ' mechanisms. In this context, there is a body of evidence that zidovudine interacts with the muscle isoform of the ADP/ATP translocator, a factor that may have had a role in the pathogenesis of myopathy associated with this drug [104–106]. It is possible that direct dose-dependent NRTI effects on a transporter preferentially distributed in adipose tissue (for example, dicarboxylate carrier [107]) could explain why these hydrophilic drugs affect adipocytes preferentially.

Host factors

While this review has focused on the contributions of NRTI and PI therapy to the lipodystrophy syndrome, we do not wish to discount the important contribution of host factors in determining susceptibility, severity and phenotypic expression of the lipodystrophy syndrome. For example, gender and racial origin appear to influence the lipodystrophy phenotype, with an increased risk of subcutaneous fat wasting among white males, while women and non-white males appear to be more prone to develop visceral fat accumulation. This finding has been reviewed recently [108]. It is pertinent to mention, however, that much of the clinical data that have been presented in both this and a previous review [1] have been obtained from white male patient populations, and may therefore not apply universally.

The role of host factors is also demonstrated in studies in which polymorphisms in genes for the β_3-adrenergic receptor [109] (involved in regulating lipolysis in visceral fat) and for tumour necrosis factor-α cytokine [110] (which is secreted by adipocytes and has been implicated in the pathogenesis of insulin resistance [111]) have been associated with altered lipodystrophy outcomes. In the future, it is likely that further host factors will emerge that significantly influence susceptibility to lipodystrophy (for example, by preventing drug-specific toxic effects such as mitochondrial toxicity) as well as the severity of metabolic and/or morphologic consequences of the syndrome.

Conclusions

There is still much to learn of the mechanisms that are involved in the development of both morphologic and metabolic components of the lipodystrophy syndrome in individuals receiving HAART, and we look forward to the further elucidation of these factors at the cellular and subcellular level. While the proposed 'adipocentric' model is necessarily simplistic in its approach to the interaction between NRTIs and PIs, at this point it is sufficient to broadly explain the clinical phenotype. There is no doubt that compensatory as well as contributory effects in other tissues will play a role, and that host factors also influence susceptibility to, and phenotypic expression of, the syndrome. Characterizing these factors will certainly provide for a more comprehensive aetiopathogenic model.

In conclusion, NRTIs and PIs have specific affects on metabolism that are removed from their antiviral activity, and are synergistic in adipose tissue. The interaction of NRTIs and PIs induces a phenotype in which fat storage is diminished while fat use is enhanced, associated with loss of the usual metabolic responses to the 'fed' state. In this setting, reduction in subcutaneous adipose tissue mass is likely to be a consequence of adipose atrophy as well as adipocyte loss, while accumulation of fat in the visceral compartment represents a response to increased fatty acid flux and insulin

resistance. Further elucidation of the mechanisms involved will aid the development of appropriate therapeutic and management strategies, as well as guide the safer use of combination antiretroviral therapies.

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