

# Mitochondrial toxicity induced by nucleoside-analogue reverse-transcriptase inhibitors is a key factor in the pathogenesis of antiretroviral-therapy-related lipodystrophy

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Highly active antiretroviral therapy (HAART) can induce a characteristic lipodystrophy syndrome of peripheral fat wasting and central adiposity. HIV-1 protease inhibitors are generally believed to be the causal agents, although the syndrome has also been observed with protease-inhibitor-sparing regimens. Here, we postulate that the mitochondrial toxicity of the nucleoside-analogue reverse-transcriptase inhibitors plays an essential part in the development of this lipodystrophy, similar to the role of mitochondrial defects in the development of multiple symmetrical lipomatosis.

Since 1996, treatment with a combination of at least three antiretroviral drugs has become the standard of care for patients with HIV-1 infection in industrialised countries. In most cases, treatment regimens include two nucleoside-analogue reverse-transcriptase inhibitors (NRTIs), and one or more protease inhibitors. The use of these new highly active antiretroviral therapies (HAART) has led to unsurpassed reductions in HIV-1-related morbidity and mortality, which in substantial proportions of patients is sustained over the long term. Unfortunately, the widespread introduction of protease-inhibitor-containing therapies in clinical practice has also been associated with an increasing recognition of unusual adverse events, of which the lipodystrophy syndrome is one of the most debilitating and worrisome.

## Lipodystrophy

Although a consensus case-definition of the lipodystrophy syndrome has not yet been formulated, it seems to be characterised by a (self-reported) wasting of peripheral fat, mostly of the distal extremities and facial region; this is often combined with a more central accumulation of fat, which is especially noticeable in the dorsocervical area ("buffalo hump"),<sup>1,2</sup> the breasts,<sup>3</sup> and inside the abdominal cavity.<sup>4</sup> Hyperlipidaemia and insulin resistance are additional characteristics of the syndrome.<sup>5</sup> The clinical presentation indicates that there are differential effects on adipose tissue at different locations (subcutaneous vs intra-abdominal fat). Although the functional characteristics of the adipose tissue in these two compartments are different,<sup>6</sup> the molecular biology of the differentiation of these two types of adipocytes has not been fully characterised in non-HIV-1-infected

individuals. This has hampered the elucidation of the HAART-related lipodystrophy syndrome so far.

In most published reports, the suggestion is that protease inhibitors are the main cause of the development of the syndrome. Carr and colleagues have, so far, offered the most comprehensive hypothetical explanation for the pathogenesis of the syndrome.<sup>7</sup> Crucial to this hypothesis is the inhibition by protease inhibitors of several host-cell proteins involved in lipid and carbohydrate metabolism; this explanation is based on the substantial degree of aminoacid sequence homology between the HIV-1 protease and these human proteins. Carr and colleagues suggested that protease inhibitors induce apoptosis of peripheral adipocytes by binding cytoplasmic retinoic-acid binding protein-1, a molecule that mediates cis-9-retinoic acid stimulation of the retinoic X receptor, normally leading to adipocyte differentiation.<sup>7</sup> In addition, protease inhibitors may inhibit the synthesis of cis-9-retinoic acid that is catalysed by cytochrome P450-3A. Within adipocyte nuclei, cis-9-retinoic acid functions as a heterodimer with peroxisome proliferator activated receptor type gamma (PPAR- $\gamma$ ).<sup>7</sup> That PPAR- $\gamma$  is preferentially expressed in peripheral rather than central fat might explain the wasting of peripheral fat in particular. Abnormal release of fat from peripheral sites would lead to hyperlipidaemia. This release is reinforced by protease-inhibitor-mediated inhibition of yet another host protein—ie, lipoprotein receptor-related protein, which is involved in hepatic and endothelial clearance of chylomicrons and triglycerides. Impaired storage of peripheral fat by default may result in fat accumulation in the more central parts of the body.<sup>7</sup>

In-vitro, protease inhibitors seem to interfere in adipocyte metabolism via altered retinoid signalling,<sup>8</sup> but further proof for Carr's hypothesis is still missing. Furthermore, it does not explain reports of the lipodystrophy syndrome in HIV-1-infected patients who never used protease inhibitors.<sup>1,3,9</sup> This suggests that alternative or at least additional pathogenic mechanisms are involved.

## Benign or multiple symmetrical lipomatosis

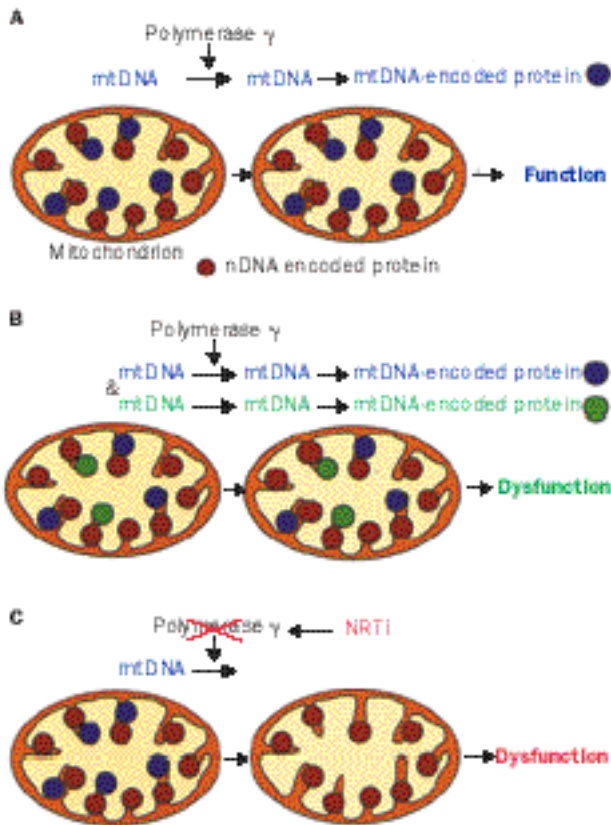
Recently, a similarity was noted between HAART-related lipodystrophy and benign or multiple symmetrical lipomatosis (MSL),<sup>10,11</sup> also called Madelung disease or

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#### Relation between mitochondrial DNA and mitochondrial function

In the normal situation (A) mitochondrial DNA (mtDNA) encodes for proteins (blue circles) in the respiratory chain, situated at the inner mitochondrial membrane. Most respiratory enzymes are encoded by nuclear DNA (nDNA) (red circles). Replication of mtDNA is regulated by the enzyme DNA polymerase  $\gamma$ . In inherited mitochondrial diseases (B), parts of mtDNA have been mutated or even deleted (green), which results in altered mtDNA-encoded proteins (green circles), leading to mitochondrial dysfunction. During NRTI treatment (C), DNA polymerase  $\gamma$  will be inhibited, leading to a depletion of mtDNA and mtDNA-encoded proteins and dysfunctioning mitochondria.

Launois-Bensaude adenolipomatosis. Clinically, this syndrome has been distinguished into two types. In MSL type 1, patients generally have a low body-mass index and show symmetrical accumulation of non-encapsulated masses of fatty tissue, especially in the subcutaneous regions of the neck and shoulders and inside the mediastinum; in addition there is pronounced atrophy of subcutaneous fat in the extremities, thereby resembling the HAART-related lipodystrophy. By contrast, patients with MSL type 2 are usually overweight and show a more diffuse lipomatosis. Since both MSL types 1 and 2 may be accompanied by hypertriglyceridaemia and insulin resistance, MSL has been termed a triglyceride storage disease.<sup>12</sup> This is supported by the observation that lipomatous tissue from patients with MSL shows a defective lipolytic response to adrenergic stimulation.<sup>13</sup> Since patients with MSL do not have abnormal intra-abdominal fat accumulation, MSL type 1 and HAART-related lipodystrophy may not be necessarily identical, but may merely represent different parts of a spectrum.

#### Aetiology

In addition to alcohol overconsumption, peripheral neuropathy is found in almost all patients with MSL,<sup>12</sup> and this feature points to the possibility that one of the main causes of MSL is mitochondrial dysfunction.<sup>14</sup> Several

reports have shown that in MSL there are point mutations at the nucleotide position 8344 in the mitochondrial DNA (mtDNA) or multiple or single mtDNA deletions, leading to an impaired function of the oxidative phosphorylation complex IV (cytochrome c oxidase).<sup>15-19</sup> Since brown adipose tissue has the highest content of mitochondria,<sup>20</sup> a defect in the enzymes of the respiratory chain can easily prompt a decrease in its high fat turnover, leading to the development of lipomas. Brown adipose tissue is particularly present in regions that are affected in MSL type I.<sup>13,21</sup>

#### Mitochondrial toxicity of NRTIs

Apart from the inherited mtDNA defects, depletion of mtDNA may also be acquired. The only enzyme that is responsible for mtDNA replication, DNA polymerase  $\gamma$ , is inhibited to a varying extent by NRTIs used in HAART.<sup>22,23</sup> Through this mechanism, NRTIs can easily induce depletion of mtDNA, resulting also in depletion of mtDNA-encoded mitochondrial enzymes and this will finally lead to mitochondrial dysfunction (figure). In fact, nearly all side-effects that have been attributed to the use of NRTIs, such as polyneuropathy, myopathy, cardiomyopathy, pancreatitis, bone-marrow suppression, and lactic acidosis, greatly resemble the spectrum of clinical manifestations seen in inherited mitochondrial diseases.<sup>23</sup>

A few studies have shown (with muscle biopsies) the occurrence of mitochondrial dysfunction during zidovudine monotherapy in selected patients with drug-induced myopathy.<sup>24,25</sup> Of the other NRTIs, mitochondrial toxicity has only been shown in vitro, when tested as single agents.<sup>23</sup> So far, there are no studies that have addressed this issue in clinical practice, but it is likely that a combination of NRTIs will synergistically give rise to any form of mitochondrial dysfunction.

#### Hypothesis

Since HAART almost always includes at least two NRTIs and since HAART-related lipodystrophy has been described in patients not taking protease inhibitors, but only NRTIs, we hypothesise that NRTIs have a key role in the pathogenesis of this syndrome. We propose that the mitochondrial toxicity of these drugs is the responsible mechanism, leading to similar metabolic disturbances as those found in MSL type I.

Protease inhibitors may very well aggravate this metabolic process through additional mechanisms, as suggested by others.<sup>7</sup> The use of NRTIs might even turn out to be the initiating essential factor, since HAART-related lipodystrophy was only observed in patients treated with protease inhibitors when they received NRTIs at the same time. The cause of HAART-related lipodystrophy would then be based on a multifactorial, cascading process, in which both NRTIs and protease inhibitors play a deleterious part.

#### Testing the hypothesis

First, a working case-definition of the syndrome needs to be established, enabling objective rating and quantification of the features. Although such a case definition was recently suggested,<sup>26</sup> during the 1st International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV (June 26-28, 1999, San Diego, USA) an attempt to reach a consensus upon the criteria was not successful. A link between the observed metabolic perturbations and the

changes in body composition was felt not to be definitely proven, and, therefore, it was felt better only to define a list with observational items. Future studies need to determine which items of the list might become criteria in the definition.

Second, prospective studies need to be done to show epidemiological evidence for the role of protease inhibitors, NRTIs, or a combination of both in the development of the syndrome. In these studies, either NRTIs or protease inhibitors have to be excluded. At present, there are only a few studies that have not included NRTIs. One such trial is the Prometheus study,<sup>27</sup> which studied a double protease inhibitor combination (saquinavir/ritonavir) with or without the NRTI stavudine. Although it was not evaluated systematically, the investigators had the impression that lipodystrophy was seen more often in patients tested with stavudine than in patients treated with protease inhibitors alone (E H Gisolf, Academic Medical Center, Amsterdam, Netherlands). Since NRTIs will remain the cornerstone of HAART in the near future, it will be difficult to study this issue in a proper, prospective fashion.

There are several studies of protease-inhibitor-spacing regimens, in which double NRTI plus non-NRTI combinations, or even triple NRTI combinations are used. These studies might disclose to what extent NRTIs alone can induce the syndrome. During the Lipodystrophy Workshop in San Diego (see above), data from observational cohorts were presented that show a strong association between the time of exposure to NRTIs and the development of the syndrome.<sup>28-30</sup> Furthermore, more cases were presented of lipodystrophy features in protease-inhibitor-naïve patients,<sup>28,31,32</sup> in one of these studies the physical features of the syndrome seemed to be indistinguishable from the lipodystrophy in patients treated with protease inhibitors, although hyperlipidaemia and insulin-resistance occurred less frequently.<sup>28</sup> Recently, Saint-Marc and colleagues claimed a special role for stavudine over and above other NRTIs in the development of lipodystrophy,<sup>33</sup> and, during the workshop, reversibility of peripheral fat wasting upon interruption of stavudine therapy only was described.<sup>34</sup>

Third, the occurrence of mitochondrial dysfunction during (combination) NRTI therapy has to be further investigated. Most likely, this needs to be done at tissue level, since the different tissue-specific pharmacodynamics of every NRTI and the different metabolic characteristics of the specific cell-types produce different tissue-specific toxicity profiles of every individual NRTI.<sup>22,23</sup> Invasive procedures, such as biopsies of muscle, nerve, or adipose tissue, are not suitable for large-scale, prospective investigations, so animal studies might better serve this purpose. Furthermore, in-vitro studies on different cell-lines, including adipocytes, might further elucidate the extent of mitochondrial dysfunction induced by NRTIs individually or in combination.

Finally, even if mitochondrial dysfunction can be shown in adipose tissue, it remains to be proven that this event leads to the clinically observed HAART-related lipodystrophy. To clarify in vivo the molecular mechanisms involved, animal experiments are inevitable: the lipodystrophy syndrome takes months to develop and short-term in-vitro experiments may therefore not be appropriate. Studies in mice with targeted gene disruptions might prove valuable in this respect.

Assays have to be developed so that the mitochondrial

dysfunction can be assessed at tissue-specific levels— not only to prove the proposed hypothesis, but also, more importantly, to detect mitochondrial dysfunction during NRTI therapy early enough to prevent deleterious side-effects. Only then will HAART have the potential to become a truly long-term successful treatment.

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## Essay

# The hunt for *Clostridium difficile*: 21-year follow-up of a stool specimen sent for culture

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“I once was lost, but now am found, was blind, but now I see.”

Most doctors have felt angry on at least one occasion upon learning that a patient's specimen, having been sent to a laboratory for an important test, has been lost in transport. They have also experienced frustration, because there has been little they could do about it; on a less emotional level, they have wondered where the specimen could have gone. Here, we report the fate of a lost stool specimen from one of our patients, who was the subject of a report in *The Lancet* in 1977 about the cause of pseudomembranous colitis.<sup>1</sup> We now know what happened to that stool. Our findings provide an interesting explanation for what was for us, but not the patient, a painful scientific mishap. We also learned that our lost specimen harboured what was destined to be the first isolate of *Clostridium difficile* received by the Centers for Disease Control and Prevention (CDC) in Atlanta, GA, USA.

In 1974, antibiotic-associated pseudomembranous colitis of unknown aetiology became a serious problem. In our 1977 paper, we reported that the stools of two patients with pseudomembranous colitis contained a cytotoxin that was neutralised by *Clostridium sordelli* antitoxin, and that an identical cytotoxin could be found in the stools of hamsters dying with antibiotic-induced caecitis.<sup>1</sup> These findings suggested to us that one or more species of toxigenic clostridia were the probable cause of the disease, but when we submitted our report we had

not yet had an opportunity to isolate the organism that produced the cytotoxin.

One of the patients in that report is the focus of this follow-up. She was a 13-year-old girl with fulminant hepatitis and severe encephalopathy. She was admitted on Feb 5, 1977, to a distant hospital affiliated with the University of Michigan Medical School. Exchange transfusions were done, and within 30 h she was fully conscious, with improving hepatic function. She developed a urinary-tract infection in hospital and was treated with ampicillin, after which she developed fever, abdominal pain, and diarrhoea. Sigmoidoscopy with biopsy on April 13, 1977, showed pseudomembranous colitis. Because of the possibility that staphylococci might have been the cause of the colitis, oral vancomycin (500 mg every 6 h) was begun and continued for 10 days. Although *Staphylococcus aureus* was not isolated from her stools, the diarrhoea, abdominal pain, and fever resolved within 48 h. She eventually recovered completely from both hepatitis and colitis.

Before treatment with vancomycin, a stool specimen had been sent to our colitis research laboratory at the University of Michigan; this stool was found to contain a cytotoxin that was neutralised by *C sordelli* antitoxin. A different stool (the focus of this report) had been sent to the clinical microbiology laboratory at the University of Michigan Hospital for isolation and identification of clostridia. However, this specimen was lost during transport. Nevertheless, our report suggested two things. First, vancomycin given orally seemed effective in the treatment of pseudomembranous colitis of unknown aetiology. Second, a cytotoxic species of *Clostridium* was the probable cause of the disease. We now know that the *C sordelli* antitoxin was neutralising the cytotoxin B of *C difficile*.

A short time after we reported our findings in 1977, several laboratories, including ours, were able to show that *C difficile* was the cause of pseudomembranous colitis in both human beings and hamsters. This organism has subsequently increased in frequency as a cause of colitis

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