Metabolic complications of antiretroviral therapy

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Mitochondrial DNA content in blood and subcutaneous fat of HIV-1 infected patients randomly allocated to zidovudine- or stavudine-based therapy: possible implications for lipodystrophy pathogenesis


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Introduction

Loss of subcutaneous fat (lipoatrophy) in the face, extremities, and buttocks, together with intra-abdominal and sometimes dorsocervical fat accumulation, is an important, potentially stigmatizing, adverse effect associated with antiretroviral treatment for HIV-1 infection. Apart from changes in body fat distribution, this lipodystrophy syndrome often includes the presence of dyslipidemia and insulin resistance.[1]

Increasing evidence suggests that both protease inhibitors (PI) and nucleoside analogue reverse transcriptase inhibitors (NRTI) contribute to the pathogenesis of the syndrome. [1-8] In addition, immune recovery resulting from suppression of HIV-1 infection may also be involved. [9]

NRTIs inhibit DNA polymerase-γ, the key cellular enzyme regulating mtDNA replication. They may thereby aggravate any prior deleterious effect on mitochondrial DNA content and possibly function resulting from HIV-1 itself, which has been suggested to occur in peripheral blood mononuclear cells (PBMC) obtained from patients and in tissue culture.[10-12] The various individual NRTIs markedly differ in their capacity to inhibit mtDNA replication and mitochondrial function both in tissue culture and animal models. [10,11,13,14] Recently, we and others have hypothesized that NRTIs may contribute to the onset of lipoatrophy by inducing mtDNA depletion within peripheral adipocytes, ultimately resulting in mitochondrial dysfunction and apoptosis of these cells. [15,16]

Several observational cohort studies [3,5,7], have indeed demonstrated the prevalence of lipodystrophy, and particularly lipoatrophy, to be higher in patients on stavudine (d4T)- compared to zidovudine (ZDV)-containing antiretroviral therapy. Similar results have been reported for patients assessed long-term after randomization to either d4T- or
ZDV-based therapy.[17,18] All of these findings were based solely on clinical assessment of lipodystrophy by treating physicians.

A number of cross-sectional studies have reported mtDNA content of subcutaneous fat biopsies to be lower in patients currently receiving NRTIs [19], and in particular dideoxynucleosides [20], than in those not currently on NRTIs, as well as in patients with as compared to without clinical lipoatrophy.[20-22] One study showed that mtDNA content was less in peripheral adipocytes from patients currently using d4T than in those on ZDV.[23] Similar relationships have thus far not been demonstrated in PBMC [20,24,25].

We comprehensively assessed the presence of lipodystrophy by both standardized clinical and radiographic means, in all traceable and consenting patients several years after their participation in a randomized open-label comparative trial of first-line combination treatment with d4T or ZDV both in combination with lamivudine (3TC). Employing a novel technique for quantification of mtDNA, mtDNA content was assessed both in PBMC and in subcutaneous adipose tissue.


Study design and participants

All patients in the current study had originally participated in an open label randomized controlled trial in treatment naïve patients of standard dose ZDV plus lamivudine (3TC) versus d4T plus 3TC, for 24 weeks with a subsequent extension to 72 weeks, the results of which have been previously published.[26,27] If plasma HIV-1 RNA at week 8 was found to be above 500 copies/ml, indinavir 800 mg tid could be added to the regimen from week 12 onwards. Two of the forty-seven randomized patients withdrew informed consent prior to the start of treatment. Recruitment into the original study lasted from July 1996 until March 1997.

Patients were eligible to participate in the current cross-sectional study if they had started randomly allocated study medication in the past. All trial participants who could be traced were approached for participation regardless of current and past treatment. Clinical assessment was performed by one study physician (MvdV) who was blinded both for the patient's current and past antiretroviral treatment history, and for any history of changes in body appearance since the initiation of treatment. The study was conducted at and approved by the institutional review board of the Academic Medical Center in Amsterdam. All subjects provided written informed consent.

Assessments

Body appearance and composition

The study physician completed a standardized questionnaire to assess the distribution of fat in different body regions (face, neck, arms, legs, buttocks, breasts and abdomen), scoring each on a seven-point scale, going from very thin (1), thin evident to others (2), moderately thin, only visible if closely looked for (3), normal (4), moderately thick, only visible if looked for (5), thick evident to others (6), and very thick (7). Fat
accumulation was defined to be present if either the neck, breasts or abdomen were scored as thick or very thick (code 6 or 7). Similarly, lipoatrophy was judged to be present when legs, arms, buttocks or face were scored as thin or very thin (code 1 or 2). A patient was considered to have clinical lipodystrophy if fat accumulation and/or lipoatrophy as defined above were present. In addition the presence of lipodystrophy was also assessed according to a recently published lipodystrophy case definition. [28]

Waist and hip circumference and skin fold thickness were measured at four sites (biceps, triceps, subscapular and suprailiacal) using a Holtain LTD\textsuperscript{C} Skinfold Caliper. Total and regional fat mass was quantified by dual-energy x-ray absorptiometry (Hologic QDR-4500W, software version whole body v8.26A: 5) providing a quantitative assessment of peripheral and truncal fat mass in kilograms. The ratio between peripheral fat mass, defined as the sum of arm and leg fat, and total fat mass (total fat mass minus head fat) was calculated to adjust for differences in body weight. A standardized single slice abdominal CT-scan through the level of the fourth lumbar vertebra was performed from which the surface of total (TAT), visceral (VAT) and subcutaneous adipose tissue (SAT) was determined and expressed in square centimeter (cm\textsuperscript{2}). The ratio between SAT: TAT was calculated to assess fat distribution.

\textit{Adherence}

Adherence to antiretroviral medication was assessed by a self-report questionnaire, as described previously. [29]
Mitochondrial DNA quantification

Nucleic acids were isolated using the Boom method [30] from viably frozen PBMC and from snap frozen subcutaneous fat biopsies, taken from the inner side of the right thigh and from the lumbar region of the back, using a 4 millimeter punch biopsy needle. Prior to isolation of nucleic acids PBMC were microscopically checked for contamination with platelets. Using standard procedure for isolation of PBMC from heparinized blood the contamination with platelets was less than 5 platelets per PBMC, a level that does not alter the result of mtDNA quantification.[31] Isolated nucleic acids equivalent to 3,000 cells were used as input in the amplification reaction. The amplification of both mtDNA and nuclear DNA (nDNA) was performed by a real-time duplex Nucleic Acid Sequence-Based Amplification (NASBA) in a single tube. [32,33] Detection of the amplification products occurred real-time by the use of mitochondrial and nuclear specific molecular beacons in a thermostated fluorimeter. Reactions with mixtures of plasmids containing mtDNA and nDNA in different ratios, equivalent from 20 to 800 copies of mtDNA per cell, were used for calibration. The mtDNA content of each sample was expressed as the number of copies of mtDNA per cell (Retina™ Mitox assay, Primagen, Amsterdam, the Netherlands).

Mitochondrial DNA was also assessed in PBMC, which had been obtained and cryopreserved in the past from all patients prior to their enrolment into the original trial.

Statistical analysis

The principle of intention-to-treat was applied in the analysis. The cumulative exposure to PI as a class, and to NVP was calculated for each patient, and expressed as cumulative exposure to either in months.
All results listed in table 1 are compared between the treatment arms using a two-sided Student's T-test with the exception of the number of patients in each arm exposed to PI and nevirapine, respectively, in which a Fisher's exact test was used. The presence of lipoatrophy as assessed by questionnaire, and lipodystrophy according to the lipodystrophy case definition, was compared between treatment arms with the Chi-square test.

For the analysis of mtDNA in PBMC and adipose tissue results were logarithmically transformed in order to obtain normal distributions. Correlation coefficients were calculated and expressed as $r^2$ to evaluate any relation between mtDNA in PBMC or fat biopsies on the one hand, and the percentage peripheral fat by DEXA scan as well as the ratio of subcutaneous adipose tissue (SAT) over total adipose tissue (TAT) by CT-scan, on the other hand. All results are expressed as medians and interquartile ranges (IQR).

**Role of the funding source**

The funding source of the study had no role in study design, data collection, data analysis, data interpretation, or in the writing of the report.
Results

Patient characteristics and treatment disposition

Twenty-eight of the 45 patients who had started randomized treatment in the past could be enrolled. Seven patients had moved and could not be traced (5 patients from the ZDV- and 2 from the d4T-arm) and 10 did not consent to the current protocol (7 patients from the ZDV- and 3 from the d4T-arm). At the time of randomization those participating in the current study did not differ with respect to gender, age, BMI, CD4-cell count and plasma HIV-1 RNA from patients not presently participating (data not shown). Seventy-seven percent of those randomized to d4T (n = 17) participated in the current study as compared to 48% of patients randomized to ZDV (n = 11) (p=0.04). These two latter groups of patients did not differ significantly in age, BMI, CD4-cell count and HIV-1 RNA viral load at the time of enrollment in the original trial (d4T-vs. ZDV: median age 40 (34 - 51) vs. 36 years (32-46); BMI 23.0 (21.3 - 24.2) vs. 23.2 kg/m² (21.6 - 24.4); CD4 – cell count 400 x 10⁶ (260 - 440) vs. 300 x 10⁶ cell/mm³ (250 – 420), and HIV-1 RNA 5.0 (4.3 – 5.1) vs. 5.0 log₁₀ copies/ml (4.4 – 5.1). All patients were clinically stable at the time of the current assessment.

In the d4T-arm the median cumulative exposure to d4T was 51 months. In 15 of the 17 patients treatment had been intensified with a protease inhibitor (PI). Two of the 17 patients in the d4T-arm continued treatment with just d4T/3TC and maintained adequate virus suppression. One of the patients in the d4T-arm, 11 months prior to the current study, switched to a ZDV-based regimen because of lipodystrophy following 47 months of treatment with d4T. At the time of the current assessments 9/15 patients in the d4T-arm were still receiving a PI-based regimen. In 5/15 of the other patients their PI had been replaced by the non-nucleoside reverse transcriptase inhibitor (NNRTI) nevirapine for a median duration of 24 months. The one
remaining patient discontinued all antiretroviral treatment 26 months prior to the current assessment.

**Figure 1** Histogram of complete antiretroviral treatment history of all 28 patients from the time of randomisation in the original clinical trial until inclusion in the current cross-sectional study.
Stavudine-arm

The end of the treatment bars indicates the time of the current assessment. Patients indicated with a vertical line stopped taking all antiretroviral therapy prior to this point, and were assessed at the time indicated by the line. LD quest: lipodystrophy according to our questionnaire, LD case def.: lipodystrophy according to lipodystrophy case definition study. Rx: antiretroviral treatment.
In the ZDV-arm (n=11) the median cumulative exposure to ZDV was 50 months (p = 0.34 when compared to the cumulative d4T-exposure in the d4T arm). In all 11 patients treatment was intensified with a PI. Two of the 11 patients in the ZDV-arm switched to a d4T-based regimen after 10 and 31 months of exposure to ZDV, respectively. At the time of the current assessment 3/11 patients were being treated with a PI-based regimen. The 8 remaining patients had replaced their PI by nevirapine (p = 0.05 when compared to the d4T arm) a median of 19 months prior to being assessed for the current study. One of these 8 patients discontinued all antiretroviral therapy 2 months prior to the current study. The complete treatment history of all patients is shown in figure 1.

**Body appearance, body composition and metabolic assessments (Table 1)**

By questionnaire, 14 out of 17 patients (82%) originally randomized to the d4T-arm were considered to have lipoatrophy, compared to only 1 out of 11 patients (9%) in the ZDV-arm (p = 0.0001). Two of the patients with lipoatrophy in the d4T-arm compared to none in the ZDV-arm were also scored as having fat accumulation. None of the patients were judged to have lipodystrophy solely because of fat accumulation without lipoatrophy. Similarly, when using the recently published lipodystrophy case definition, significantly more patients allocated to d4T were scored as having lipodystrophy. (88% vs. 45%, p = 0.03). The presence or absence of lipodystrophy according to both methods is shown for each individual patient in figure 1. With respect to patients in whom PIs had been replaced by nevirapine, all 5 patients in the d4T arm were scored as having lipoatrophy, compared to only one of 8 patients in the ZDV-arm.

Patients randomized to the d4T-arm had both significantly less peripheral fat by DEXA scan (p= 0.005), and less SAT CT-scan (p = 0.04). Consistently, they also had lower peripheral over total fat (p=0.04), lower SAT/ TAT ratios (p = 0.005), and smaller skin-folds by
antropometry at the level of the biceps and triceps ($p = 0.002$ and $p = 0.02$, respectively). There was no relation between the cumulative exposure to PI and NVP, respectively, (figure 2) and the amount of peripheral fat by CT- and DEXA-scan. Treatment adherence did not differ significantly between patients in the two groups. (data not shown).

**Figure 2** Assessment of peripheral fat both by DEXA and CT scan in relation to cumulative exposure to protease inhibitor (PI) and nevirapine (NVP), respectively.

SAT: subcutaneous adipose tissue; TAT: total adipose tissue; %: percentage; CT scan data available from 16 patients in the d4T arm and 11 in the ZDV arm.
Table 1: Body appearance, body composition and metabolic assessments of participants from current study

<table>
<thead>
<tr>
<th></th>
<th>D4T-arm (n = 17)</th>
<th>ZDV-arm (n = 11)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>44 (39 - 55)</td>
<td>40 (37 - 50)</td>
<td>0.4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.8 (21.8 - 23.8)</td>
<td>23.0 (21.0 - 24.7)</td>
<td>0.98</td>
</tr>
<tr>
<td>Patients still on randomized NRTI backbone (n)</td>
<td>15</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>- cum. exp. to d4T (months/pt.)</td>
<td>51 (49 - 54)</td>
<td>32 (22 - 43)</td>
<td></td>
</tr>
<tr>
<td>- cum. exp. to ZDV (months/pt.)</td>
<td>11</td>
<td>50 (45 - 53)</td>
<td></td>
</tr>
<tr>
<td>Patients exposed to PIs (n)</td>
<td>15</td>
<td>11</td>
<td>0.51</td>
</tr>
<tr>
<td>- cum. exp. to PIs (months/pt.)</td>
<td>45 (24 - 48)</td>
<td>31 (21 - 53)</td>
<td></td>
</tr>
<tr>
<td>Patients exposed to NVP (n)</td>
<td>5</td>
<td>8</td>
<td>0.05</td>
</tr>
<tr>
<td>- cum. exp. to NVP (months/pt.)</td>
<td>24 (17 - 25)</td>
<td>19 (17 - 26)</td>
<td></td>
</tr>
<tr>
<td>% pt. HIV-1 RNA &lt;50 c/ml</td>
<td>71%</td>
<td>64%</td>
<td>0.7</td>
</tr>
<tr>
<td>CD4-cell count (x10⁶/mm³)</td>
<td>690 (510 - 780)</td>
<td>620 (450 - 710)</td>
<td>0.4</td>
</tr>
<tr>
<td>Platelet count (x 10⁹/l)</td>
<td>226 (176 - 242)</td>
<td>219 (202 - 259)</td>
<td>0.44</td>
</tr>
<tr>
<td>No (%) pt with lipodystrophy according to questionnaire</td>
<td>14 (82%)</td>
<td>1 (9%)</td>
<td>0.0001</td>
</tr>
<tr>
<td>No (%) pt with lipodystrophy according to case def score</td>
<td>15 (88%)</td>
<td>5 (45%)</td>
<td>0.03</td>
</tr>
<tr>
<td>Waist to Hip ratio</td>
<td>0.94 (0.91 - 0.97)</td>
<td>0.95 (0.93 - 1.04)</td>
<td>0.42</td>
</tr>
<tr>
<td>biceps circumference (mm)</td>
<td>41 (36 - 46)</td>
<td>60 (44 - 75)</td>
<td>0.002</td>
</tr>
<tr>
<td>triceps circumference (mm)</td>
<td>52 (44 - 65)</td>
<td>73 (58 - 94)</td>
<td>0.02</td>
</tr>
<tr>
<td>suprailiacal circumference (mm)</td>
<td>81 (68 - 101)</td>
<td>146 (111 - 186)</td>
<td>0.001</td>
</tr>
<tr>
<td>Subscapular circumference (mm)</td>
<td>142 (118 - 158)</td>
<td>132 (105 - 191)</td>
<td>0.28</td>
</tr>
<tr>
<td>SAT (cm²)</td>
<td>75 (54 - 95) (n = 16)</td>
<td>131 (105 - 149)</td>
<td>0.04</td>
</tr>
<tr>
<td>VAT (cm²)</td>
<td>142 (91 - 208) (n = 16)</td>
<td>136 (78 - 202)</td>
<td>0.48</td>
</tr>
<tr>
<td>SAT:TAT</td>
<td>0.35 (0.30 - 0.44) (n = 16)</td>
<td>0.50 (0.41 - 0.62)</td>
<td>0.005</td>
</tr>
<tr>
<td>peripheral fat in kg (DEXA)</td>
<td>2.4 (1.9 - 3.2)</td>
<td>4.8 (3.2 - 6.3)</td>
<td>0.005</td>
</tr>
<tr>
<td>peripheral:total fat (DEXA)</td>
<td>0.33 (0.27 - 0.38)</td>
<td>0.37 (0.34 - 0.49)</td>
<td>0.04</td>
</tr>
<tr>
<td>% total fat DEXA</td>
<td>13.2 (12.6 - 16.8)</td>
<td>18.4 (14.0 - 25.7)</td>
<td>0.17</td>
</tr>
<tr>
<td>mtDNA content / cell in PBMC at baseline</td>
<td>362 (300 - 464)</td>
<td>322 (248 - 452)</td>
<td>0.39</td>
</tr>
<tr>
<td>mtDNA content / cell in PBMC current study</td>
<td>96 (84 - 118)</td>
<td>120 (108 - 147)</td>
<td>0.02</td>
</tr>
<tr>
<td>mtDNA content / cell in fat biopsies from the back</td>
<td>571 (440 - 760) (n = 15)</td>
<td>707 (542 - 802) (n = 8)</td>
<td>0.23</td>
</tr>
<tr>
<td>mtDNA content / cell in fat biopsies from the leg</td>
<td>488 (387 - 748) (n = 15)</td>
<td>702 (599 - 748) (n = 8)</td>
<td>0.12</td>
</tr>
<tr>
<td>lactate (mmol/l)</td>
<td>1.3 (1.0 - 1.9)</td>
<td>1.1 (0.7 - 1.3)</td>
<td>0.34</td>
</tr>
</tbody>
</table>

D4T: stavudine; ZDV: zidovudine; n: number of participants; yrs: years; kg: kilogram; m²: meter square; cum. exp.: cumulative exposure; pt: patient; c/ml: copies per milliliter; mm³: cubic millimeter; pmol/l: picomol per liter; SAT: subcutaneous adipose tissue; VAT: visceral adipose tissue; TAT: total adipose tissue; SAT:TAT: the ratio of SAT over TAT; kg: kilogram; DEXA: dual-energy x-ray absorptiometry; mtDNA: mitochondrial DNA; PBMC: peripheral blood mononuclear cells; mmol/l: millimol per liter; t: time; min.: minutes; pmol/l: picomol per liter; Results expressed as medians with interquartile ranges.
Mitochondrial DNA

The median mtDNA content in PBMC cryopreserved prior to initiation of antiretroviral therapy did not differ between treatment arms for patients included in the current study. (table 1) Likewise, at randomisation there was no difference in PBMC mtDNA content between patients who were and were not included in the current study (data not shown; p =0.97). In both arms mtDNA in PBMC at the time of the present study was significantly lower compared to before the start of treatment. In the d4T arm the calculated proportional mtDNA decrease in PBMC was significantly greater when compared to the ZDV arm (73% (67-79) versus 63% (56 -73), p =0.01, respectively), resulting in 96 copies/PBMC in the d4T arm and 120 copies/PBMC in the ZDV arm (p = 0.02). The amount of mtDNA per cell in the subcutaneous adipose tissue biopsies taken both from the thigh (p=0.12) and back (p=0.23) did not differ significantly between the d4T- and the ZDV-arm. The patients with -as opposed to those without- lipoatrophy according to our questionnaire, had significantly lower mtDNA content in PBMC (p = 0.002), but not in subcutaneous fat biopsies taken from either the thigh or back (Figure 3).

A significant, but modest, inverse relation was found between the amount of mtDNA in PBMC ($r^2 = 0.30$, $p = 0.003$) as well as in biopsies from the thigh ($r^2 = 0.31$, $p = 0.007$), but not from the lower back, on the one hand, and the severity of lipoatrophy assessed by CT-scan (SAT/TAT) on the other hand. Similar relations both for PBMC ($r^2 = 0.15$, $p = 0.04$) and thigh biopsies ($r^2 = 0.17$, $p = 0.049$) were observed when fat distribution was assessed by DEXA scan and expressed as the percentage peripheral of total body fat.

The mtDNA content in PBMC correlated significantly with mtDNA content in the biopsies taken from the back ($r^2 = 0.36$, $p = 0.002$), but not with those taken from the thigh ($r^2 = 0.076$, $p = 0.2$). There was no significant relation between mtDNA content of fat biopsies taken from
the thigh and back, respectively ($r^2 = 0.14, p = 0.08$).

**Figure 3** Mitochondrial DNA content in PBMC, as well as in thigh and back subcutaneous adipose tissue biopsies at the time of the current study, both according to randomly allocated treatment and according to the presence or absence of lipoatrophy as determined by standardized questionnaire.

Subcutaneous fat biopsies from the thigh and back were available from 15/17 patients in the d4T arm and from 8/11 patients in the ZDV-arm.

PBMC: peripheral blood mononuclear cells; leg: subcutaneous fat biopsies taken from the thigh; back: subcutaneous fat biopsies taken from the lumbar region of the back; d4T: stavudine; ZDV: zidovudine; y-axis mitochondrial DNA copies per cell; atrophy: the presence of lipoatrophy according to the questionnaire.
Discussion

Previous studies have found NRTIs to contribute to the development of ART-associated lipodystrophy, and lipoatrophy in particular. These reports were based on assessment of changes in body appearance by physicians and patients. [1-8,17,18] In most of these studies, the use of stavudine was associated with a greater risk of developing lipoatrophy, when compared to that of zidovudine. Our study confirms these observations in a group of patients who on average 4 years before had been randomly allocated to initiate antiretroviral treatment containing stavudine or zidovudine, and who at the time of assessment had been exposed for the same length of time to either of these two NRTIs. A single physician, blind to patients' prior and current antiretroviral treatment history, judged significantly more patients allocated to stavudine as having lipodystrophy and more specifically lipoatrophy. Importantly, clinical judgement was confirmed by objective measurements of body fat distribution. DEXA- and CT-scan both demonstrated patients who had been randomized to stavudine- as opposed to zidovudine-containing therapy to have significantly less peripheral fat, both in absolute terms and when measured relative to total body fat. The difference between patients allocated to stavudine or zidovudine was likewise noted when patients were assessed according to a recently published validated case definition of lipodystrophy [28]. Interestingly, when using this definition more patients (5 on zidovudine but only one on stavudine) were scored as having lipodystrophy than when judged by one of the participating physicians (MvdV). This may imply that the validated case definition has a greater ability to diagnose patients with lipodystrophy of lesser severity. Our finding that this applied particularly to patients on zidovudine seems to be consistent with recently presented results from two prospective longitudinal studies showing that objectively measured loss of peripheral fat is a gradually progressive phenomenon, which does occur both in patients on zidovudine- and stavudine-containing ART, but is less severe and of slower onset in the former.[34,35]
Lipoatrophy severity, assessed both by DEXA- and CT-scan, showed a statistically significant inverse correlation with the mtDNA content in subcutaneous adipose tissue from the thigh and in PBMC. This could be interpreted as evidence that NRTI-induced mitochondrial DNA depletion of adipose tissue is causally linked to the development of lipoatrophy. However, both in view of the modest correlation observed and the lack of a significant difference in mtDNA content of adipose tissue from a clinically markedly affected site such as the thigh between patients with or without lipoatrophy, one could also argue that any such causal relationship could at most be partial. Similar reasoning may be applied when interpreting the lack of difference in mtDNA content of adipose tissue from patients allocated to stavudine or zidovudine, in spite of the lipoatrophy prevalence being highly significantly different between both groups. Differing from our results, two studies did report a lower mtDNA content of adipose tissue in patients with as opposed to those without lipodystrophy. [19,22] Those findings may however have been biased by the fact that in both studies patients with lipodystrophy had been exposed significantly longer to NRTIs than those without lipodystrophy.

In contrast to what was found in adipose tissue, the mtDNA in PBMC was significantly lower, both in those with compared to without lipoatrophy (p=0.002) and in those allocated to stavudine as opposed to zidovudine (p=0.02). MtDNA in PBMC had decreased significantly in both treatment groups compared to before the initiation of antiretroviral therapy, with the proportional decrease from baseline being 73% for patients on stavudine versus 63% on zidovudine (p=0.01).

Our study has several possible limitations. First, the cross-sectional nature does not allow an assessment of changes in either fat distribution or mtDNA content of adipose tissue from before treatment. Second, only 62% of patients recruited into the original clinical trial were included in the current study. They did however not differ
Lipoatrophy in relation to mitochondrial DNA

significantly before the start of treatment from those not presently included with respect to demographic characteristics and markers of HIV-1 disease progression. Furthermore, more patients were included who had originally been allocated to stavudine than to zidovudine (77 versus 48%). This difference however largely resulted from more patients in the ZDV-arm having moved who thereby could not be traced (21% versus 9% in the d4T - arm). Third, more patients in the d4T-arm maintained the PI in their regimen and did not switch to nevirapine, as compared to those in the ZDV-arm (p =0.05). In view of earlier reports suggesting that PIs as a class are involved in the development of lipodystrophy[1,4,5,7], this could have contributed to the difference in lipodystrophy prevalence observed between both patient groups. There was however no significant difference in the median duration of exposure to PIs between the two patient groups, and no relation was demonstrated between the duration of exposure to either PI or NVP and peripheral fat mass assessed by CT- and DEXA-scan (Figure 2). Nevertheless, given the cross-sectional nature of the study we cannot rule out that the different degree of replacing PI by NVP may have contributed to the difference in lipoatrophy observed between both groups. However, the finding that all 5 patients in the d4T-arm who replaced PI by nevirapine were judged to have lipoatrophy as compared to only one of 8 patients in the ZDV-arm, does support the notion that d4T is associated with a higher risk of lipoatrophy development than zidovudine. Finally, mtDNA was assessed in subcutaneous adipose tissue samples without the prior removal of cells other than adipocytes, including stromal and vascular cells. The relative abundance of such cells together with the proliferation of mitochondria within remaining adipocytes in subcutaneous adipose tissue from patients on stavudine, as recently reported by Nolan et al[23], may have confounded the assessment of mtDNA content within actual adipocytes in our study.

In conclusion, our study in a group of patients who had been randomly allocated to treatment provides objective confirmation for regimens containing stavudine to be associated with a greater risk of lipoatrophy
compared to those containing zidovudine. They also indicate that mtDNA depletion in PBMC and lipoatrophy are likely to both be NRTI-associated phenomena, and to generally be more severe in patients on stavudine. The lack of observing a significantly reduced mtDNA content in adipose tissue from patients allocated to stavudine and from those with lipoatrophy may have been confounded by a relative preponderance of stromal-vascular tissue in the subcutaneous tissue samples from these patients, combined with compensatory mitochondrial proliferation in their remaining fewer adipocytes. Preferential mitochondrial DNA depletion within adipocytes of patients on stavudine having undergone apoptosis thereby resulting in lipoatrophy may thus have been masked. However, our results may also indicate that the difference in risk of lipoatrophy between NRTIs may not solely be explained by differences in mitochondrial DNA depletion directly at the level of peripheral adipose tissue.
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