

Prevalence of lipoatrophy and mitochondrial DNA content of blood and subcutaneous fat in HIV-1-infected patients randomly allocated to zidovudine- or stavudine-based therapy

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Introduction: Mitochondrial toxicity resulting from mitochondrial DNA (mtDNA) depletion is suggested to be involved in the pathogenesis of lipodystrophy.

Methods: We cross-sectionally assessed lipodystrophy both clinically and radiographically in patients who, 4 years before, had been enrolled in a randomized comparative trial of stavudine- or zidovudine-based therapy. mtDNA content was measured in peripheral blood mononuclear cells (PBMCs) and subcutaneous adipose tissue from the thigh and back.

Results: Twenty-eight of the 45 patients enrolled in the original trial were included. Despite comparable exposure to stavudine or zidovudine (51 and 50 months, respectively), lipoatrophy prevalence by intent-to-treat analysis was significantly greater in stavudine recipients (82 vs 9%, $P=0.0001$). Likewise, those allocated to stavudine had significantly less peripheral fat. In an analysis restricted to patients who had remained on randomly allocated nucleoside reverse transcriptase inhibitors (NRTIs), mtDNA in PBMCs decreased after the start of treatment in both groups ($P<0.0001$) (-73% for stavudine and -67% for zidovudine, $P=0.11$), resulting in

significantly lower levels in patients with lipoatrophy ($P=0.007$). The mtDNA content in subcutaneous adipose tissue from the thigh, but not from the back, was significantly lower in patients allocated to stavudine compared to zidovudine ($P=0.01$). mtDNA in adipose tissue from either location did not differ significantly between those with or without lipoatrophy.

Discussion: This study objectively confirms that regimens containing stavudine are associated with a greater risk of lipoatrophy than those containing zidovudine. mtDNA in PBMCs markedly declined with both treatments and was lowest in patients with lipoatrophy. The lack of difference in mtDNA in adipose tissue from patients with as opposed to without lipoatrophy may have been masked by a relative preponderance of stromal and vascular tissue in the subcutaneous tissue samples from these patients, combined with compensatory mitochondrial proliferation in remaining adipocytes. However, our findings may also suggest that the different risk of lipoatrophy observed between NRTIs cannot solely be explained by differences in mtDNA depletion directly at the level of peripheral adipose tissue.

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 dyslipidaemia and insulin resistance [1].

Increasing evidence suggests that both protease inhibitors (PIs) and nucleoside reverse transcriptase inhibitors (NRTIs) contribute to the pathogenesis of the syndrome [1–8].

NRTIs inhibit DNA polymerase- γ , the key cellular enzyme regulating mitochondrial DNA (mtDNA) replication. They may thereby aggravate any prior deleterious effect on mtDNA content and possibly function resulting from HIV-1 itself, which has been suggested to occur in peripheral blood mononuclear cells (PBMCs) [9]. The relevance, however, of these findings in peripheral blood cells to possible tissue-specific mitochondrial toxicity within adipose tissue is less certain [10]. The various individual NRTIs markedly differ in their capacity to inhibit mtDNA replication and mitochondrial function, both in tissue culture and animal models [11–14]. Recently, others and we 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 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 both prospectively [17] as well as long term after randomization to either d4T- or ZDV-based therapy [18,19].

A number of cross-sectional studies have reported mtDNA content of subcutaneous fat biopsies to be lower in patients currently receiving NRTIs [20] and in particular dideoxynucleosides [21], than in those not currently on NRTIs, as well as in patients with as compared to without clinical lipoatrophy [21–23]. One study showed that mtDNA content was less in peripheral adipocytes from patients currently using d4T than in those on ZDV [10]. Similar relationships have thus far not been demonstrated in PBMC [21,24,25].

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 PBMCs and in subcutaneous adipose tissue.

Methods

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 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 three times a day could be added to the regimen from week 12 onwards. Two of the 47 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.

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 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[®] 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 kg. 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 cm². The SAT/TAT ratio was calculated to assess fat distribution.

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 PBMCs 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 mm punch biopsy needle. PBMCs were cryopreserved by performing a programmed temperature decline with liquid nitrogen as a cooling agent, a procedure designed to yield viable cells after thawing. Prior to isolation of nucleic acids, PBMCs were microscopically checked for contamination with platelets. Counting of platelets was performed using both automated procedures (both with an optical and an impedance method) and visually by microscopy. We did not find that platelet counts before and after viable freezing were different. Using standard procedure for isolation of PBMCs from heparinized blood the contamination with platelets was less than 5 platelets/PBMC, a level that does not alter the result of mtDNA quantification [31]. Isolated nucleic acids equivalent to 3000 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 [31,32]. 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).

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

Statistical analysis

All analyses, except those concerning the assessment of mtDNA, were conducted applying the of intent-to-treat principle. The cumulative exposure to PIs as a class, and to nevirapine (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 PIs and NVP, 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 χ^2 test.

With respect to the assessment of mtDNA content in PBMCs and adipose tissue, and its correlation with measures of fat distribution, the analyses were restricted to those patients who had maintained their randomly allocated NRTI therapy up until the time of the current investigation.

This approach was taken in order to avoid any potential confounding that may have resulted from switching or withdrawal of NRTIs on mtDNA content as has recently been reported to occur in peripheral blood cells [33]. For the analysis of mtDNA results were logarithmically transformed in order to obtain normal distributions. Correlation coefficients were calculated and expressed as r^2 to evaluate any relationship between mtDNA in PBMCs or fat biopsies on the one hand, and the percentage peripheral fat by DEXA scan as well as the SAT/TAT ratio 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 (five patients from the ZDV- and two from the d4T-arm) and 10 did not consent to the current protocol (seven patients from the ZDV- and three 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

Table 1. Body appearance, body composition and metabolic assessments of participants from current study

	d4T-arm (<i>n</i> =17)	ZDV-arm (<i>n</i> =11)	<i>P</i> -value
Age (years)	44 (39–55)	40 (37–50)	0.4
BMI (kg/m ²)	22.8 (21.8–23.8)	23.0 (21.0–24.7)	0.98
Patients still on randomized NRTI backbone (<i>n</i>)	15	8	
Cumulative exposure to d4T (months/patient)	51 (49–54)	32 (22–43)	
Cumulative exposure to ZDV (months/patient)	11	50 (45–53)	
Patients exposed to PIs (<i>n</i>)	15	11	0.51
Cumulative exposure to PIs (months/patient)	45 (24–48)	31 (21–53)	
Patients exposed to NVP (<i>n</i>)	5	8	0.05
Cumulative exposure to PIs (months/patient)	24 (17–25)	19 (17–26)	
% Patient HIV-1 RNA <50 copies/ml	71%	64%	0.7
CD4-cell count (×10 ⁶ /mm ³)	690 (510–780)	620 (450–710)	0.4
Platelet count (×10 ⁹ /l)	226 (176–242)	219 (202–259)	0.44
Number (%) patients with lipodystrophy according to questionnaire	14 (82%)	1 (9%)	0.0001
Number (%) patients with lipodystrophy according to case def score	15 (88%)	5 (45 %)	0.03
Waist-to-hip ratio	0.94 (0.91–0.97)	0.95 (0.93–1.04)	0.42
Biceps circumference (mm)	41 (36–46)	60 (44–75)	0.002
Triceps circumference (mm)	52 (44–65)	73 (58–94)	0.02
Suprailiacal circumference (mm)	81 (68–101)	146 (111–186)	0.001
Subscapular circumference (mm)	142 (118–158)	132 (105–191)	0.28
SAT (cm ²)	75 (54–95) (<i>n</i> =16)	131 (105–149)	0.04
VAT (cm ²)	142 (91–208) (<i>n</i> =16)	136 (78–202)	0.48
SAT/TAT	0.35 (0.30–0.44) (<i>n</i> =16)	0.50 (0.41–0.62)	0.005
peripheral fat in kg (DEXA)	2.4 (1.9–3.2)	4.8 (3.2–6.3)	0.005
peripheral/total fat (DEXA)	0.33 (0.27–0.38)	0.37 (0.34–0.49)	0.04
% total fat DEXA	13.2 (12.6–16.8)	18.4 (14.0–25.7)	0.17
mtDNA content/cell in PBMCs at baseline	362 (300–464)	322 (248–452)	0.39
mtDNA content/cell in PBMCs current study*	92 (75–118) (<i>n</i> =15)	116 (101–141) (<i>n</i> =8)	0.05
mtDNA content/cell in fat biopsies from the back*	564 (440–685) (<i>n</i> =13)	753 (679–833) (<i>n</i> =6)	0.10
mtDNA content/cell in fat biopsies from the leg*	454 (371–738) (<i>n</i> =13)	707 (698–867)(<i>n</i> =6)	0.01
Lactate (mmol/l)	1.3 (1.0–1.9)	1.1 (0.7–1.3)	0.34

d4T, stavudine; ZDV, zidovudine; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; TAT, total adipose tissue; SAT/TAT, the ratio of SAT over TAT; DEXA, dual-energy X-ray absorptiometry; mtDNA, mitochondrial DNA; PBMCs, peripheral blood mononuclear cells.
 * Analysis limited to patients still on original randomly allocated NRTI therapy at the time of the current study. Results expressed as medians with interquartile ranges.

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 enrolment 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 400×10⁶ (260–440) vs 300×10⁶ 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 nine of 15 patients in the d4T-arm were still receiving a PI-based regimen. In five of 15 of the other patients their PI had been replaced by the non-nucleoside reverse transcriptase inhibitor (NNRTI) nevirapine (NVP) for a median duration of 24 months. The one remaining patient discontinued all antiretroviral treatment 26 months prior to the current assessment.

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 three of 11 patients were being treated with a PI-based regimen. The eight remaining patients had replaced their PI by NVP (*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 eight patients discontinued all antiretroviral therapy 2 months prior to the current study. The complete treatment history of all patients is shown in Figure 1.

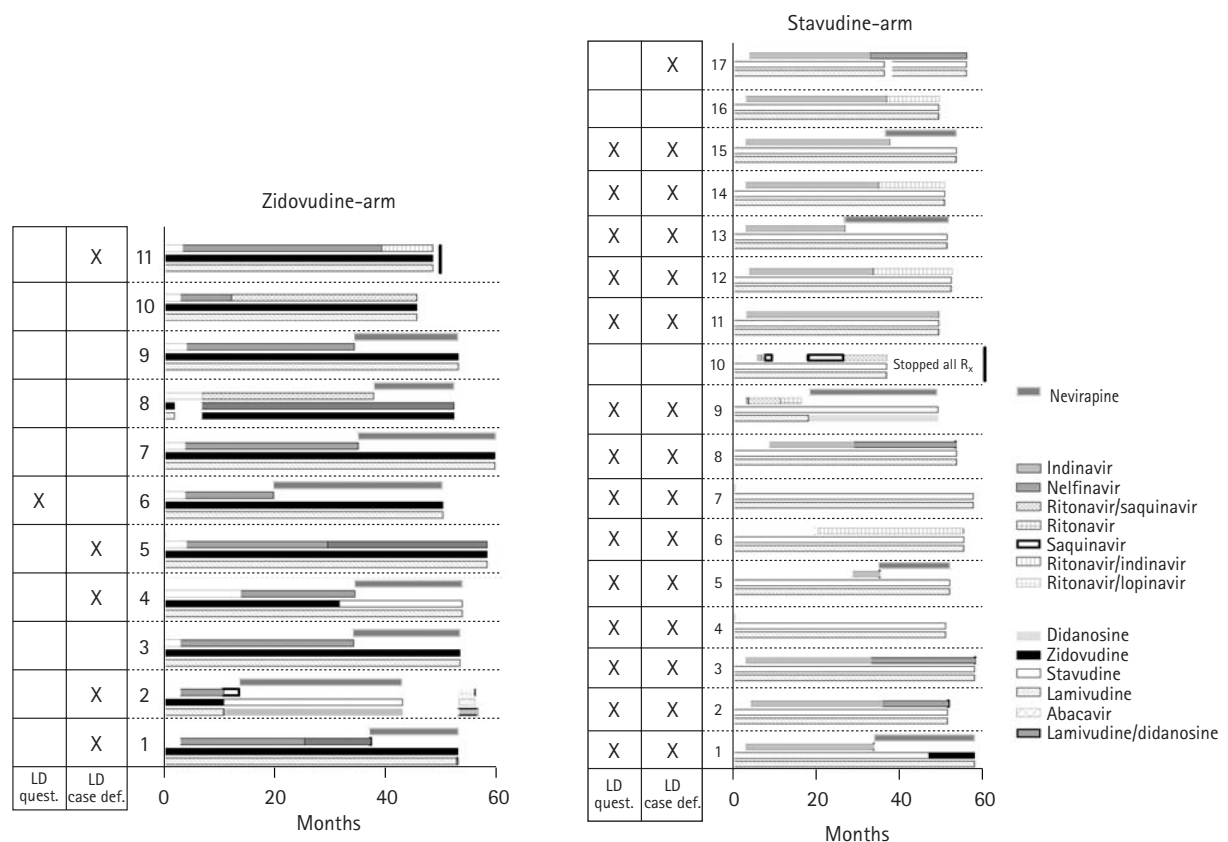
Body appearance, body composition and metabolic assessments (Table 1)

By questionnaire, 14 of 17 patients (82%) originally randomized to the d4T-arm were considered to have lipodystrophy, compared to only out of 11 patients (9%) in the ZDV-arm ($P=0.0001$). Two of the patients with lipodystrophy 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 lipodystrophy. 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 NVP, all five patients in the d4T-arm were scored as having lipodystrophy, compared to only one of eight patients in the ZDV-arm.

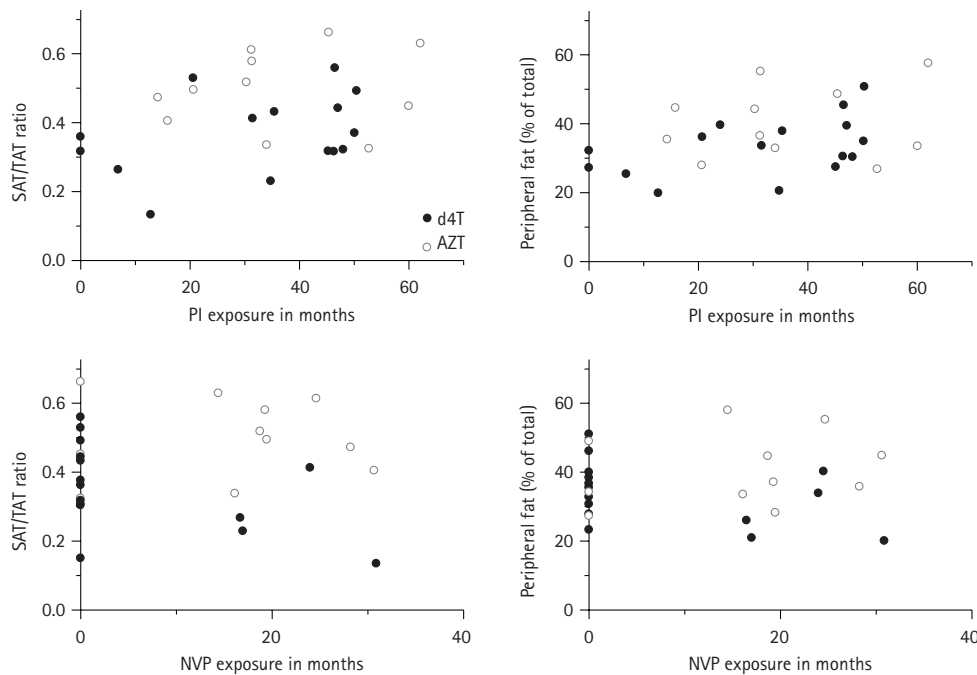
Patients randomized to the d4T-arm had both significantly less peripheral fat by DEXA-scan ($P=0.005$), and less SAT by CT-scan ($P=0.04$). Consistently, they also had lower peripheral over total fat ($P=0.04$), lower SAT/TAT ratios ($P=0.005$), and smaller skin-folds by antropometry at the level of the biceps and triceps ($P=0.002$ and $P=0.02$, respectively). There was no relationship 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 1. Histogram of complete antiretroviral treatment history of all 28 patients from the time of randomization in the original clinical trial until inclusion in the current cross-sectional study



The end of the treatment bars indicates the time of the current assessment. Patients indicated with | stopped taking all antiretroviral therapy prior to this point, and were assessed at the time indicated by |. LD quest, lipodystrophy according to our questionnaire; LD case def., lipodystrophy according to lipodystrophy case definition study; R_t, antiretroviral treatment.

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



SAT, subcutaneous adipose tissue; TAT, total adipose tissue; PI, protease inhibitor; NVP, nevirapine. CT-scan data available from 16 patients in the d4T-arm and 11 in the ZDV-arm.

Mitochondrial DNA

The median mtDNA content in PBMCs cryopreserved prior to initiation of antiretroviral therapy did not differ between treatment arms for patients included in the current study (Table 1). Likewise, at randomization 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 for patients who had maintained their original randomly allocated NRTI therapy, mtDNA in PBMCs at the time of the present study was significantly lower compared to before the start of treatment (a decline from 300-something to 100-something, $P<0.0001$). In the d4T arm the calculated proportional median mtDNA decrease in PBMCs was 73% (66–80) versus 67% (59–76) in the ZDV-arm ($P=0.11$), resulting in 92 copies/PBMC in the d4T-arm and 116 copies/PBMC in the ZDV-arm ($P=0.05$). The amount of mtDNA per cell in the SAT biopsies taken from the thigh was significantly lower in the d4T-arm (454 copies/cell) compared to the ZDV-arm (707 copies/cell, $P=0.01$), whereas this did not differ significantly in the SAT taken from the back. The patients with – as opposed to those without – lipoatrophy according to our questionnaire, had significantly lower mtDNA content in PBMCs ($P=0.007$), but not in subcutaneous fat biopsies taken from either the thigh or back (Figure 3).

A significant, but modest, inverse relationship was

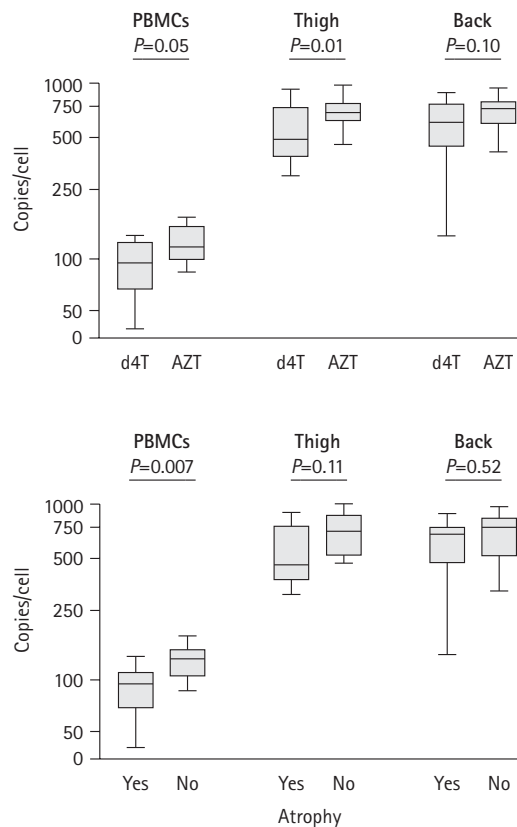
found between the amount of mtDNA in PBMCs ($r^2=0.29$, $P=0.009$), as well as in biopsies from the thigh ($r^2=0.35$, $P=0.01$), but not from the lower back, on the one hand, and the severity of lipoatrophy assessed by CT-scan (SAT/TAT) on the other hand. When fat distribution expressed as percentage peripheral of total body fat was assessed by DEXA-scan, a similar relationship was found with mtDNA content of PBMCs ($r^2=0.22$, $P=0.02$) and mtDNA in thigh ($r^2=0.22$, $P=0.04$) but not for lumbar ($r^2=0.01$, $P=0.63$) adipose tissue biopsies.

The mtDNA content in PBMCs correlated significantly with mtDNA content in the biopsies taken from the back ($r^2=0.39$, $P=0.004$), but not with those taken from the thigh ($r^2=0.063$, $P=0.30$). There was no significant relation between mtDNA content of fat biopsies taken from the thigh and back, respectively ($r^2=0.11$, $P=0.16$).

Discussion

Previous studies have found NRTIs to contribute to the development of antiretroviral therapy-associated lipodystrophy, and lipoatrophy in particular [1–8,17–19]. In most of these studies, the use of d4T was associated with a greater risk of developing lipoatrophy, when compared to that of ZDV. Our study confirms these observations in a group of patients who

Figure 3. Mitochondrial DNA content in PBMCs, as well as in thigh and back subcutaneous adipose tissue biopsies at the time of the current study in the patients on randomized treatment, and according to the presence or absence of lipoatrophy as determined by standardized questionnaire



PBMCs, 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.

on average 4 years before had been randomly allocated to initiate antiretroviral treatment containing d4T or ZDV, 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 d4T 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 d4T- as opposed to ZDV-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 d4T or ZDV 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

(five on ZDV but only one on d4T) 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 ZDV 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 ZDV- and d4T-containing antiretroviral therapy, but is less severe and of slower onset in the former [17,34].

Restricting the analysis to those patients who had maintained their originally allocated NRTI therapy, lipoatrophy severity, assessed both by DEXA and CT scan, showed a statistically significant inverse correlation with the mtDNA content in PBMCs. A similar correlation was found between mtDNA content of SAT from the thigh and severity of fat loss measured by CT and DEXA scan. These results could be interpreted as evidence that NRTI-induced mtDNA 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. Differing from our results, two studies did report a lower mtDNA content of adipose tissue in patients with as opposed to those without lipodystrophy [20,23]. 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.

The mtDNA in PBMCs was significantly lower in those patients with compared to without lipoatrophy ($P=0.007$) and borderline significant lower in those allocated to d4T as opposed to ZDV ($P=0.05$). mtDNA in PBMCs had decreased significantly in both treatment groups compared to before the initiation of antiretroviral therapy ($P<0.0001$); however, this decrease did not differ between the two groups ($P=0.11$).

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 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 d4T than to ZDV (77 vs 48%). This difference however, largely resulted from more patients in the ZDV-arm having moved who thereby could not be traced (21 vs 9% in the d4T-arm). Third, more patients in the d4T-arm maintained the PI in their regimen and did not switch to NVP, 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 relationship 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 five patients in the d4T-arm who replaced PI by NVP were judged to have lipoatrophy as compared to only one of eight patients in the ZDV-arm, does support the notion that d4T is associated with a higher risk of lipoatrophy development than ZDV. Finally, mtDNA was assessed in SAT 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 d4T, as recently reported by Nolan *et al.* [10], 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 d4T to be associated with a greater risk of lipoatrophy compared to those containing ZDV. They also indicate that mtDNA depletion in PBMCs and lipoatrophy are likely to both be NRTI-associated phenomena, and to generally be more severe in patients on d4T. The lack of observing a significantly reduced mtDNA content in adipose tissue from patients with as opposed to without 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 mtDNA depletion within adipocytes having undergone apoptosis in patients with 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 mtDNA depletion directly at the level of peripheral adipose tissue.

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