

## Skeletal muscle mitochondrial DNA content and aerobic metabolism in patients with antiretroviral therapy-associated lipoatrophy

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**Objectives:** To assess whether mitochondrial dysfunction in skeletal muscle characterizes antiretroviral therapy (ART)-associated lipoatrophy (LA).

**Methods:** A cross-sectional study comparing HIV-infected, antiretroviral-treated patients with LA ( $n=5$ ; LA+) and without LA ( $n=5$ ; non-LA) was conducted. Positron emission tomography was used to measure blood flow, oxygen extraction and oxygen consumption in quadriceps femoris muscle during rest and aerobic exercise. Mitochondrial DNA (mtDNA) was quantified by PCR. Body composition was measured by dual-energy X-ray absorptiometry and magnetic resonance imaging. All data are given as means  $\pm$  SEM.

**Results:** Compared with the non-LA group, the LA+ group had significantly less limb fat and more intra-abdominal fat, but similar leg muscle mass. The LA+ group versus the non-LA group had reduced mtDNA content per nucleus in adipose tissue ( $173 \pm 38$  versus  $328 \pm 62$ ;  $P=0.067$ ), but not in skeletal muscle ( $2606 \pm 375$  versus  $2842 \pm 309$ ;  $P=0.64$ ). Perfusion in resting muscle ( $34 \pm 7$  versus  $28 \pm 6$  mL/kg/min in the LA+ group versus the non-LA group;  $P=0.5$ ), and the mean absolute ( $277 \pm 30$  versus  $274 \pm 43$  mL/kg/min, respectively;  $P=0.95$ ) and relative ( $10.6 \pm 2.5$ - versus  $11.9 \pm 1.5$ -fold change, respectively;  $P=0.67$ ) increases in perfusion during exercise were similar between the groups. Oxygen consumption at rest ( $2.2 \pm 0.7$  versus  $2.1 \pm 0.3$  mL/kg/min in the LA+ group versus the non-LA group;  $P=0.9$ ), and the mean absolute ( $14.6 \pm 1.7$  versus  $24.3 \pm 8.8$  mL/kg/min, respectively;  $P=0.3$ ) and relative ( $10.3 \pm 2.8$ - versus  $11.7 \pm 2.4$ -fold change, respectively;  $P=0.73$ ) exercise-induced increases in oxygen consumption were similar between the groups. The oxygen extraction fraction was comparable between the groups, both at rest and during exercise. Plasma lactate concentrations remained unchanged in both groups during exercise.

**Conclusions:** HIV-infected patients with ART-associated LA have similar mtDNA content in skeletal muscle and comparable skeletal muscle aerobic exercise metabolism to antiretroviral-treated non-lipoatrophic patients.

**Keywords:** lipodystrophy, muscle biopsy, positron emission tomography

### Introduction

The prognosis of HIV-infected patients treated with combination antiretroviral therapy (ART) appears to approach that of the

general population.<sup>1</sup> However, ART is also associated with long-term metabolic side effects, such as lipodystrophy, i.e. loss of subcutaneous fat [lipoatrophy (LA)] with or without accumulation of intra-abdominal fat, dyslipidaemia and insulin resistance.<sup>2</sup>

Drug-induced mitochondrial toxicity appears to be a key feature in the pathophysiology of ART-associated LA.<sup>3</sup> Nucleoside analogue reverse transcriptase inhibitors (NRTIs) inhibit mitochondrial DNA polymerase  $\gamma$ , leading to the depletion of mitochondrial DNA (mtDNA).<sup>4</sup> In keeping with these *in vitro* data, several studies have shown mtDNA to be reduced in lipotrophic subcutaneous adipose tissue of antiretroviral-treated patients.<sup>5,6</sup> This may impair mitochondrial function and, for example, the translation of mtDNA-encoded components of the respiratory chain complex may decrease.

At present it is unknown how uniformly different human tissues are affected by the NRTI-induced mitochondrial toxicity. Data regarding the mtDNA content of peripheral blood mononuclear cells are conflicting, showing both unchanged<sup>7,8</sup> and decreased<sup>9</sup> mtDNA content in patients with ART-associated lipodystrophy. The mtDNA content has even been shown to be increased in CD4+ lymphocytes in patients with ART-associated lipodystrophy.<sup>10</sup> Data are scarce and inconsistent on whether NRTI-induced LA (i.e. mitochondrial toxicity in adipose tissue) is associated with mitochondrial toxicity also in skeletal muscle, a tissue rich in mitochondria.

The NRTI zidovudine was widely used as single drug therapy in the early 1990s, before current combination therapies were available. Although zidovudine-associated myopathy is a known complication of high-dose zidovudine monotherapy,<sup>11</sup> these patients have not been reported to have lipodystrophy. The latter was first reported as a complication of ART in 1997–8<sup>12,13</sup> and has been associated with the use of combination therapies, possibly implying a different pathophysiology. A recent animal model has shown that zidovudine, but not zalcitabine, induces mitochondrial myopathy with mtDNA depletion.<sup>14</sup> In addition, a recent microarray study demonstrated significant dose-related effects of zidovudine on the expression of genes associated with apoptosis, fatty acid metabolism, mitochondrial genome maintenance and various mitochondrial membrane transporters in the skeletal muscle of infant mice.<sup>15</sup> Interestingly, although the number of patients receiving zidovudine as part of ART dramatically increased during the late 1990s and although up to 50% of patients on ART may develop lipodystrophy,<sup>16,17</sup> the prevalence of skeletal muscle complications has not increased similarly. Conversely, data from France suggest that there has been a marked decrease in the number of muscle biopsies performed in HIV-infected individuals in neuromuscular centres since 1998.<sup>18</sup>

The production of adenosine triphosphate (ATP) in skeletal muscle is dependent on mitochondrial function. Therefore, significant mitochondrial dysfunction, potentially caused by ART, should impair the aerobic exercise capacity of skeletal muscle. Skeletal muscle could possibly compensate this impairment by increasing blood flow and/or oxygen consumption. Positron emission tomography (PET) is the most advanced scintigraphic imaging technique in humans that allows non-invasive studies of metabolism and perfusion *in vivo*.<sup>19</sup>

In the current study, we aimed to evaluate whether skeletal muscle is affected in ART-associated LA by measuring the mtDNA content in skeletal muscle, and by using the unique features of PET to directly measure blood flow and oxygen consumption in skeletal muscle during rest and aerobic exercise in patients with and without ART-associated LA.

## Materials and methods

### Study design

This is a cross-sectional study comparing HIV-infected, antiretroviral-treated patients with LA (LA+ group) and HIV-infected, antiretroviral-treated patients without LA (non-LA group).

The purpose, nature and potential risks of the study were explained to the study subjects before their informed consent was obtained. The study protocol was approved by the ethics committees of Helsinki (clinical studies other than PET) and Turku (PET) University Central Hospitals.

### Subjects

The patients were recruited from the HIV outpatient clinic of Helsinki University Central Hospital. Inclusion criteria included: male gender; age >18 years; clinically stable HIV infection; and  $\geq 2$  year duration of combination ART prior to study entry. In addition, patients in the LA+ group had self-reported symptoms of loss of subcutaneous fat with or without increased abdominal girth. Patients in the non-LA group had used ART without developing LA. The presence or absence of symptoms of lipodystrophy was confirmed by the study physician (J. S.) prior to entry into the study.

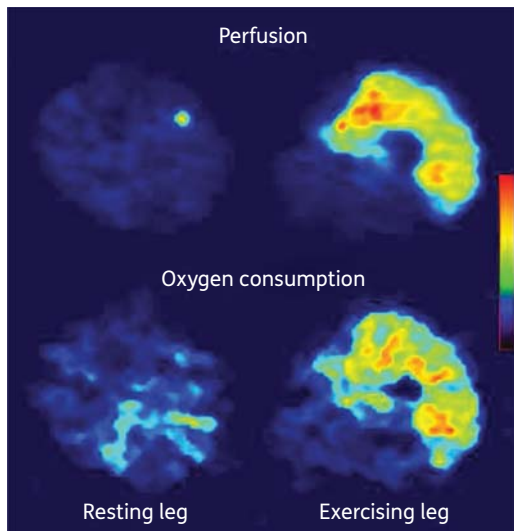
### Body composition

Limb fat, truncal fat, total body fat and lean body mass were measured using dual-energy X-ray absorptiometry (DEXA; Lunar Prodigy, Madison, WI, USA). Intra-abdominal fat and subcutaneous fat of the abdomen were quantified by analysing a total of 16 T1-weighted trans-axial magnetic resonance imaging (MRI) scans, reaching from 8 cm above to 8 cm below the fourth and fifth lumbar interspace (field of view 375×500 mm<sup>2</sup>, slice thickness 10 mm, breath-hold repetition time 138.9 ms, echo time 4.1 ms), as previously described.<sup>20</sup> MRI scans were analysed by a single investigator using image analysis software (Alice 3.0, Parexel, Waltham, MA, USA). Investigators analysing body composition data were blinded to patient disposition in the LA+ or non-LA groups.

### PET

After measuring maximal isometric knee-extension contraction force (MVC), PET scanning for the measurement of blood flow and oxygen consumption in skeletal muscle was performed. The subjects were instructed to avoid exercise during the last 3 days before the measurement. Moreover, they were asked to avoid caffeinated beverages for 24 h and to fast overnight for 10 h before the experiment.

One hour before the PET measurement, two catheters were inserted, one into an antecubital vein for infusion of saline and injection of tracer, and the other into the opposite radial artery for blood sampling. Thereafter, subjects were attached to the PET scanner, as described before.<sup>21</sup> While adjusting the dynamometer, the subject performed a 5 min familiarization and warm-up with the dynamometer. After the transmission scan, two 12 min dynamic knee-extension exercise periods with a 10 min recovery between the sets were performed with the right leg in a continuous manner with a constant knee extension frequency (15 contractions per min) and resistance ( $7 \pm 0\%$  of MVC; range 2.0–6.0 kg) while the left leg was at rest.<sup>22</sup> Muscle perfusion and oxygen uptake were measured in quadriceps femoris muscle (Figure 1), as described earlier, during the first and second exercise bouts.<sup>22</sup> The positron-emitting tracers were produced in a standardized manner<sup>23</sup> and an ECAT 931/08 tomograph (Siemens/CTI, Knoxville, TN, USA) was used for emission scanning. All PET data were collected and analysed, as previously shown.<sup>24,25</sup>



**Figure 1.** Examples of parametric perfusion and oxygen uptake PET images during dynamic knee-extension exercise. The red colour corresponds to high perfusion and oxygen uptake, and the dark colour corresponds to low perfusion and oxygen uptake. The figure illustrates significantly increased perfusion and oxygen uptake in the right exercising quadriceps femoris muscle during the knee-extension exercise, which specifically activates this muscle, whereas the levels of perfusion and oxygen uptake remain low in the resting left leg. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

### Skeletal muscle and adipose tissue biopsies

Skeletal muscle biopsies from the vastus lateralis muscle were taken using Bergstrom needles under local lidocaine anaesthesia. The biopsy sample was immediately frozen in liquid nitrogen. Surgical biopsies from subcutaneous adipose tissue were taken under local lidocaine anaesthesia from midway between the iliac crest and the umbilicus. The biopsy was immediately frozen in liquid nitrogen for further analysis.

### mtDNA quantification

Genomic DNA was extracted from adipose tissue and skeletal muscle with the QIAamp DNA isolation kit (Qiagen, Hilden, Germany). mtDNA and nuclear DNA (nDNA) copy numbers were determined by quantitative PCR using the ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). We amplified the mtDNA ATP-6 gene between nucleotide positions 8981 and 9061 with the forward primer 5'-ACCAATAGCCCTGGC CGTAC-3' and the backward primer 5'-GGTGGCGCTTCCAATTAGGT-3'. mtDNA was quantified with a FAM-fluorophore-labelled probe (5'-6FAM-CCTAACCGCTAACATTACTGCAGGCCACC-TAMRA-3'). For the detection of nDNA, we selected exon 8 of the GAPDH gene between nucleotide positions 4280 and 4342, using the forward primer 5'-CGGGGCTCTCCAGA ACATC-3' and the backward primer 5'-ATGACCTTGCCACAGCCT-3'. In this case, we used a VIC-fluorophore-labelled probe (5'-VIC-CCCTGCCT CTAAGGCGCTGCC-TAMRA-3').

Each 25  $\mu$ L reaction contained 25 ng of genomic DNA, 100 nM of probe, 200 nM of primers and Taq-man Universal Master Mix (Applied Biosystems, Foster City, CA, USA). Amplifications of mitochondrial and nuclear products were separately performed in optical 96-well plates (Applied Biosystems, Foster City, CA, USA). An initial incubation at 50°C for 2 min was followed by 10 min at 95°C and 40 denaturing steps at 95°C (15 s), alternating with combined annealing/extension at 60°C (1 min). All samples were run in triplicate. Absolute mtDNA and nDNA

copy numbers were calculated using serial dilutions of plasmids with known copy numbers.

### Laboratory parameters

Serum free insulin was determined by time-resolved fluoroimmunoassay using an Insulin Kit (AUTOdelfia, Wallac, Turku, Finland). Plasma glucose concentrations were measured by a hexokinase method using an auto-analyser (Roche Diagnostics Hitachi 917, Hitachi Ltd, Tokyo, Japan). The homeostasis model assessment of insulin resistance (HOMA) was calculated from the formula: fasting glucose (mmol/L)  $\times$  fasting insulin (mU/L)/22.5.<sup>26</sup> Serum total and high-density lipoprotein (HDL) cholesterol as well as triglyceride concentrations were determined with respective enzymatic kits from Roche Diagnostics using the Roche Diagnostics Hitachi 917 autoanalyser. Plasma lactate concentrations were measured using an enzymatic colorimetric method (Cobas Integra 400, Roche Diagnostics GmbH, Mannheim, Germany). The CD4+ cell count was determined using a flow cytometric apparatus (FACSort/FACSCalibur, Becton Dickinson, San José, CA, USA). The HIV viral load was measured using the HPS Cobas TaqMan 48n HIV-1 Test (Roche Diagnostics, Branchburg, NJ, USA), with a detection limit of 1.7 log<sub>10</sub> copies/mL.

### Statistical analysis

The unpaired t-test was used for comparison of differences between the study groups after logarithmic transformation when necessary. Correlations were calculated using Spearman's rank correlation coefficient. All data are given as means  $\pm$  SEM.

## Results

### Physical and biochemical characteristics (Table 1)

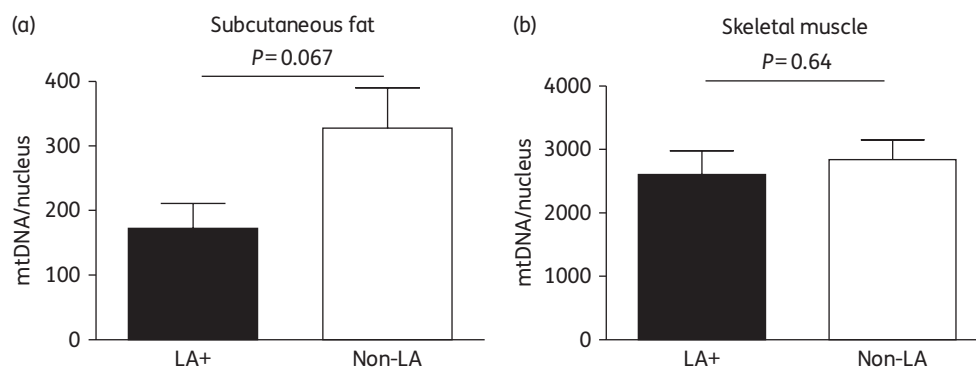
All participants ( $n=10$ ) were male and the groups were comparable with respect to age, body weight and body mass index.

**Table 1.** Characteristics of the study groups: HIV-infected, antiretroviral-treated patients with LA (LA+) and without LA (non-LA)

Variable	LA+ ( $n=5$ )	Non-LA ( $n=5$ )	P value
Age (years)	51 $\pm$ 5	47 $\pm$ 6	0.7
Body weight (kg)	69.1 $\pm$ 5.0	78.6 $\pm$ 3.8	0.2
Body mass index (kg/m <sup>2</sup> )	22.4 $\pm$ 1.5	24.5 $\pm$ 1.0	0.3
Total limb fat (g)	1120 $\pm$ 450	5740 $\pm$ 740	0.0008
Intra-abdominal fat (cm <sup>3</sup> )	2220 $\pm$ 360	960 $\pm$ 140	0.012
Leg lean mass (g)	18940 $\pm$ 1220	19160 $\pm$ 1440	0.9
Plasma glucose (mmol/L)	8.1 $\pm$ 2.2	5.2 $\pm$ 0.2	0.2
Serum insulin (mU/L)	13.0 $\pm$ 2.3	4.6 $\pm$ 0.7	0.008
HOMA	4.1 $\pm$ 0.9	1.1 $\pm$ 0.2	0.01
Serum triglycerides (mmol/L)	3.5 $\pm$ 0.3	1.7 $\pm$ 0.4	0.008
Serum cholesterol (mmol/L)	5.2 $\pm$ 0.7	4.9 $\pm$ 0.4	0.7
Serum LDL cholesterol (mmol/L)	2.1 $\pm$ 0.5	2.5 $\pm$ 0.5	0.6
Serum HDL cholesterol (mmol/L)	1.1 $\pm$ 0.1	1.7 $\pm$ 0.1	0.02
Haemoglobin (g/L)	133 $\pm$ 6	147 $\pm$ 7	0.2
MVC (N)	49 $\pm$ 5	56 $\pm$ 9	0.5

HOMA, homeostasis model assessment of insulin resistance [fasting glucose (mmol/L)  $\times$  fasting insulin (mU/L)/22.5]; LDL, low-density lipoprotein; HDL, high-density lipoprotein; MVC, maximal isometric knee-extension contraction force; N, newton.

Data are means  $\pm$  SEM.



**Figure 2.** Amount of mtDNA per nucleus in subcutaneous adipose tissue (a) and skeletal muscle (b) in HIV-infected, antiretroviral-treated patients with LA (black bars) and without LA (white bars). Error bars indicate SEM.

The patients in the non-LA group had 5-fold more limb fat than the LA+ group. Conversely, the LA+ group had twice as much intra-abdominal fat as the non-LA group. The lean mass of the legs was almost identical between the groups.

Patients in the LA+ group had significantly higher serum insulin concentrations and HOMA indices than the non-LA group. The LA+ group also had other biochemical features of insulin resistance, including increased serum triglyceride and decreased serum HDL cholesterol concentrations. Blood haemoglobin concentrations were similar between the groups.

### HIV-related characteristics

The time since diagnosis of the HIV infection was  $12.3 \pm 2.6$  years in the LA+ group versus  $6.2 \pm 1.8$  years ( $P=0.09$ ) in the non-LA group. The duration of combination ART with at least three different drugs was longer in the LA+ group than in the non-LA group ( $6.2 \pm 0.6$  versus  $2.9 \pm 0.5$  years;  $P=0.003$ ). The cumulative exposure to thymidine analogues (including also monotherapy prior to the era of combination therapy) was longer in the LA+ group than in the non-LA group ( $8.8 \pm 1.3$  versus  $2.7 \pm 0.6$  years;  $P=0.003$ ). The most recent viral load was below the detection limit in 9 out of 10 study participants. One patient had a viral load of 252 copies/mL; his metabolic profile was not different when compared with the other study subjects. The most recent CD4 count was almost identical between the groups ( $480 \pm 121$  versus  $488 \pm 63 \times 10^6/L$  in the LA+ versus the non-LA group;  $P=0.96$ ).

Two patients in the LA+ group were receiving zidovudine and three were taking stavudine. In the non-LA group, four patients were taking zidovudine and one patient did not take any thymidine-analogue NRTI. The single patient not receiving a thymidine-analogue NRTI at the time of the study had previously been treated with zidovudine and stavudine. He did not show a different metabolic pattern from the rest of the non-LA group. Four patients in the LA+ group and one patient in the non-LA group were receiving a protease inhibitor, and one versus four patients were taking a non-nucleoside reverse transcriptase inhibitor, respectively.

### mtDNA quantification (Figure 2)

The mtDNA content in subcutaneous adipose tissue in the LA+ group was approximately half of that in the non-LA group,

whereas the mtDNA content in skeletal muscle was almost identical between the two study groups.

### Skeletal muscle perfusion and oxygen consumption

The MVC was similar between the groups (Table 1).

Perfusion in resting ( $P=0.5$ ) and exercising ( $P=0.9$ ) quadriceps femoris muscle were similar between the study groups (Figure 3a). Similarly, the mean absolute ( $277 \pm 30$  versus  $274 \pm 43$  mL/kg/min in the LA+ versus non-LA group;  $P=0.95$ ) and relative ( $10.6 \pm 2.5$ - versus  $11.9 \pm 1.5$ -fold change, respectively;  $P=0.67$ ) increases in perfusion over resting values in response to exercise were similar between the groups.

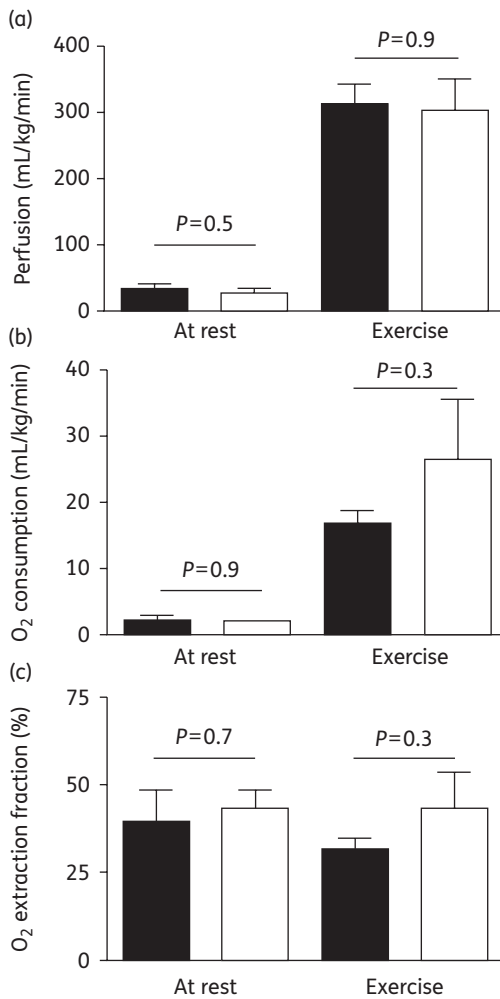
Oxygen consumption in skeletal muscle at rest was identical ( $P=0.9$ ) between the groups (Figure 3b). During exercise, oxygen consumption ( $P=0.3$ ) was also comparable between the groups, although the interindividual variation was greater than at rest. The mean absolute ( $14.6 \pm 1.7$  versus  $24.3 \pm 8.8$  mL/kg/min in the LA+ versus non-LA group;  $P=0.3$ ) and relative ( $10.3 \pm 2.8$ - versus  $11.7 \pm 2.4$ -fold change, respectively;  $P=0.73$ ) exercise-induced increases in oxygen consumption were not significantly different between the groups (Figure 3b). Likewise, there were no significant differences in the oxygen extraction fraction between the groups at rest ( $P=0.7$ ) or during exercise ( $P=0.3$ ) (Figure 3c).

### Plasma lactate concentrations

Plasma lactate levels were higher in the LA+ versus the non-LA group at rest before both periods of exercise (period 1,  $1.8 \pm 0.2$  versus  $1.0 \pm 0.2$  mmol/L;  $P=0.02$  and period 2,  $1.9 \pm 0.2$  versus  $1.1 \pm 0.2$  mmol/L;  $P=0.03$ ). Plasma lactate concentrations remained similarly higher in the LA+ versus the non-LA group at the end of exercise during both periods (period 1,  $1.8 \pm 0.2$  versus  $1.2 \pm 0.1$  mmol/L;  $P=0.03$  and period 2,  $1.8 \pm 0.2$  versus  $1.0 \pm 0.1$  mmol/L;  $P=0.01$ ). There was no significant change in lactate concentration from baseline (at rest) through to the end of the exercise period in either group.

### Correlations among all study participants

The mtDNA content in subcutaneous adipose tissue correlated with the amount of limb fat ( $r=0.70$ ,  $P=0.04$ ), whereas the amount of mtDNA in skeletal muscle did not correlate with the

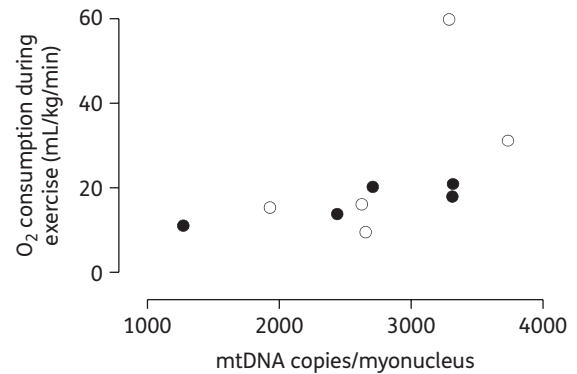


**Figure 3.** Perfusion (a), oxygen consumption (b) and oxygen extraction fraction (c) at rest and during exercise in quadriceps femoris muscle of HIV-infected, antiretroviral-treated patients with LA (black bars) and without LA (white bars). Error bars indicate SEM.

amount of leg lean mass ( $r=0.3$ ,  $P=0.5$ ). The HOMA index tended to correlate inversely with the mtDNA content in subcutaneous adipose tissue ( $r=-0.59$ ,  $P=0.08$ ), but not with the mtDNA content in skeletal muscle ( $r=-0.37$ ,  $P=0.3$ ). The HOMA index also tended to correlate inversely with limb fat mass ( $r=-0.65$ ,  $P=0.07$ ).

MVC tended to correlate with the amount of skeletal muscle mtDNA ( $r=0.60$ ,  $P=0.07$ ). Both MVC and mtDNA in skeletal muscle correlated positively with perfusion ( $r=0.68$ ,  $P=0.03$  and  $r=0.71$ ,  $P=0.03$ , respectively) and with oxygen consumption ( $r=0.72$ ,  $P=0.02$  and  $r=0.77$ ,  $P=0.01$ , respectively) during exercise (Figure 4). There were no significant correlations between mtDNA in skeletal muscle or MVC with perfusion rate or oxygen consumption at rest.

The cumulative exposure to thymidine-analogue NRTI therapy correlated inversely with the amount of subcutaneous fat (limb fat  $r=-0.78$ ,  $P=0.03$ ) and positively with insulin resistance (HOMA  $r=0.72$ ,  $P=0.03$ ), but not with skeletal muscle perfusion or oxygen consumption at rest or during exercise.



**Figure 4.** Relationship between the amount of mtDNA in skeletal muscle and oxygen consumption ( $r=0.77$ ,  $P=0.01$ ) during exercise in quadriceps femoris muscle of HIV-infected, antiretroviral-treated patients with LA (filled circles) and without LA (open circles).

## Discussion

In the present study, the amount of mtDNA in skeletal muscle was not decreased in HIV-infected, antiretroviral-treated patients with LA when compared with HIV-infected, antiretroviral-treated patients without LA. Furthermore, oxygen consumption measured by PET scanning in skeletal muscle during standardized exercise was similar between these two study groups. These data imply that drug-induced mitochondrial toxicity causing LA does not affect all human tissues in a similar fashion and that skeletal muscle remains spared in patients with ART-associated LA.

The mtDNA content in skeletal muscle was not different between the LA+ and the non-LA groups, although the LA+ group had lower mtDNA content in the subcutaneous adipose tissue. The observation of low mtDNA content in lipotrophic adipose tissue is in agreement with previous data from different groups.<sup>5,6</sup> The content of mtDNA in skeletal muscle in the context of lipodystrophy has been reported in a few previous studies. Our finding is consistent with the study by Haugaard *et al.*,<sup>27</sup> who found similar mtDNA content in skeletal muscle between lipodystrophic and non-lipodystrophic HIV-infected patients. In the same study, there was no difference in skeletal muscle mtDNA content in patients taking versus not taking stavudine,<sup>27</sup> which is the drug most strongly associated with a loss of mtDNA content in subcutaneous fat. In contrast, Vittecoq *et al.*<sup>28</sup> reported low mtDNA copy numbers in skeletal muscle in lipodystrophic patients when compared with healthy controls. In their study, the inclusion criterion for the lipodystrophic group was the presence of at least two severe adverse effects. Twenty out of 21 patients in this study also had either neuropathy or amyotrophy and, hence, the observed abnormalities may be linked with these other complications of therapy rather than lipodystrophy.<sup>28</sup> In addition to these studies, Zaera *et al.*<sup>29</sup> measured mitochondrial protein content per milligram of skeletal muscle. Mitochondrial protein contents did not differ between HIV-infected lipodystrophic, HIV-infected non-lipodystrophic and healthy control subjects.<sup>29</sup>

Mitochondrial RNA (mtRNA) has been found to be decreased in adipose tissue of HIV-negative subjects after 2 weeks of exposure to NRTIs, even in the absence of depletion of

mtDNA.<sup>30</sup> The transcription of mtRNA has not been studied in skeletal muscle of HIV-infected patients and it was not evaluated in the present study.

Some aspects of mitochondrial function in skeletal muscle have been evaluated by different *ex vivo* methods in HIV-infected patients. The enzyme activity of mitochondrial respiratory complex I has been reported to be unchanged<sup>29,31</sup> or decreased<sup>28</sup> in skeletal muscle of lipodystrophic patients as compared with controls. The activity of complex II has been reported to be unchanged in two studies,<sup>29,31</sup> whereas the activities of complexes III and IV have been reported to be lower than in control subjects.<sup>28,29,31</sup> The activity of citrate synthase (a citric acid cycle enzyme) and hydroxyacyl-coenzyme A dehydrogenase (a mitochondrial enzyme catalysing the oxidation of fatty acids to acetyl-coenzyme A) were not different at rest or during exercise between lipodystrophic (or hyperlactataemic) HIV-infected patients versus healthy controls.<sup>32</sup> Also, the muscle glycogen content and the concentrations of IMP, AMP, ADP and ATP were similar between the groups.<sup>32</sup> It is, however, difficult to evaluate how much lipodystrophy *per se* contributed to the reported findings due to the heterogeneity of the study populations, e.g. in some studies the lipodystrophic patients had to present with additional complications and the control groups varied from healthy controls to HIV-infected treatment-naïve patients with uncontrolled viral replication.<sup>28,29,31,32</sup> HIV infection *per se* may play an important confounding effect, as was demonstrated in a study showing increased apoptosis in skeletal muscle in patients with HIV compared with non-infected persons; however, among the HIV-infected patients the apoptosis rate was similar in those with and without lipodystrophy.<sup>33</sup>

Recently, significant differences in mitochondrial function have been described in skeletal muscle in different anatomical sites. When HIV-negative patients with type 2 diabetes were compared with healthy controls, reduced mitochondrial function in diabetic patients was only present in the lower and not the upper limb skeletal muscle.<sup>34</sup> No study has yet evaluated mitochondrial function in the upper limb skeletal muscle in HIV-infected patients.

We evaluated whether skeletal muscle aerobic metabolism, which is partly dependent on myocellular mitochondrial function but also on oxygen delivery (perfusion), is affected in HIV-infected, antiretroviral-treated patients with lipodystrophy compared with HIV-infected, antiretroviral-treated patients without lipodystrophy. The PET technique used in the study allows direct *in vivo* measurement of muscle perfusion and oxygen consumption.<sup>19</sup> To our knowledge, this is the first study using PET scanning for the determination of the integrity of skeletal muscle exercise capacity in the context of ART-associated lipodystrophy.

Oxygen consumption was similar between the LA+ and non-LA groups, both at rest and during standardized exercise. In addition, muscle perfusion, which affects muscle oxygen supply, and oxygen extraction were similar between the study groups. The exercise intensity (7% of maximal force) was kept low enough in order not to exceed the aerobic capacity. The low concentration of blood lactate in both groups during exercise is consistent with the aerobic exercise intensity. The ability of the LA+ group to perform the same workload by consuming the same amount of oxygen without any increase in blood lactate as the non-LA group implies similar mitochondrial respiratory chain function, i.e. ATP production.

A recent DEXA modelling study suggests that increased resting energy expenditure in patients with LA could be explained by a hypermetabolic state in skeletal muscle.<sup>35</sup> However, the direct measurements of blood perfusion and oxygen consumption in skeletal muscle in the current study do not support this model.

In the present study, the blood oxygen carrying capacity, as determined from the haemoglobin concentration, was similar between the groups. Mitochondrial efficiency to produce ATP is also dependent on physical fitness. In the current study, the maximal voluntary isometric contraction force of knee extensors, which indirectly indicates physical fitness, was similar between the groups, and the leg lean tissue mass was also almost identical between the groups.

Previous studies evaluating the skeletal muscle performance in patients with ART-associated lipodystrophy have used incremental exercise tests until exhaustion. Our findings are in line with those by Roge *et al.*,<sup>32</sup> who found no evidence of significant damage to skeletal muscle mitochondrial function in lipodystrophic patients compared with healthy controls. In this study, the lipodystrophic patients had higher blood lactate concentrations at rest, but there was no significant difference in the maximal lactate levels or in the decline in blood lactate during the recovery period.<sup>32</sup> In contrast, in the study by Chaplain *et al.*,<sup>31</sup> lipodystrophic patients reached the lactic acidosis threshold at a lower workload and had lower maximum power output than antiretroviral-treated non-lipodystrophic patients; findings were considered to be due to mitochondrial dysfunction. However, these results may be confounded by the fact that the lipodystrophic patients were less fit than the non-lipodystrophic subjects.<sup>31</sup>

Further indirect evidence for the lack of substantial and at least irreversible mitochondrial toxicity in skeletal muscle in patients with ART-associated lipodystrophy comes from physical training interventions. In a randomized study with lipodystrophic patients, the peak oxygen uptake increased significantly in the exercise and diet group, whereas there was no change in the diet-only group.<sup>36</sup> Other studies have similarly shown improvement in exercise tolerance and aerobic fitness in patients with ART-associated lipodystrophy after training programmes during ongoing ART.<sup>37,38</sup> Furthermore, both endurance and strength training have improved skeletal muscle glucose uptake measured by clamp studies in patients with ART-associated lipodystrophy.<sup>39</sup> These data demonstrating an improvement in skeletal muscle function contrast the data from adipose tissue, where several studies have shown continuous worsening of LA during ongoing thymidine analogue NRTI (tNRTI)-containing ART.<sup>40-42</sup> In contrast, significant improvement in LA has been shown in clinical trials after switching away from a thymidine analogue.<sup>43</sup> This clinical improvement has been associated with a statistically significant increase in mtDNA content in adipose tissue, but not in skeletal muscle.<sup>44</sup> This again implies a different response in adipose tissue versus skeletal muscle.

During recent years, genetic studies have been performed to evaluate susceptibility to LA and/or mitochondrial damage during ART. Haemochromatosis gene polymorphism (HFE 187C→G) and mitochondrial haplotype J have been associated with a reduced risk of developing LA.<sup>45</sup> No genetic studies were performed in the present study.

The limitations of the current study include its small size due to complex methodology. Furthermore, the results do not

exclude the possibility that some antiretroviral agents may cause damage in skeletal muscle under certain circumstances, e.g. 1 month exposure to stavudine as compared with placebo in healthy volunteers decreased the mtDNA content in skeletal muscle and insulin sensitivity, as measured by hyperinsulinaemic euglycaemic clamp, without changes in body composition.<sup>46</sup> Our study focused on the potential inter-relations between LA and skeletal muscle, and we did not include a healthy unexposed control group. Therefore, we cannot exclude the possibility that there were some identical changes in skeletal muscle in both the LA+ and non-LA groups as compared with healthy subjects. Our results, however, clearly imply that those pathophysiological changes leading to LA in adipose tissue do not occur in a similar fashion and extent in skeletal muscle. Since none of the patients had any clinical signs of myopathy, these results cannot be generalized to patients with the well-characterized condition of zidovudine-associated myopathy. Intermuscular adipose tissue, the role of which is under investigation in HIV-related metabolic abnormalities,<sup>47</sup> was not evaluated in the present study.

In conclusion, data from the current study imply that although HIV-infected patients with ART-associated LA have decreased mtDNA content in subcutaneous fat, they have similar mtDNA content in skeletal muscle and comparable skeletal muscle aerobic exercise metabolism as HIV-infected, antiretroviral-treated non-LA patients. NRTIs seem to cause mitochondrial defects in adipose tissue, but to spare skeletal muscle function in these patients. The tissue specificity of mitochondrial toxicity in ART-associated LA is reminiscent of other mitochondrial diseases, which in general demonstrate tissue specificity.<sup>48,49</sup>

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## Transparency declarations

None to declare.

## References

- Bhaskaran K, Hamouda O, Sannes M *et al.* Changes in the risk of death after HIV seroconversion compared with mortality in the general population. *JAMA* 2008; **300**: 51–9.
- Grinspoon S, Carr A. Cardiovascular risk and body-fat abnormalities in HIV-infected adults. *N Engl J Med* 2005; **352**: 48–62.
- Brinkman K, Smeitink JA, Romijn JA *et al.* Mitochondrial toxicity induced by nucleoside-analogue reverse-transcriptase inhibitors is a key factor in the pathogenesis of antiretroviral-therapy-related lipodystrophy. *Lancet* 1999; **354**: 1112–5.
- Kakuda TN. Pharmacology of nucleoside and nucleotide reverse transcriptase inhibitor-induced mitochondrial toxicity. *Clin Ther* 2000; **22**: 685–708.
- Shikuma CM, Hu N, Milne C *et al.* Mitochondrial DNA decrease in subcutaneous adipose tissue of HIV-infected individuals with peripheral lipotrophy. *AIDS* 2001; **15**: 1801–9.
- Walker UA, Bickel M, Lutke Volksbeck SI *et al.* Evidence of nucleoside analogue reverse transcriptase inhibitor-associated genetic and structural defects of mitochondria in adipose tissue of HIV-infected patients. *J Acquir Immune Defic Syndr* 2002; **29**: 117–21.
- Rabing Christensen E, Stegger M, Jensen-Fangel S *et al.* Mitochondrial DNA levels in fat and blood cells from patients with lipodystrophy or peripheral neuropathy and the effect of 90 days of high-dose coenzyme Q treatment: a randomized, double-blind, placebo-controlled pilot study. *Clin Infect Dis* 2004; **39**: 1371–9.
- Gerschenson M, Shiramizu B, LiButti DE *et al.* Mitochondrial DNA levels of peripheral blood mononuclear cells and subcutaneous adipose tissue from thigh, fat and abdomen of HIV-1 seropositive and negative individuals. *Antivir Ther* 2005; **10** Suppl 2: M83–9.
- van der Valk M, Casula M, Weverlingz GJ *et al.* Prevalence of lipotrophy and mitochondrial DNA content of blood and subcutaneous fat in HIV-1-infected patients randomly allocated to zidovudine- or stavudine-based therapy. *Antivir Ther* 2004; **9**: 385–93.
- Cossarizza A, Riva A, Pinti M *et al.* Increased mitochondrial DNA content in peripheral blood lymphocytes from HIV-infected patients with lipodystrophy. *Antivir Ther* 2003; **8**: 315–21.
- Arnaudo E, Dalakas M, Shanske S *et al.* Depletion of muscle mitochondrial DNA in AIDS patients with zidovudine-induced myopathy. *Lancet* 1991; **337**: 508–10.
- Hengel RL, Watts NB, Lennox JL. Benign symmetric lipomatosis associated with protease inhibitors. *Lancet* 1997; **350**: 1596.
- Miller KD, Jones E, Yanovski JA *et al.* Visceral abdominal-fat accumulation associated with use of indinavir. *Lancet* 1998; **351**: 871–5.
- Lebrecht D, Deveaud C, Beauvoit B *et al.* Uridine supplementation antagonizes zidovudine-induced mitochondrial myopathy and hyperlactatemia in mice. *Arthritis Rheum* 2008; **58**: 318–26.
- Desai VG, Lee T, Moland CL *et al.* Effect of short-term exposure to zidovudine (AZT) on the expression of mitochondria-related genes in skeletal muscle of neonatal mice. *Mitochondrion* 2009; **9**: 9–16.
- Carr A, Cooper DA. Adverse effects of antiretroviral therapy. *Lancet* 2000; **356**: 1423–30.
- Schambelan M, Benson CA, Carr A *et al.* Management of metabolic complications associated with antiretroviral therapy for HIV-1 infection: recommendations of an international AIDS society—USA panel. *J Acquir Immune Defic Syndr* 2002; **31**: 257–75.
- Authier FJ, Chariot P, Gherardi RK. Skeletal muscle involvement in human immunodeficiency virus (HIV)-infected patients in the era of highly active antiretroviral therapy (HAART). *Muscle Nerve* 2005; **32**: 247–60.
- Nuutila P, Kalliokoski K. Use of positron emission tomography in the assessment of skeletal muscle and tendon metabolism and perfusion. *Scand J Med Sci Sports* 2000; **10**: 346–50.
- Sutinen J, Häkkinen AM, Westerbacka J *et al.* Rosiglitazone in the treatment of HAART-associated lipodystrophy—a randomized double-blind placebo-controlled study. *Antivir Ther* 2003; **8**: 199–207.
- Laaksonen MS, Kalliokoski KK, Kyröläinen H *et al.* Skeletal muscle blood flow and flow heterogeneity during dynamic and isometric exercise in humans. *Am J Physiol Heart Circ Physiol* 2003; **284**: H979–86.

- 22 Laaksonen MS, Kivelä R, Kyröläinen H et al. Effects of exhaustive stretch-shortening cycle exercise on muscle blood flow during exercise. *Acta Physiol (Oxf)* 2006; **186**: 261–70.
- 23 Sipilä H, Clark JC, Peltola O et al. An automatic [ $^{15}\text{O}$ ]H $_2$ O production system for heart and brain studies. *J Labelled Compd Rad* 2001; **44**: S1066–8.
- 24 Nuutila P, Raitakari M, Laine H et al. Role of blood flow in regulating insulin-stimulated glucose uptake in humans. Studies using bradykinin, [ $^{15}\text{O}$ ]water, and [ $^{18}\text{F}$ ]fluoro-deoxy-glucose and positron emission tomography. *J Clin Invest* 1996; **97**: 1741–7.
- 25 Kalliokoski KK, Kemppainen J, Larmola K et al. Muscle blood flow and flow heterogeneity during exercise studied with positron emission tomography in humans. *Eur J Appl Physiol* 2000; **83**: 395–401.
- 26 Matthews DR, Hosker JP, Rudenski AS et al. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; **28**: 412–9.
- 27 Haugaard SB, Andersen O, Pedersen SB et al. Depleted skeletal muscle mitochondrial DNA, hyperlactatemia, and decreased oxidative capacity in HIV-infected patients on highly active antiretroviral therapy. *J Med Virol* 2005; **77**: 29–38.
- 28 Vittecoq D, Jardel C, Barthelemy C et al. Mitochondrial damage associated with long-term antiretroviral treatment: associated alteration or causal disorder? *J Acquir Immune Defic Syndr* 2002; **31**: 299–308.
- 29 Zaera MG, Miro O, Pedrol E et al. Mitochondrial involvement in antiretroviral therapy-related lipodystrophy. *AIDS* 2001; **15**: 1643–51.
- 30 Mallon PW, Unemori P, Sedwell R et al. *In vivo*, nucleoside reverse-transcriptase inhibitors alter expression of both mitochondrial and lipid metabolism genes in the absence of depletion of mitochondrial DNA. *J Infect Dis* 2005; **191**: 1686–96.
- 31 Chapplain JM, Beillot J, Begue JM et al. Mitochondrial abnormalities in HIV-infected lipotrophic patients treated with antiretroviral agents. *J Acquir Immune Defic Syndr* 2004; **37**: 1477–88.
- 32 Roge BT, Calbet JA, Moller K et al. Skeletal muscle mitochondrial function and exercise capacity in HIV-infected patients with lipodystrophy and elevated p-lactate levels. *AIDS* 2002; **16**: 973–82.
- 33 Miro O, Lopez S, Fernandez-Sola J et al. Short communication: HIV infection, antiretrovirals, and apoptosis: studies on skeletal muscle. *AIDS Res Hum Retroviruses* 2005; **21**: 702–5.
- 34 Rabol R, Larsen S, Hojberg PM et al. Regional anatomic differences in skeletal muscle mitochondrial respiration in type 2 diabetes and obesity. *J Clin Endocrinol Metab* 2010; **95**: 857–63.
- 35 Kosmiski LA, Ringham BM, Grunwald GK et al. Dual-energy X-ray absorptiometry modeling to explain the increased resting energy expenditure associated with the HIV lipotrophy syndrome. *Am J Clin Nutr* 2009; **90**: 1525–31.
- 36 Terry L, Sprinz E, Stein R et al. Exercise training in HIV-1-infected individuals with dyslipidemia and lipodystrophy. *Med Sci Sports Exerc* 2006; **38**: 411–7.
- 37 Jones SP, Doran DA, Leatt PB et al. Short-term exercise training improves body composition and hyperlipidaemia in HIV-positive individuals with lipodystrophy. *AIDS* 2001; **15**: 2049–51.
- 38 Thoni GJ, Fedou C, Brun JF et al. Reduction of fat accumulation and lipid disorders by individualized light aerobic training in human immunodeficiency virus infected patients with lipodystrophy and/or dyslipidemia. *Diabetes Metab* 2002; **28**: 397–404.
- 39 Lindegaard B, Hansen T, Hvid T et al. The effect of strength and endurance training on insulin sensitivity and fat distribution in human immunodeficiency virus-infected patients with lipodystrophy. *J Clin Endocrinol Metab* 2008; **93**: 3860–9.
- 40 Mallon PW, Miller J, Cooper DA et al. Prospective evaluation of the effects of antiretroviral therapy on body composition in HIV-1-infected men starting therapy. *AIDS* 2003; **17**: 971–9.
- 41 Nolan D, Hammond E, James I et al. Contribution of nucleoside-analogue reverse transcriptase inhibitor therapy to lipotrophy from the population to the cellular level. *Antivir Ther* 2003; **8**: 617–26.
- 42 Pozniak AL, Gallant JE, DeJesus E et al. Tenofovir disoproxil fumarate, emtricitabine, and efavirenz versus fixed-dose zidovudine/lamivudine and efavirenz in antiretroviral-naïve patients: virologic, immunologic, and morphologic changes—a 96-week analysis. *J Acquir Immune Defic Syndr* 2006; **43**: 535–40.
- 43 Carr A, Workman C, Smith DE et al. Abacavir substitution for nucleoside analogs in patients with HIV lipotrophy: a randomized trial. *JAMA* 2002; **288**: 207–15.
- 44 McComsey GA, Paulsen DM, Lonergan JT et al. Improvements in lipotrophy, mitochondrial DNA levels and fat apoptosis after replacing stavudine with abacavir or zidovudine. *AIDS* 2005; **19**: 15–23.
- 45 Hulgán T, Tebas P, Canter JA et al. Hemochromatosis gene polymorphisms, mitochondrial haplogroups, and peripheral lipotrophy during antiretroviral therapy. *J Infect Dis* 2008; **197**: 858–66.
- 46 Fleischman A, Johnsen S, Systrom DM et al. Effects of a nucleoside reverse transcriptase inhibitor, stavudine, on glucose disposal and mitochondrial function in muscle of healthy adults. *Am J Physiol Endocrinol Metab* 2007; **292**: E1666–73.
- 47 Bacchetti P, Grunfeld C, Heymsfield SB et al. Intermuscular adipose tissue is decreased in HIV infection. In: *Abstracts of the Eleventh International Workshop on Adverse Drug Reactions and Co-Morbidities in HIV, Philadelphia, PA, 2009*. *Antivir Ther* 2009; **14** Suppl 2: A3. Abstract 001.
- 48 Goto Y. Clinical and molecular studies of mitochondrial disease. *J Inherit Metab Dis* 2001; **24**: 181–8.
- 49 Mazat JP, Rossignol R, Malgat M et al. What do mitochondrial diseases teach us about normal mitochondrial functions...that we already knew: threshold expression of mitochondrial defects. *Biochim Biophys Acta* 2001; **1504**: 20–30.