MAJOR ARTICLE

Gene Expression and Immunohistochemistry in Adipose Tissue of HIV Type 1–Infected Patients with Nucleoside Analogue Reverse-Transcriptase Inhibitor–Associated Lipoatrophy

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Background. Long-term use of both zidovudine (AZT) and stavudine (d4T) is associated with lipoatrophy, but it occurs possibly through different mechanisms.

Methods. Surgical biopsy specimens of subcutaneous adipose tissue were obtained from 18 human immunodeficiency virus type 1 (HIV-1)–infected lipoatrophic patients (the LA+ group) who were treated with either zidovudine (the AZT+LA+ group; $n = 10$) or stavudine (the d4T+LA+ group; $n = 8$) and from 10 nonlipoatrophic HIV-1–infected patients (the LA− group) who received antiretroviral therapy. Mitochondrial DNA (mtDNA) copy numbers, gene expression, and immunohistochemistry data were analyzed.

Results. mtDNA copy numbers were significantly reduced in the LA+ group, compared with the LA− group, and in the d4T+LA+ group, compared with the AZT+LA+ group. The ratio of mtDNA-encoded cytochrome *COX3* to nuclear DNA–encoded *COX4* expression was significantly lower in the LA+ group than in the LA− group. Compared with the LA− group, the LA+ group had significantly lower expression of genes involved in adipogenesis *(SREBP1c* and *CEBPB)*, lipid (fatty acid synthase), and glucose *(GLUT4)* metabolism. Expression of genes involved in mitochondrial biogenesis *(PGC1B)*, apoptosis *(FAS)*, inflammation *(IL1B)*, oxidative stress *(PCNA* and *SOD1)*, and lamin B was significantly higher in the LA+ group than in the LA− group. The d4T+LA+ group had significantly lower expression of genes involved in mitochondrial biogenesis *(POLG1)*, energy metabolism (the *COX3/COX4* ratio), adipogenesis *(SREBP1c* and *CEBPA)*, perilipin, and hexokinase than did the AZT+LA+ group. There were 7-fold more macrophages in adipose tissue specimens obtained from patients in the LA+ group, compared with the LA− group.

Conclusions. Lipoatrophy is characterized by mtDNA depletion, inflammation, and signs of apoptosis. Changes were more profound in the d4T+LA+ group than in the AZT+LA+ group.

Abnormalities in body fat distribution (lipodystrophy) are highly prevalent in human immunodeficiency virus type 1 (HIV-1)–infected patients who are receiving long-term treatment with antiretroviral drugs. Lipoatrophy (LA; i.e., loss of subcutaneous fat) is a clinical feature of the lipodystrophy syndrome and is associated with metabolic complications [1, 2].

It has been suggested that nucleoside analogue reverse-transcriptase inhibitor (NRTI)–induced mitochondrial toxicity is the main pathophysiological mechanism responsible for LA via inhibition of the mitochondrial DNA (mtDNA) polymerase γ (POLG) [3]. Consistent with this hypothesis, mtDNA depletion

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has been described in adipocyte cultures treated with antiretroviral drugs and in tissue samples obtained from lipoatrophic patients, along with a decrease of mitochondrial respiratory chain subunits and increased production of reactive oxygen species [4]. Clinical trials have linked LA with long-term use of the thymidine nucleoside analogues zidovudine (AZT) and stavudine (d4T) [5].

In vitro studies show a hierarchy of different NRTIs that interact with POLG [6, 7]. Although the active form of d4T is efficiently incorporated in the nascent mtDNA by POLG and causes chain termination, AZT has virtually no effect on mtDNA chain elongation [8]. Nevertheless, AZT has been associated with mtDNA depletion [4], adipocyte apoptosis [4, 9], and clinical LA [10]. This implies that AZT induces its mitochondrial toxicity via a POLG-independent mechanism.

Treatment with d4T results in more-severe LA than does treatment with AZT [5]. We hypothesized that there may be differences between AZT- and d4T-treated lipoatrophic patients with regard to mtDNA copy numbers and expression of mitochondria-encoded genes, as well as in nuclear transcripts involved in mitochondrial biogenesis, adipogenesis, and metabolic and endocrine function. Currently, there are little human data comparing these 2 NRTIs in this regard [11]. In the present study, we compared mtDNA content and gene expression in highly active antiretroviral therapy (HAART)–treated patients with and without LA. Moreover, we conducted a comparison between AZT- and d4T-associated LA. Finally, an immunohistochemical analysis was performed to compare the inflammatory state and vitality of lipoatrophic adipose tissue in AZT- and d4T-associated LA.

MATERIALS AND METHODS

Patients. After approval by the ethics review committee and provision of written and informed from the patients, clinically stable HIV-1–infected adult patients were recruited from the HIV outpatient clinic of the Helsinki University Central Hospital (Helsinki, Finland). Lipoatrophic patients had to be treated with HAART for at least 18 months, with no changes in the regimen for the 12 weeks before the study, and had to be taking either d4T or AZT. LA was defined as self-reported and investigator-confirmed loss of subcutaneous fat, with or without increased abdominal girth or breast size or development of a buffalo hump. Patients with LA were participants in a study examining the effects of uridine on LA, but all present examinations were performed before the uridine and/or placebo intervention [12]. Nonlipoatrophic patients had to have been taking HAART for a minimum of 18 months, with no changes in the regimen for the 12 weeks prior to the study, and to have not developed symptoms of LA (self-reported and clinician-confirmed) while receiving antiretroviral therapy. HIV-1–infected patients with LA who were taking HAART (hereafter referred to as "the LA+ group") were compared with those who had not developed LA while receiving HAART (hereafter referred to as "the LA− group"). We subdivided the LA+ group into a subgroup of patients who were taking d4T (hereafter referred to as "the d4T+LA+ group") or AZT (hereafter referred to as "the AZT+LA+ group").

Measures of body composition. Limb, truncal, and total body fat were measured using dual-energy x-ray absorptiometry (Lunar Prodigy). Intra-abdominal and abdominal subcutaneous fat were quantified by analyzing 16 T1-weighted trans-axial magnetic resonance images, as described elsewhere [13].

Subcutaneous fat biopsies. After administration of local anaesthesia with lidocaine, subcutaneous fat biopsy specimens were surgically taken from the midpoint between the iliac crest and the umbilicus. Part of the biopsy specimen was immediately snap-frozen in liquid nitrogen, and 100 mg of tissue from each patient was subsequently processed with trizol for RNA extraction. Another part of the biopsy was formalin fixed and paraffin embedded for subsequent immunohistochemical analyses.

RNA extraction. One hundred mg of adipose tissue per patient was homogenized using the Geneclean isolation kit (Bio101 systems, Obiogene). RNA was extracted with the RNeasy Lipid Tissue Kit (Qiagen) in accordance with the manufacturer's instructions. The quantity and integrity of RNA were verified using RNA 6000 nanochips (Agilent2100 Bioanalyser). For each sample 1.5 μg of RNA was used for reverse transcription, employing 400 U of Superscript II (Invitrogen) and 100 μM of oligo$(dT)_{12–18}$ (Invitrogen) as primer.

Real-time quantification of gene expression. Primer pairs were designed in an intron-spanning fashion to avoid unspecific amplification of contaminating genomic DNA, using the universal probe library from Roche (http://www.universalprobelibrary.com). The complete list of primer sequences is available in table 1, which appears only in the electronic version of the *Journal*. Gene expression was quantified using the LightCycler 480 (Roche) on a 384-well plate. Ten-μL reactions contained 5 μL of SYBR Green I Master mix (Roche), 50 ng of cDNA template, and 0.5 μM of each primer. Target genes were run in duplicate on 1 plate, which included all patient samples plus standard dilution curve of a glyceraldehyde-3-phosphate dehydrogenase plasmid (6 dilutions from a factor of 10^2 to 10^7) plus a no-template control. Cycling conditions were as follows: activation, 95°C for 10 s; and 40 amplification cycles at 95°C for 10 s, 52°C for 5 s, and 72°C for 12 s. Polymerase chain reaction conditions were optimized for linearity of amplification for all primers in a dilution series. Melting curve analysis

Table 1. Primer sequences of the studied genes.

This table is available in its entirety in the online edition of the *Journal of Infectious Diseases*

was performed to ensure that all investigated genes were represented by a single peak, indicating specificity. Gene expression was calculated from the real-time polymerase chain reaction efficiency [14] in relation to the mean of 3 housekeeping genes *(ACTB, 36B4,* and *B2M)* that are commonly used [15]. Nonregulation of the housekeeping genes was validated using geNorm, version 3.4 (PrimerDesign) [16].

Gene expression analysis. All studied genes, with their full and abbreviated names and brief function descriptions, are provided in table 2.

mtDNA copy numbers. Genomic DNA was extracted from adipose tissue using the QIAamp DNA isolation kit (Qiagen). mtDNA and nuclear DNA (nDNA) copy numbers were determined by quantitative polymerase chain reaction using the ABI 7700 sequence detection system (Applied Biosystems). We amplified the mtDNA-encoded ATP synthase 6 gene between nucleotide positions 8981 and 9061. mtDNA was quantified with a FAM fluorophore–labelled probe (5′–6FAM-CCTAACC-GCTAACATTACTGCAGGCC ACC-TAMRA-3′). For the detection of nDNA, we selected exon number 8 of the glyceraldehyde-3-phosphate dehydrogenase gene between nucleotide positions 4280 and 4342 and used a VIC fluorophore–labelled probe (5′–VIC-CCCTGCCTCTACTGGCGCTGCC-TAMRA-3′). Each 25-μL reaction contained 25 ng of genomic DNA, 100 nM of probe, 200 nM of primers, and TaqMan Universal Master Mix (Applied Biosystems). Amplifications of mitochondrial and nuclear products were separately performed in optical 96-well plates (Applied Biosystems). An initial incubation at 50°C for 2 min was followed by 10 min at 95°C and 40 denaturing steps at 95°C for 15 s, alternating with combined annealing and/or extension at 60°C for 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.

Immunohistochemistry. Adipose tissue biopsy specimens were used for immunohistochemical analysis. CD68 served as a marker for macrophages and perilipin (PLIN) as a marker for viable adipocytes [17]. Sectioning was performed using a

Table 2. Full and abbreviated names of genes included in the study and the function of the genes.

Abbreviation	Gene	Function
16SRNA	16S ribosomal RNA	Mitochondrial transcription
36B4	Acidic ribosomal phosphoprotein P0	Housekeeping gene
ACTB	Actin β	Housekeeping gene
B2M	β2 Microglobulin	Housekeeping gene
CEBPA	CCAAT/enhancer binding protein–α	Early adipocyte differentiation
CEBPB	CCAAT/enhancer binding protein–β	Adipocyte differentiation
COX3	Cytochrome c oxidase subunit III	Respiratory chain subunit
COX4	Cytochrome c oxidase subunit IV	Respiratory chain subunit
FAS	Factor of apoptotic stimulus	Regulation of apoptosis
FASN	Fatty acid synthase	Lipid metabolism
GLUT4	Glucose transporter 4	Glucose metabolism
GPX1	Glutathione peroxidase transcript variant 1	Scavenging of reactive oxygen species
HEXOK1	Hexokinase 1	Glucose metabolism
IL1B	Interleukin-1β	Proinflammatory cytokine
IL6	Interleukin-6	Proinflammatory cytokine
LMNA	Lamin A/C	Nuclear DNA maturation, chromatin organization
LMNB	Lamin B	Marker of cell proliferation
p53	Tumor protein p53	Regulation of apoptosis
PCNA	Proliferating cell nuclear antigen	Marker of cell proliferation
PGC1B	Peroxisome proliferator–activated gamma coactivator 1β	Mitochondrial transcription
PLIN	Perilipin	Lipid metabolism
POLG1	Polymerase γ (catalytic subunit)	Mitochondrial biogenesis
POLG2	Polymerase γ (accessory subunit)	Mitochondrial biogenesis
PPARG	Peroxisome proliferator–activated receptor γ	Lipogenesis
PPARG2	Peroxisome proliferator–activated receptor γ subunit 2	Lipogenesis
SOD1	Superoxide dismutase 1 (cytosolic)	Scavenging of reactive oxygen species
SREBP1c	Sterol regulatory element–binding protein 1c	Lipogenesis
TFAM	Mitochondrial transcription factor A	Mitochondrial transcription
TNFA	Tumor necrosis factor–α	Proinflammatory cytokine

standard protocol for formalin-fixed paraffin-embedded tissue blocks. Consecutive serial sections were treated with xylene, descending ethanol dilution series, and distilled water to dewax the tissue samples. Thereafter, sections were microwave-treated in 10 mM of citrate buffer (pH, 6.0) and washed alternately with distilled water, hydrogen peroxide, and phosphate-buffered saline with 0.25% Triton X-100 (pH, 7.2) to inactivate endogenous staining. Nonspecific staining was reduced by applying normal goat serum (ratio, 1:5; Dako) to the sections for 30 min. Samples were then incubated for 1 h at room temperature with mouse monoclonal anti-CD68 (ratio, 1:200; Novocastra Laboratories) or guinea pig polyclonal anti-PLIN (ratio, 1:1000; Acris Antibodies GmbH). For negative controls of CD68 staining, mouse monoclonal isotypic control (ratio, 1:200; Abcam) was used as a primary antibody. For PLIN-negative control, the primary antibody was omitted. After rinsing in phosphate-buffered saline–Triton X-100 buffer, sections were incubated with biotinylated anti-mouse (ratio, 1:1500; Vector Laboratories) or anti-guinea pig (ratio, 1:1500; Abcam) secondary antibodies. Avidin-biotin peroxidase complexes (Vector Laboratories) were added followed by visualization with 3,3-diaminobenzidine tetrachloride (Vector Laboratories). After washes with distilled water, ascending ethanol series, and xylene, sections were counterstained with Harris hematoxylin (Histolab). For each sample, the number of macrophages, crown-like structures and PLIN-free cells in the entire section were counted using light microscopy and normalized for the total section area. Macrophages were identified as CD68-positive cells, and crown-like structures were defined as 1 PLIN-free adipocyte surrounded by $\geqslant 3$ macrophages [17]. Measurement of total section area using arbitrary units was performed using Adobe Photoshop Elements, version 1.0.1 (Adobe Systems).

Statistical analysis. Demographic and clinical parameters among the study groups were compared using Fisher's exact test for categorical variables and unpaired *t* test or the Wilcoxon-Mann-Whitney test for continuous variables, as appropriate. Correlations were calculated using the Pearson's product-moment coefficient. *P* values were not adjusted for multiple comparisons. For statistical analyses, we used Sigma Stat for Windows software, version 3.0 (Jandel Corporation); GraphPad Prism, version 3.02 (GraphPad Software); and Lotus 1–2-3 of Lotus SmartSuite Release 9.5 (Lotus Development Corporation, IBM Corporation). Data are presented as mean ± standard deviation. Two-tailed *P* values <.05 were considered to be statistically significant.

RESULTS

Demographic and HIV-1 characteristics of the patients. The LA+ and LA− groups (table 3) were comparable with respect to age, sex, duration of HIV-1 infection, $CD4^+$ T cell count, and HIV-1 RNA load. The LA+ group had significantly less total limb fat and a longer history of antiretroviral therapy than did the LA− group.

With regard to the AZT+ and d4T+LA+ subgroups (table 3), the treatment-related characteristics were similar. The d4T+LA+ group had a significantly lower body mass index and less limb, truncal, and total fat, compared with the AZT+LA+ group. The amount of intra-abdominal fat was similar between the LA subgroups.

Of the 18 patients with lipoatrophy, 10 received zidovudine (AZT+LA+) and 8 received stavudine (d4T+LA+). In the AZT+LA+ group, all patients received lamivudine, and 2 patients also received abacavir. All patients in the AZT+LA+ group also received a protease inhibitor (PI); 2 patients received a double-boosted PI regimen, and 2 received a PI plus a non-NRTI (NNRTI). The following PIs and NNRTIs were prescribed: ritonavir-boosted lopinavir ($n = 8$), amprenavir ($n = 2$), indinavir ($n = 2$), efavirenz ($n = 1$), and nevirapine ($n = 1$). In the d4T+LA+ group, 7 patients received lamivudine and 1 patient received tenofovir plus abacavir. All 8 patients received a PI (1 patient received a PI and an NNRTI). The following PIs and NNRTIs were prescribed: ritonavir-boosted lopinavir ($n = 3$), nelfinavir ($n = 3$), ritonavir-boosted indinavir ($n = 1$), ritonavir-boosted saquinavir ($n = 1$), and efavirenz ($n = 1$). In the LA− group, all patients received lamivudine, and 9 patients received zidovudine. In this group, 2 patients received a PI (1 received ritonavir-boosted lopinavir, and 1 received nelfinavir), and 8 patients received an NNRTI (4 received efavirenz, and 4 received nevirapine).

Fat histology. In adipose tissue specimens obtained from patients in the LA+ group, there were 7-fold more macrophages than specimens from the LA− group (14.1 ± 13.1 vs. 2.3 ± 1.9 macrophages per 100,000 arbitrary area units; $P = .01$) (figure 1). The number of macrophages did not differ between the LA subgroups. The number of crown-like structures and PLIN-free cells did not differ between the LA+ and LA− groups or between the LA subgroups (figure 2).

mtDNA copy numbers. The mean amount of mtDNA was significantly lower in the LA+ group than in the LA− group (238 ± 129 vs. 585 ± 558 copies/cell; $P = .009$). Furthermore, the d4T+LA+ group had a significantly lower mean mtDNA copy number, compared with did the AZT+LA+ group (139 ± 59 vs. 317 ± 115 copies/cell; $P = .001$).

Gene transcripts related to mitochondrial function. Expression of several genes involved in the supply of respiratory chain subunits—namely, the mtDNA-encoded cytochrome c oxidase subunit 3 *(COX3)* and *16SRNA* and the nDNA-encoded *COX4*. *COX3* transcripts were slightly, but not significantly, lower in the LA+ group than in the LA− group, whereas *COX4* transcripts were significantly higher in the LA+ group (table 4). These changes resulted in a significantly lower *COX3/*

Table 3. Human immunodeficiency virus type 1 (HIV-1)–related and body composition characteristics of the study groups.

Characteristic	LA+ group (n = 18)	LA− group (n = 10)	P[a]	d4T+LA+ group (n = 8)	AZT+LA+ group (n = 10)	P[b]
Demographic and HIV-1–related characteristics						
Patient age, years	46.7 ± 10.0	43.3 ± 11.0	.42	49.3 ± 11.9	44.6 ± 8.2	.34
No. of female patients	3	1	>.99	1	2	>.99
Duration of HIV-1 infection, years	9.9 ± 4.6	6.9 ± 3.6	.09	8.2 ± 3.4	11.2 ± 4.9	.18
Duration of HAART, years	5.9 ± 1.8	4.2 ± 1.8	.02	6.0 ± 1.5	5.8 ± 2.1	.86
No. of patients with an HIV-1 RNA level <50 copies/mL	15	10	.53	7	8	>.99
$CD4^+$ T cell count, cells/mL	548 ± 270	543 ± 187	.96	592 ± 248	513 ± 294	.56
Receipt of zidovudine	10	9	.10	0	10	<.001
Receipt of stavudine	8	0	.03	8	0	<.001
Receipt of PI(s)	18	2	<.001	8	10	NA
Receipt of NNRTI(s)	3	8	<.001	1	2	>.99
Body composition						
Weight, kg	73.6 ± 13.3	75.0 ± 10.8	.79	66.6 ± 10.0	79.2 ± 13.3	.04
BMI	23.5 ± 3.4	23.5 ± 2.8	.97	21.5 ± 2.6	25.1 ± 3.2	.02
Total limb fat, g	3243 ± 2610	5750 ± 2767	.03	1554 ± 1474	4594 ± 2572	.009
Total truncal fat, g	9546 ± 4961	9377 ± 4858	.93	6772 ± 2495	11,766 ± 5416	.03
Total fat, g	13,223 ± 7444	15,683 ± 7661	.43	8656 ± 3891	16,876 ± 7716	.02
Intra-abdominal fat, cm^3	2171 ± 1237	854 ± 567	.004	1901 ± 803	2388 ± 1507	.42

NOTE. Data are mean ± standard deviation, unless otherwise indicated. AZT+LA+ group, HIV-1–infected patients with lipoatrophy whose HAART regimen included zidovudine; BMI, body mass index (calculated as weight in kilograms divided by the square of height in meters); d4T+LA+ group, HIV-1–infected patients with lipoatrophy whose HAART regimen included stavudine; HAART, highly active antiretroviral therapy; LA+ group, HIV-1–infected patients with lipoatrophy who were taking HAART; LA− group, HIV-1–infected patients who had not developed lipoatrophy while receiving HAART; NA, not applicable; NNRTI, nonnucleoside reverse-transcriptase inhibitor; PI, protease inhibitor.

[a] LA+ group vs. LA− group.

[b] d4T+LA+ group vs. AZT+LA+ group.

COX4 ratio in the LA+ than the LA− group (table 4). The d4T+LA+ group had significantly lower expression of *COX3* and *16SRNA*, compared with the AZT+LA+ group (table 5). Also, the *COX3/COX4* ratio was significantly lower in the d4T+LA+ group than in the AZT+LA+ group (table 5).

mtRNA per mtDNA template. To calculate the relative number of RNA transcripts per molecule of mtDNA template, we normalized mtDNA-encoded genes for the amount of mtDNA molecules. Relative to the mtDNA copies per cell, transcription of mtDNA-encoded genes was significantly higher in the LA+ group than in the LA− group (table 6). This effect was more pronounced (although the difference was statistically insignificant) in the d4T+LA+ group than in the AZT+LA+ group (table 6).

Gene transcripts involved in mitochondrial biogenesis. Polymerase γ, an enzyme consisting of 2 subunits *(POLG1* and *POLG2)* and mitochondrial transcription factor A *(TFAM)*, which provides the replication primer, play a key role in the regulation of mtDNA replication [18]. PPARG coactivator 1β *(PGC1B)* stimulates mitochondrial gene transcription via activation of *TFAM* [18] and enhances transcription of the peroxisome proliferator–activated receptor γ *(PPARG)*, thus representing a link between mitochondrial biogenesis and adipose cell function [19].

POLG1 and *POLG2* transcription did not differ between the LA+ and the LA− groups (table 4). Expression of *POLG1*, but not of *POLG2*, was significantly lower in the d4T+LA+ group than in the AZT+LA+ group (table 5). *TFAM* expression was comparable between all groups. The *PGC1B* transcripts were 7-fold higher in the LA+ group than in the LA− group (table 4). There was no significant difference between the AZT+LA+ and d4T+LA+ groups with regard to *PGC1B* (table 5).

Adipogenesis. *SREBP1c* expression was lower in the LA+ group than in the LA− group (table 4). Among all patients, there was an inverse correlation between the expression of *SREBP1c* and *PGC1B* ($r = -0.58$; $P = .002$) and a positive correlation between *SREBP1c* expression and mtDNA copy number ($r = 0.55$; $P = .014$). *CEBPA* and *SREBP1c* transcript levels were lower in the d4T+LA+ group than in the AZT+LA+ group (table 5).

Lamin B *(LMNB)* expression was significantly increased in the LA+ group, compared with the LA− group (table 4). Expression of lamin A/C *(LMNA)* and *LMNB* was comparable between the LA subgroups (table 5).

Lipid and glucose metabolism. The LA+ group had fewer fatty acid synthase *(FASN)* and glucose transporter 4 *(GLUT4)* transcripts did than the LA− group (table 4). *PLIN* and *HEXOK1* transcripts were significantly lower in the d4T+LA+

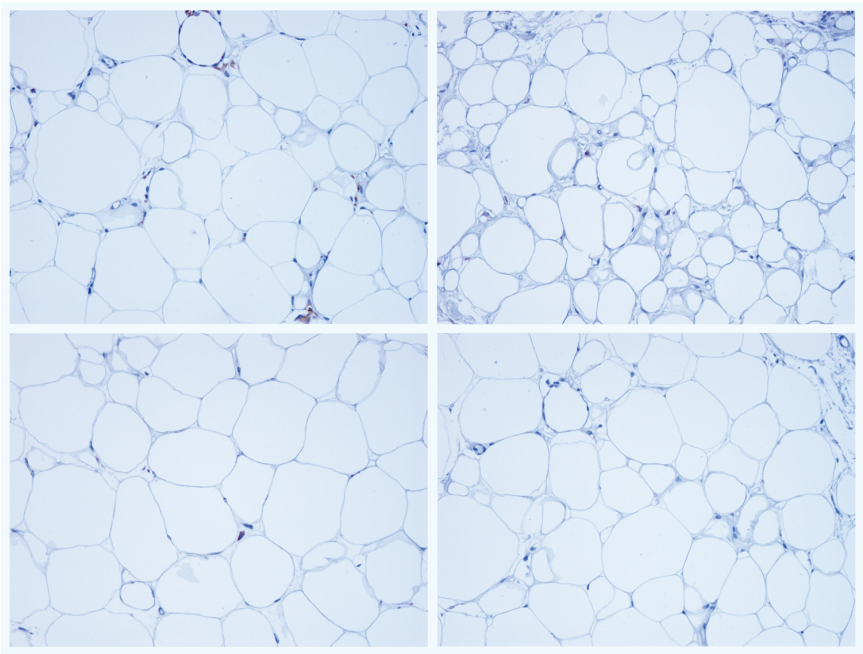

Figure 1. Immunohistochemical staining with CD68. Macrophages are identified as CD68-positive cells *(brown)* in the adipose tissue. Original magnification, ×300. Representative images of zidovudine-associated lipoatrophy *(upper left),* stavudine-associated lipoatrophy *(upper right),* no lipoatrophy *(lower left),* and a CD68-negative control *(lower right).*

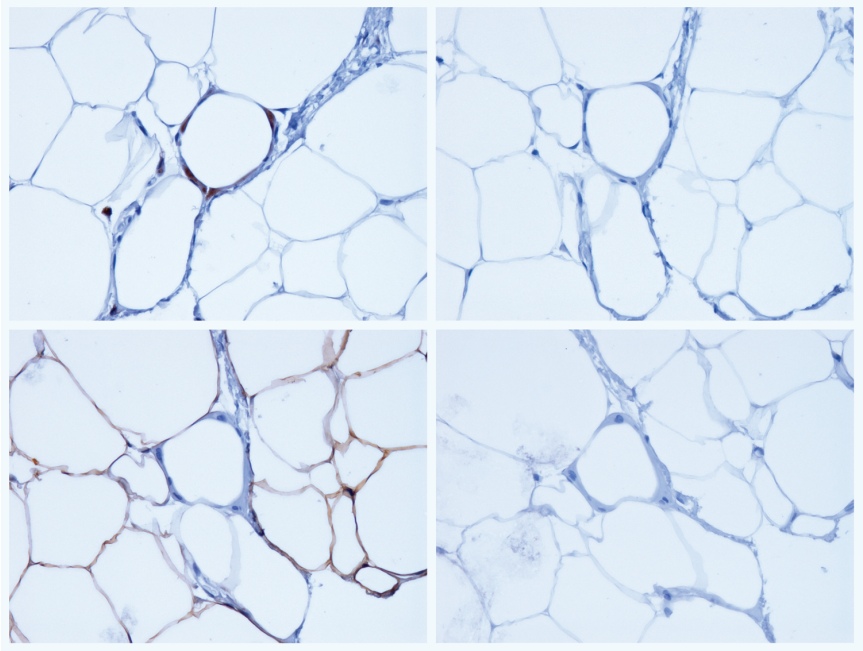

Figure 2. Immunohistochemical stainings with CD68 and perilipin. Macrophages are identified as CD68-positive cells *(brown)* in the adipose tissue. Original magnification, ×600. Images are of a representative crown-like structure (CLS) in a CD68-positive stain *(upper left),* the same CLS in the CD68-negative control *(upper right),* the same CLS in the perilipin stain *(lower left),* and the perilipin-negative control of the same structure *(lower right).* Note that the macrophage-encircled adipocyte, CLS, is lacking the perilipin immunoactivity in its membrane (blue instead of normal, viable brown), a feature in literature classified as a sign of a nonviable adipocyte.

Table 4. Relative expression of gene transcripts in comparison with the mean of 3 housekeeping genes (actin β [ACTB]; β-2 microglobulin [B2M], and ribosomal phosphoprotein P0 [36B4]) for patients with versus patients without lipatrophy.

Gene	Mean ± standard deviation		*P*	Direction of change[c]
	LA+ group[a]	LA− group[b]		
Housekeeping genes *ACTB, B2M,* and *36B4*	1.05 ± 0.36	1.00 ± 0.17	.62	…
Mitochondrial energy metabolism				
COX4	2.53 ± 0.87	1.37 ± 0.42	.001	Increase
COX3/COX4-ratio	0.33 ± 0.16	0.89 ± 0.35	<.001	Decrease
COX3	0.87 ± 0.41	1.28 ± 0.73	.07	…
16SRNA	0.70 ± 0.34	0.72 ± 0.33	.90	…
Mitochondrial biogenesis				
PGC1B	6.74 ± 6.94	0.95 ± 0.32	.02	Increase
POLG2	1.24 ± 0.28	1.09 ± 0.12	.16	…
TFAM	1.30 ± 0.50	1.22 ± 0.16	.65	…
POLG1	1.21 ± 0.44	1.19 ± 0.12	.89	…
Adipogenesis				
LMNB	1.39 ± 0.75	0.61 ± 0.43	.01	Increase
SREBP1c	0.40 ± 0.32	1.98 ± 1.15	<.001	Decrease
CEBPB	0.96 ± 0.48	1.43 ± 0.65	.046	Decrease
CEBPA	1.96 ± 1.16	2.74 ± 1.24	.12	…
PPARG2	1.72 ± 0.85	1.40 ± 0.31	.29	…
PPARG	1.82 ± 1.07	1.48 ± 0.44	.36	…
LMNA	1.26 ± 0.55	1.05 ± 0.18	.40	…
Lipid and glucose metabolism				
FASN	3.64 ± 3.79	9.67 ± 2.82	<.001	Decrease
GLUT4	1.42 ± 1.15	2.87 ± 1.68	.015	Decrease
HEXOK1	1.21 ± 0.54	1.42 ± 0.26	.27	…
PLIN	1.03 ± 1.04	1.37 ± 0.58	.38	…
Apoptosis, inflammation and oxidative stress				
IL1B	0.39 ± 0.15	0.12 ± 0.07	<.001	Increase
FAS	6.01 ± 3.08	2.38 ± 1.16	.002	Increase
SOD1	2.03 ± 0.84	1.36 ± 0.17	.03	Increase
PCNA	1.81 ± 0.38	1.49 ± 0.38	.046	Increase
IL6	0.35 ± 0.60	0.01 ± 0.02	.12	…
GPX1	1.84 ± 1.07	1.31 ± 0.36	.16	…
TNFA	0.80 ± 1.23	0.23 ± 0.21	.19	…
p53	1.02 ± 0.35	1.09 ± 0.26	.57	…

NOTE. *CEBPA* and *CEBPB,* CCAAT/enhancer binding protein α and β; *COX3* and *COX4,* cytochrome c oxidase subunit 3 and 4; *FAS,* factor of apoptotic stimulus; *FASN,* fatty acid synthase; *GLUT4,* glucose transporter 4; *GPX1,* glutathione peroxidase 1; *HEXOK1,* hexokinase 1; *IL1B* and *IL6,* interleukin-1β and -6; *LMNA* and *LMNB,* lamin A/C and B; *p53,* tumor protein p53; *PGC1B,* peroxisome proliferative-activated receptor gamma coactivator 1β; *PCNA,* proliferating cell nuclear antigen; *PLIN,* perilipin; *POLG1,* polymerase γ (catalytic subunit); *POLG2,* polymerase γ (accessory subunit); *PPARG* and *PPARG2,* peroxisome proliferative-activated receptor γ and γ subunit 2; *16SRNA,* 16S ribosomal RNA; *SOD1,* superoxide dismutase 1; *SREBP1c,* sterol element binding transcription factor 1c; *TFAM,* mitochondrial transcription factor A; *TNFA,* tumor necrosis factor–α.

[a] Human immunodeficiency virus type 1 (HIV-1)–infected patients with lipoatrophy who were taking highly active antiretroviral therapy.

[b] HIV-1–infected patients who had not developed lipoatrophy while receiving highly active antiretroviral therapy.

[c] Direction of change in gene expression is indicated as the LA+ group versus the LA− group.

group than in the AZT+LA+ group (table 5). Among all patients, *GLUT4* expression correlated positively with both *FASN* ($r = 0.73$; $P < .001$) and *HEXOK1* ($r = 0.64$; $P < .001$).

Apoptosis, inflammation, and oxidative stress. The factor of apoptotic stimulus *(FAS)* receptor is a key regulator of apoptosis due to extrinsic stress signals. Because the extrinsic stress signals do not affect the mRNA concentrations of p53 but, rather, regulate p53 posttranslationally [20], the gene was used as a control for stress-related apoptotic mRNA alterations. We also analyzed superoxide dismutase 1 *(SOD1)* and glutathione

Table 5. Relative expression of gene transcripts in comparison with the mean of 3 housekeeping genes (actin β [ACTB]; β-2 microglobulin [B2M], and ribosomal phosphoprotein P0 [36B4]) for patients with lipoatrophy who received zidovudine versus stavudine.

Gene	Mean ± standard deviation		*P*	Direction of change[c]
	d4T+LA+ group[a]	AZT+LA+ group[b]		
Housekeeping genes *ACTB, B2M,* and *36B4*	1.06 ± 0.37	1.04 ± 0.36	.49	…
Mitochondrial energy metabolism				
COX3/COX4-ratio	0.24 ± 0.09	0.43 ± 0.16	.02	Decrease
COX3	0.64 ± 0.24	1.06 ± 0.43	.03	Decrease
16SRNA	0.50 ± 0.19	0.86 ± 0.36	.03	Decrease
COX4	2.77 ± 0.89	2.29 ± 0.86	.60	…
Mitochondrial biogenesis				
POLG1	0.93 ± 0.38	1.41 ± 0.37	.01	Decrease
TFAM	1.38 ± 0.67	1.24 ± 0.32	.57	…
POLG2	1.20 ± 0.16	1.26 ± 0.34	.64	…
PGC1B	6.52 ± 6.89	6.90 ± 7.34	.96	…
Adipogenesis				
CEBPA	1.29 ± 0.58	2.50 ± 1.25	.02	Decrease
SREBP1c	0.25 ± 0.37	0.47 ± 0.28	.04	Decrease
LMNA	1.09 ± 0.60	1.44 ± 0.45	.14	…
CEBPB	0.78 ± 0.34	1.11 ± 0.54	.15	…
PPARG2	1.43 ± 0.74	1.95 ± 0.90	.18	…
PPARG	1.58 ± 1.03	2.01 ± 1.11	.48	…
LMNB	1.50 ± 0.79	1.33 ± 0.76	.67	…
Lipid and glucose metabolism				
HEXOK1	0.86 ± 0.51	1.48 ± 0.40	.007	Decrease
PLIN	0.53 ± 0.71	1.52 ± 1.13	.04	Decrease
GLUT4	0.90 ± 0.52	1.78 ± 1.34	.11	…
FASN	3.00 ± 3.59	4.22 ± 4.07	.26	…
Apoptosis, inflammation and oxidative stress				
GPX1	1.35 ± 0.41	2.18 ± 1.27	.10	…
PCNA	1.94 ± 0.46	1.70 ± 0.27	.10	…
SOD1	1.71 ± 0.58	2.29 ± 0.95	.16	…
FAS	6.65 ± 2.98	5.51 ± 3.22	.34	…
TNFA	0.42 ± 0.61	1.09 ± 1.53	.38	…
p53	0.97 ± 0.45	1.05 ± 0.27	.45	…
IL6	0.27 ± 0.28	0.42 ± 0.83	.66	…
IL1B	0.38 ± 0.18	0.40 ± 0.14	.85	…

NOTE. *CEBPA* and *CEBPB,* CCAAT/enhancer binding protein α and β; *COX3* and *COX4,* cytochrome c oxidase subunit 3 and 4; *FAS,* factor of apoptotic stimulus; *FASN,* fatty acid synthase; *GLUT4,* glucose transporter 4; *GPX1,* glutathione peroxidase 1; *HEXOK1,* hexokinase 1; *IL1B* and *IL6,* interleukin-1β and -6; *LMNA* and *LMNB,* lamin A/C and B; *PCNA,* proliferating cell nuclear antigen; *p53,* tumor protein p53; *PGC1B,* peroxisome proliferative-activated receptor gamma coactivator 1β; *PLIN,* perilipin; *POLG1,* polymerase γ (catalytic subunit); *POLG2,* polymerase γ (accessory subunit); *PPARG* and *PPARG2,* peroxisome proliferative-activated receptor γ and γ subunit 2; *16SRNA,* 16S ribosomal RNA; *SOD1,* superoxide dismutase 1; *SREBP1c,* sterol element binding transcription factor 1c; *TFAM,* mitochondrial transcription factor A; *TNFA,* tumor necrosis factor-α.

[a] Human immunodeficiency virus type 1 (HIV-1)–infected patients with lipoatrophy whose highly active antiretroviral regimen included stavudine.

[b] HIV-1–infected patients with lipoatrophy whose highly active antiretroviral regimen included zidovudine.

[c] Direction of change in gene expression is indicated as the d4t+LA+ group versus the AZT+LA+ group.

peroxidase 1 *(GPX1)* expression as indicators of oxidative stress [21,22]. The proliferating cell nuclear antigen *(PCNA)* was evaluated as a marker of adipocyte cycling.

The expression of *FAS*, but not of *p53*, was increased in the LA+ group, compared with the LA− group (table 4). The level of *SOD1* and *PCNA* gene expression was higher in the LA+ group than in the LA− group (table 5). Of the inflammatory cytokines, expression of *IL1B* was greater in the LA+ group than in the LA− group (table 4). *IL1B* transcripts correlated inversely with mtDNA copy numbers ($r = -0.42$; $P = .005$).

Table 6. Expression of mitochondrial DNA-encoded transcripts in the patient groups.

Gene	Mean ± standard deviation			Mean ± standard deviation		
	LA+ group (n = 18)	LA− group (n = 10)	P^a	d4T+LA+ group (n = 8)	AZT+LA+ group (n = 10)	P^b
16SRNA RNA	0.34 ± 0.18	0.15 ± 0.05	.004	0.42 ± 0.23	0.28 ± 0.08	.07
COX3 RNA	0.42 ± 0.20	0.25 ± 0.08	.02	0.52 ± 0.24	0.35 ± 0.11	.058

NOTE. AZT+LA+ group, HIV-1–infected patients with lipoatrophy whose highly active antiretroviral therapy (HAART) regimen included zidovudine; *COX3,* cytochrome c oxidase subunit 3; d4T+LA+ group, HIV-1–infected patients with lipoatrophy whose HAART regimen included stavudine; LA+ group, HIV-1–infected patients with lipoatrophy who were taking HAART; LA− group, HIV-1–infected patients who had not developed lipoatrophy while receiving HAART; *16SRNA,* 16S ribosomal RNA.

[a] For the LA+ group versus the LA− group.

[b] For the d4t+LA+ group versus the AZT+LA+ group.

There were no statistically significant differences in the expression of markers of apoptosis, inflammation, and oxidative stress between the AZT+LA+ group and the d4T+LA+ group (table 5).

DISCUSSION

We compared gene expression and immunohistochemical signs of inflammation in subcutaneous abdominal adipose tissue biopsy specimens obtained from HIV-1–infected patients with and without HAART-associated lipoatrophy. We were particularly interested in comparing the lipoatrophic patients using either AZT or d4T.

In keeping with previous studies, we found mtDNA to be depleted in the LA+ group, compared with the LA− group [23, 24]. In addition, the mtDNA-encoded *COX3* expression was decreased in patients with lipoatrophy, especially in those with d4T-associated lipoatrophy. nDNA-encoded *COX4* expression was increased in both treatment subgroups. *POLG1* expression, unlike that of *POLG2,* was lower in the d4T+LA+ group than in the AZT+LA+ group. This may, in part, explain the more severe mtDNA depletion in the d4T+LA+ group than in the AZT+LA+ group.

Mitochondrial toxicity may induce oxidative stress in adipose tissue, consequently contributing to impaired adipocyte differentiation, increased inflammation, and activation of apoptosis [25]. Accordingly, in the current study, we found a positive correlation between mtDNA copy number and *SREBP1c* expression (an important transcription factor in adipogenesis) and an inverse correlation between mtDNA copy number and the expression of *IL1B.*

An increase in mitochondrial gene expression relative to the number of mtDNA copies per cell in the LA+ group may reflect an attempt to compensate for mtDNA depletion. The markedly higher gene expression of *PGC1B* supports this line of reasoning. Expression of *TFAM*, however, was not altered in our LA subgroups, although in healthy individuals, even short-term exposure to AZT and d4T is associated with up-regulation of *PGC1B* and *TFAM* mRNA [26].

With respect to expression of genes involved in adipogenesis, we confirmed decreased *SREBP1c* expression in the LA+ group, compared with the LA− group [27, 28]. We now extended this finding to both LA subgroups. Expression of *PPARG* was unchanged, as was reported in some [29] but not all [27, 30] previous studies.

Expression of *CEBPB* was significantly lower in the LA+ group than in the LA− group. *CEBPA* followed the same trend. Down-regulation of these genes was more prominent in the d4T+LA+ group than in the AZT+LA+ group. These findings are in accordance with previous reports of decreased expression of *CEPBA* and *CEBPB* in patients with HAART-associated lipoatrophy [27, 28].

We found *GLUT4* gene expression to be decreased in the LA+ group, compared with the LA− group, which is in keeping with our own previous findings [31] and with those of others [27]. Expression of *GLUT4* did not significantly differ between the LA subgroups, although it tended to be lower in the d4T+LA+ group than in the AZT+LA+ group.

LMNA and *LMNB* expression was analyzed because it has been postulated that PI treatment may alter *LMNA* maturation and, thus, inhibit translocation of *SREBP1c* from the cytoplasm to the nucleus in LA [32]. We could not confirm decreased *LMNA* gene expression in LA. We found significantly higher expression of *LMNB* in the LA+ group than in the LA− group. The relationship between altered lamin maturation and stability as well as the reduced *SREBP1c* translocation through nuclear pores, which was previously observed with PI exposure [32], has also been recently challenged [33].

Adipose tissue in lipoatrophic patients has been suggested to be chronically inflamed [34]. An increased number of foamy histiocytes [35] and immunohistochemically identified CD68-positive macrophages has been reported in adipose tissue specimens obtained from lipoatrophic patients [34]. However, in

these studies, patients with LA were compared with HIV-1–negative subjects. We have previously described increased adipose tissue inflammation in a different group of HIV-1–positive, HAART-treated, lipodystrophic patients who were compared with HIV-1–positive, HAART-treated, nonlipodystrophic patients [36]. In the current study, these findings are confirmed.

Proinflammatory cytokines impair adipocyte metabolism and induce insulin resistance and apoptosis in adipose tissue [37]. Apoptosis in the context of HAART-associated LA has previously been described in vitro [4] and in vivo [38]. We found that *FAS* was increased in the LA+ group, compared with the LA− group. The expression of *p53* did not differ between the groups, which is consistent with the predominantly posttranscriptional activation of the *p53* network [20]. With regard to *TNFA*—a trigger of the extrinsic pathway of apoptosis—there was no difference between the d4T+LA+ and the AZT+LA+ groups, although there was a trend towards increased *TNFA* expression in the AZT+LA+ group, as previously observed [38]. The increase of *IL1B* and *TNFA* transcripts could originate from either recruited macrophages or inflamed adipocytes [39, 40].

Compared with the LA+ group, no significant up-regulation of *SOD1* was evident in the LA− group, and *GPX1* followed the same pattern. In comparison, for the treatment subgroups, both of those genes tended to be more prevalent in the AZT+LA+ group. This finding may reflect the particular propensity of AZT to induce reactive oxygen species production [41].

The present study has several limitations. As with any cross-sectional study, only an association, not a causal relationship, can be demonstrated. Because of the limited group size, negative results should be interpreted with caution. It should also be emphasized that mRNA profiles do not equal protein expression and that statistical analyses were not corrected for multiple comparisons. It is also possible that the imbalance in the use of PIs between the LA+ and the LA− groups may have affected the results. Although in vitro models have shown PI-induced inhibition of adipocyte differentiation both by PPARγ-dependent [42] and PPARγ-independent [43] mechanisms, cessation of PI therapy, as opposed to cessation of NRTI therapy, does not lead to improvement of lipoatrophy [44].

In summary, the present study demonstrates alterations in gene expression in adipose tissue of patients with HAART-associated LA, compared to HAART-treated, nonlipoatrophic patients. The results confirm previous findings of mtDNA depletion, inflammation, and disturbances in adipogenesis in lipoatrophic fat. Furthermore, an excessive number of macrophages in lipoatrophic adipose tissue was demonstrated. When comparing the d4T- and the AZT-treated lipoatrophic patients, more severe mtDNA depletion and decrease in gene expression of mtDNA-encoded *COX3*, nuclear adipogenic transcription factors, and *PLIN* was found in patients with d4T-associated lipoatrophy.

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