

Mitochondrial proliferation, DNA depletion and adipocyte differentiation in subcutaneous adipose tissue of HIV-positive HAART recipients

Craig S Pace¹, Annalise M Martin¹, Emma L Hammond¹, Cyril D Mamotte², David A Nolan¹ and Simon A Mallal^{1,2*}

¹Centre for Clinical Immunology and Biomedical Statistics, Royal Perth Hospital and Murdoch University, Western Australia

²Department of Clinical Immunology and Biochemical Genetics, Royal Perth Hospital, Perth, Western Australia

*Corresponding author: Tel: +61 89 244 2899; Fax: +61 89 244 2920; E-mail: S.Mallal@murdoch.edu.au

Objectives: To examine the *in vivo* effects of highly active antiretroviral therapy (HAART) regimens on adipose tissue mitochondrial DNA (mtDNA) depletion, mitochondrial organellar proliferation, and markers of adipocyte differentiation and phenotype.

Design and methods: DNA and mRNA quantification using real-time PCR methods was performed on adipose tissue samples from 31 HIV-infected individuals, of whom 11 were treatment-naïve and 20 were receiving HAART. mtDNA depletion was measured as mtDNA copies/cell, and mitochondrial proliferation by quantification of mitochondrial protein mass. Regulation of mitochondrial biogenesis was assessed by NRF-1 and mtTFA mRNA. PPAR γ , UCP2 and UCP1 mRNA expression was used to assess adipocyte differentiation and phenotype. **Results:** Stavudine-based HAART recipients ($n=10$) displayed significant mtDNA depletion (12.8% of control, $P<0.001$), mildly increased mitochondrial protein mass (2.6-fold of control, $P=0.032$) and decreased expression of PPAR γ (53.9% of control, $P=0.021$), UCP2 (62.2% of control, $P=0.024$) and UCP3 (51.8% of control, $P=0.047$)

mRNA compared with controls. Zidovudine-based HAART recipients ($n=7$) also displayed significant mtDNA depletion (34.45% of control, $P=0.031$), increased mitochondrial protein mass (5.7-fold of control, $P=0.009$), and markedly increased UCP1 (18-fold of control, $P=0.009$) mRNA. Elevated UCP1 mRNA expression was found to be associated with non-stavudine (zidovudine or abacavir), protease inhibitor (PI)-containing HAART (95-fold of non-stavudine, non-PI-containing HAART, $P=0.006$).

Conclusion: Differential effects of stavudine and zidovudine therapy on mtDNA depletion and expression of adipocyte differentiation markers PPAR γ and UCP2 were observed, consistent with increased adipose tissue toxicity associated with stavudine therapy. Increased UCP1 mRNA, a marker of brown adipose tissue phenotype, was associated with non-stavudine, PI-containing HAART, and may represent an adaptive response to the increased fatty acid flux associated with PI therapy, and may contribute to the increased resting energy expenditure reported in such patients.

Introduction

A common adverse effect of antiretroviral therapy, the 'lipodystrophy syndrome' is characterized by peripheral lipoatrophy and/or visceral lipohypertrophy, dyslipidaemia and insulin resistance, and is considered a major determinant of therapy adherence [1,2]. Historically, the emergence of lipodystrophy coincided temporally with the introduction of protease inhibitors (PIs) for the management of HIV infection and led to the conjecture that the PI class of drugs was the causative agent [3]. However, lipoatrophy has also been observed in PI-naïve individuals [4,5]. There is now increasing evidence that each of the clinical characteristics of the lipodystrophy syndrome mentioned above arise via distinct aetiopathogenic mechanisms rather than a single cause [3].

Research into the mechanisms of nucleoside reverse transcriptase inhibitor (NRTI) toxicity and lipoatrophy has focused on depletion of mitochondrial DNA (mtDNA). Several *in vivo* studies have shown significant mtDNA depletion in subcutaneous adipose tissue of lipodystrophic NRTI recipients [6-8], strongly suggesting NRTI-mediated mtDNA depletion has a role in the pathogenesis of lipoatrophy [9]. This is supported by clinical studies demonstrating that the choice of NRTI therapy (stavudine versus zidovudine) is the dominant determinant of the severity of lipoatrophy [10,11], and by *in vitro* studies indicating that NRTIs such as stavudine, didanosine and zalcitabine are capable of inducing mtDNA depletion at pharmacological doses [12,13]. While zidovudine has the ability to cause some degree of mtDNA depletion [12],

there is also evidence to suggest that this drug may contribute to mitochondrial dysfunction through alternative mechanisms [14].

The observation that HIV PI therapy contributes to hyperlipidaemia and insulin resistance has prompted numerous *in vitro* studies into the effects of PIs on adipogenesis, the results of which are conflicting. While several studies have demonstrated impaired adipogenesis using suprapharmacological PI concentrations, few studies have demonstrated impaired adipogenesis using pharmacologically relevant PI concentrations [15,16]. In addition, there appear to be distinct differences in the ability of each individual PI to impair adipogenesis, although the clinical relevance of this is not known [17]. Several animal models of lipodystrophy suggest that the insulin resistance and metabolic alterations observed are causally related to the initial loss of subcutaneous adipose tissue [18], although the relevance of these findings to antiretroviral therapy-associated lipodystrophy has not yet been established [19].

In light of this information, we sought to examine the *in vivo* effects of antiretroviral therapy combinations on mtDNA depletion, mitochondrial organellar proliferation and adipocyte differentiation. Previous studies have noted some brown adipocyte-like characteristics in subcutaneous adipose tissue of NRTI-treated individuals [20,21] and NRTI-exposed cell lines [14]. We therefore also examined the influence of the drugs on expression of a brown adipocyte marker (uncoupling protein 1, UCP1). We found that stavudine use was associated with mtDNA depletion, mitochondrial proliferation and decreased expression of peroxisome proliferator activator receptor- γ (PPAR γ), UCP2 and UCP3 mRNA, suggestive of impaired adipocyte differentiation. Zidovudine use was also associated with mtDNA depletion and mitochondrial proliferation, though significantly less mtDNA depletion than observed with stavudine. Non-stavudine, PI-containing HAART was associated with markedly increased expression (mRNA) of the brown adipocyte marker, UCP1, that may constitute an adaptive response to the increased fatty acid flux associated with PI therapy and may contribute to the increased resting energy expenditure observed in such patients.

Materials and methods

Patient selection

Thirty-one male Caucasian patients from the Western Australian HIV Cohort [22] who had subcutaneous adipose tissue biopsies were selected and grouped according to their HAART regimen at the time of biopsy. To be included in this study, individuals had to be on the antiretroviral class/agent for which they were

grouped for a minimum of 6 months. To assess the influence of PI therapy, individuals were grouped into PI-containing HAART regimens (PI+, $n=7$) and non-PI-containing HAART (non-PI, $n=13$) regimens (Table 1). Similarly, individuals were also grouped into stavudine-based HAART recipients ($n=10$), zidovudine-based HAART recipients ($n=7$) and abacavir-based HAART recipients ($n=3$) to assess the influence of these NRTIs on the aforementioned parameters (Table 2). Informed consent and approval for the use of human genetic material was obtained from all individuals studied and the Royal Perth Hospital ethics committee, respectively. The control group consisted of 11 HIV-seropositive male Caucasians with similar age and BMI who had never received antiretroviral therapy.

Subcutaneous adipose tissue biopsies

Subcutaneous adipose biopsies were obtained from the supra-iliac region following a 5 cm surgical incision down to the adipose layer and direct dissection of adipose tissue; dissected tissues were immediately frozen in liquid nitrogen prior to storage at -70°C .

Quantification of mitochondrial DNA copies/adipocyte

Adipocytes were isolated from adipose tissue by incubation in Hanks balanced salt solution (pH 7.0) containing 3% collagenase solution (Type 1, Sigma C0130) and 1.5% bovine serum albumin at 37°C for 80 min followed by differential centrifugation, as previously described [20]. Total DNA was extracted from isolated adipocytes using QIAamp DNA MIDI Kit (Qiagen, Inc., Chatsworth, Calif., USA) according to the manufacturer's protocol.

Quantification of mitochondrial protein mass

Mitochondrial protein mass was quantified by the Bradford method, as previously described [20].

Quantitation of UCP1, UCP2, PPAR γ , mtTFA and NRF-1 mRNA expression by real-time PCR

Total RNA was extracted from adipose tissue using Ultraspec[®] (Fisher Biotec, Australia) according to the manufacturer's protocol. Total RNA concentration

Table 1. Demographics of the naive, non-PI and PI+ groups

Parameter	Naive	Non-PI	PI+	P-value
Number (n)	11	13	7	-
Time on regimen*	-	23.5 \pm 19.5	42.3 \pm 20.2	0.058
Age (years)	49.0 \pm 11.0	46.6 \pm 10.3	45.9 \pm 8.5	0.780
BMI (kg/m^2)	24.8 \pm 4.2	23.7 \pm 3.6	21.0 \pm 1.8	0.099

All values are expressed as mean \pm SD; *months on particular regimen at time of biopsy.

Table 2. Demographics of the naive, stavudine, zidovudine and abacavir groups

Parameter	Naive	Stavudine	Zidovudine	Abacavir	P-value
Number (n)	11	10	7	3	–
Time on regimen*	–	32.2 ±18.9	20.86 ±17.1	44.7 ±34.4	0.058
Age (years)	49.0 ±11.0	48.3 ±9.5	43.9 ±6.3	45.7 ±17.0	0.740
BMI (kg/m ²)	24.8 ±4.2	22.8 ±3.2	24.1 ±2.7	19.3 ±3.2	0.122

All values are expressed as mean ±SD; *months on particular regimen at time of biopsy.

was measured at 260 nm using a Beckman DU 530 Life Science UV/Vis spectrophotometer (USA); 260:280 ratios between 1.7 and 2.0 were considered satisfactorily pure for subsequent reactions. First strand cDNA was synthesized from 1 µg of total RNA using Omniscript (Qiagen, USA) according to the manufacturer's protocol and stored at –20°C.

The sequence of all oligonucleotide primers and fluorogenic probes used in this study are shown in Table 3. Those used for quantification of human UCP1, UCP2, UCP3 and glyceraldehyde-6-phosphate-dehydrogenase (GAPDH) were designed using Primer Express™ software v. 1.5 (Applied Biosystems, Foster City, Calif., USA; ABI), while those used for PPAR γ were obtained from Xin *et al.* [23], and those for mitochondrial transcription factor A (mtTFA) and nuclear respiratory factor-1 (NRF-1) were obtained from Miranda *et al.* [24]. The specificity of all oligonucleotides was assessed using BLASTN (<http://www.ncbi.nlm.nih.gov/BLAST/>) and CLUSTAL W v.1.8 (<http://clustalw.genome.ad.jp/>). Where possible, primer binding sites were designed to flank intron–exon boundaries; when this was not possible, forward and reverse primer binding sites were designed in separate exons. All probes were labelled at the 5' end with 6-carboxy-fluorescein (FAM) and at the 3' end with 6-carboxy-tetramethyl-rhodamine (TAMRA).

Quantitative PCR was carried out in duplicate for each sample on a PE7700 Sequence Detection System (ABI). Each 25 µl reaction contained 50 ng of cDNA, 450 nmol of each forward and reverse primer, 2.5 µl of 10X PCR buffer (Gibco, USA), 2 mM MgCl₂, 0.5 µl of 50X ROX reference dye (Invitrogen, USA), 0.5 µl of 40 mM dNTPs, 1U of Platinum® Taq DNA polymerase (Gibco, USA) and sterile, DEPC-treated water pH 8.0. For quantification of UCP1, UCP2, UCP3, PPAR γ and GAPDH cDNA, 200 nM of probes was included in the PCR reaction, and for quantification of mtTFA and NRF-1 cDNA, 0.5 µl of 20X SYBR green was included in each reaction. All reactions were carried out using the following cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Real-time amplifications were analysed using the Sequence Detector software v. 1.5 (ABI) and indirect quantification of the target mRNA was calculated from a standard curve generated by amplification of purified PCR products. The

end-point of this assay is the crossing threshold (CT), which represents the number of PCR cycles required to enter the logarithmic amplification phase. As a linear increase in CT thus represents a logarithmic increase in PCR products, all values are expressed and analysed on the logarithmic scale (Figures 1 and 2). For each sample, results were normalized by dividing the amount of target cDNA by the amount of GAPDH. Due to the use of the non-specific dsDNA binding dye, SYBR green, exclusive amplification of mtTFA and NRF-1 was verified by melting curve analysis.

Statistical analysis

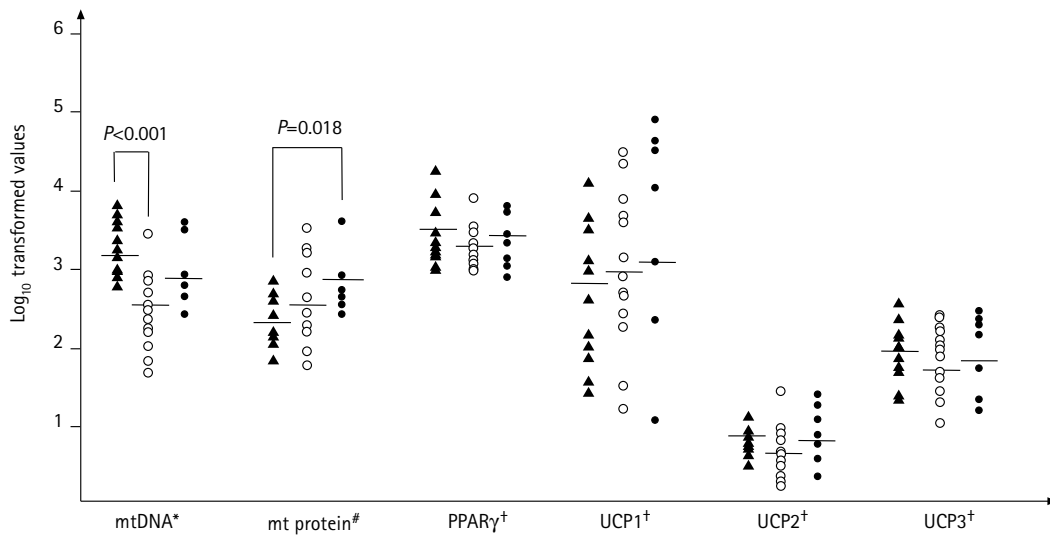
All values are expressed as log transformed data to better approximate normality. For parametric data, means were compared by ANOVA for multiple

Table 3. Oligonucleotide primers and probe sequences used in real-time quantitative PCR

Gene	Amplicon size
UCP1	240 bp
Forward	5'-CTGCCACTCCTCCAGTCGTT-3'
Reverse	5'-CCGCCTCTCTCAGGATCGGCCT-3'
Probe	5'-CCGCCTCTCTCAGGATCGGCCT-3'
UCP2	288 bp
Forward	5'-GACCTATGACCTCATCAAGG-3'
Reverse	5'-ATAGGTGACGAACATCACCACG-3'
Probe	5'-ACAGATGACCTCCCTTGCCACTTCACT-3'
UCP3	311 bp
Forward	5'-ATGGACGCCTACAGAACCAT-3'
Reverse	5'-CTGGGCCACCATCTTTATCA-3'
Probe	5'-CCTGTCCAAGGAACCTTTGCCCAACA-3'
PPAR γ	109bp
Forward	5'-TTTCACTATGGAGTTCATGCTTG-3'
Reverse	5'-TTTTTGTGGATCCGACAGTTAAGA-3'
Probe	5'-CAAGGGTTTCTTCCGGAGAACAATCAG-3'
GAPDH	226 bp
Forward	5'-GAAGGTGAAGGTCGGAGTC-3'
Reverse	5'-GAAGATGGTGATGGGATTTTC-3'
Probe	5'-CAAGCTTCCCCTTCTCAGCC-3'
mtTFA	441 bp
Forward	5'-TATCAAGATGCTTATAGGGC-3'
Reverse	5'-ACTCCTCAGCACCATATTTT-3'
NRF-1	643 bp
Forward	5'-GGAGTGATGCCGCACAGAA-3'
Reverse	5'-CGCTGTAAAGCGCCATAGTG-3'

Bp, base pairs; note mtTFA and NRF-1 were quantified using the dsDNA binding dye, SYBR green, and as such, did not require fluorogenic probes.

Figure 1. Recipients of non-PI-containing HAART and PI-containing HAART displayed mtDNA depletion and elevated mitochondrial protein, respectively



Horizontal bar indicates mean; ▲, HIV-positive, HAART-naïve individuals; ○, non-PI-containing HAART recipients; ●, PI-containing HAART recipients; *mtDNA copies/adipocyte; # mitochondrial protein; † copies/GAPDH copies.

comparisons. For non-parametric data, the Mann-Whitney test was used. The threshold for significance was set at $P=0.05$. Comparisons between groups were performed on standard statistical software (Excel and SPSS), and differences between study groups were expressed as percentage and fold differences using absolute (untransformed) values that were derived from the log transformed output data. These values (that is, 10^x where x denotes the log transformed value), therefore, estimate the median values for untransformed data.

Results

The effects of HAART on mitochondrial proliferation, mtDNA depletion, and adipocyte differentiation and phenotype were assessed in subcutaneous adipose tissue biopsies from 31 HIV-seropositive patients on various HAART regimens; gene expression at the mRNA level was compared according to HAART regimen. Parameters that were significantly influenced by NRTIs and PI as a class, or by stavudine, zidovudine or abacavir individually, are displayed in Figures 1 and 2, respectively.

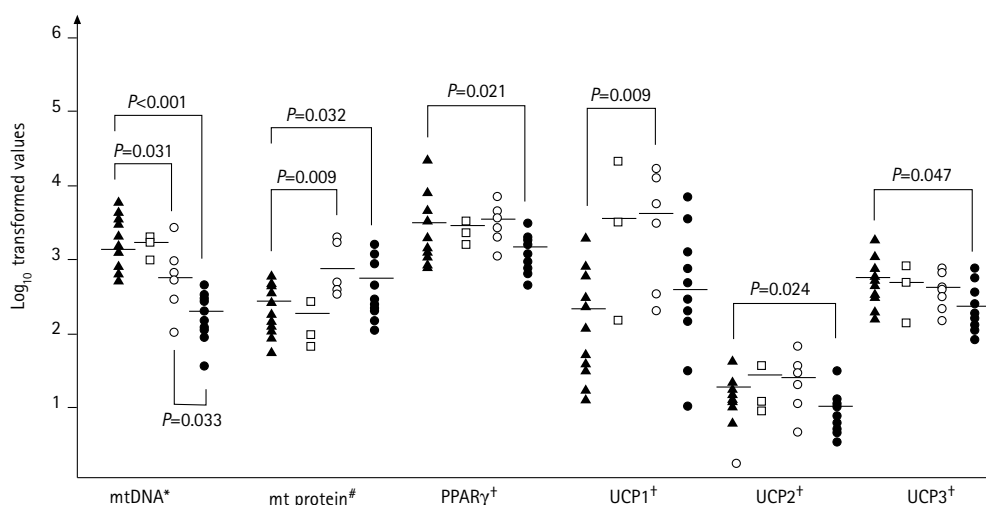
Expression of GAPDH mRNA within subcutaneous adipose tissue was similar amongst non-PI HAART recipients, PI+ HAART recipients and controls ($P=0.95$). In addition, there did not appear to be an effect of stavudine, zidovudine or abacavir on the level of GAPDH mRNA expression ($P=0.17$), suggesting GAPDH is a suitable endogenous control gene within this system, consistent with findings by Gorzelnik and colleagues [25].

Effect of NRTI therapy versus NRTI and PI combination therapy on mitochondrial proliferation, mtDNA, and adipocyte differentiation and phenotype

As presented in Figure 1, patients on non-PI-containing HAART regimens (non-PI) displayed significantly reduced mtDNA/adipocyte (19.3% of controls, $P<0.001$), suggesting that NRTI therapy alone is sufficient to induce mtDNA depletion. The addition of PIs to NRTI-based HAART regimens (PI+) also tended towards significant mtDNA depletion (43.1% of controls, $P=0.062$). Median mtDNA/adipocyte was not significantly different amongst non-PI and PI regimens ($P=0.132$).

While the PI+ group had significantly increased median mitochondrial protein mass compared to controls (3.3-fold, $P=0.018$), there was no significant difference in the median mitochondrial protein mass of PI+ and non-PI regimens (1.4-fold, $P=0.315$), thus PI therapy did not appear to have an independent significant effect on mitochondrial protein mass. Using linear regression analysis to investigate these associations further, choice of a specific NRTI – zidovudine ($P=0.010$) or stavudine ($P=0.047$), but not abacavir ($P=0.604$) – was found to be the dominant determinant of mitochondrial protein mass. Use of PI therapy did not contribute significantly to the model after adjustment for the effect of these variables ($P=0.604$). In addition, expression of mtTFA and NRF-1 mRNA – key transcription factors involved in mtDNA replication and mitochondrial organellar biogenesis [28,29] – were similar amongst non-PI and PI+ HAART recipients, and

Figure 2. Stavudine and zidovudine recipients displayed significant mtDNA depletion and elevated mitochondrial protein mass. However, stavudine recipients also displayed reduced expression of PPAR γ , UCP2 and UCP3 mRNA, while zidovudine recipients display significantly elevated expression of UCP1 mRNA



Horizontal bar indicates mean; ▲, HIV-positive, HAART-naïve individuals; □, abacavir-based HAART recipients; ○, zidovudine-based HAART recipients; ●, stavudine-based HAART recipients; *mtDNA copies/adipocyte; # mitochondrial protein; † copies/GAPDH copies.

in controls ($P=0.352$ and 0.589 , respectively). Similarly, there were no significant differences in expression of PPAR γ ($P=0.649$), UCP2 ($P=0.514$) or UCP3 ($P=0.663$) mRNA in comparisons of PI+ and non-PI regimens.

Expression of a brown adipocyte-specific marker, UCP1, was only associated with PI therapy once stavudine recipients were excluded from the analysis (Figure 3). Patients receiving either zidovudine or abacavir, and a PI, expressed UCP1 mRNA at levels 96-fold higher than patients receiving either zidovudine or abacavir, and no PI ($P=0.006$), 1500-fold higher than patients receiving stavudine and PI ($P=0.007$), 108-fold higher than patients receiving stavudine no PI ($P=0.007$) and 228-fold higher than naives ($P<0.001$). No significant effect of PIs was observed in stavudine-based HAART recipients ($P=0.189$).

Effects of stavudine, zidovudine and abacavir on mitochondrial proliferation, mtDNA, and adipocyte differentiation and phenotype

Current use of stavudine or zidovudine therapy was associated with significantly reduced mtDNA/adipocyte (12.8% of controls, $P<0.001$ and 34.4% of controls, $P=0.031$, respectively). However, the mtDNA depletion associated with stavudine therapy was significantly more severe than that associated with zidovudine therapy (37.3% of zidovudine values, $P=0.033$), as presented in Figure 2.

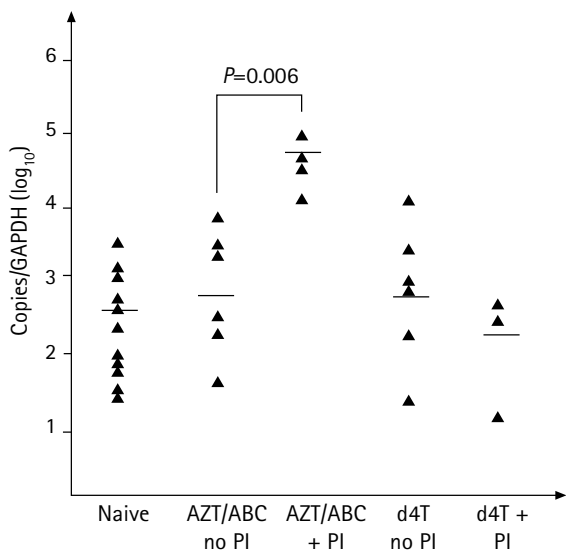
In addition, stavudine and zidovudine were associated with significantly increased mitochondrial protein mass (2.6-fold, $P=0.032$ and 5.7-fold,

$P=0.009$, respectively) compared to controls. However, only stavudine-based HAART recipients had significantly reduced median expression of adipocyte differentiation markers PPAR γ (54% of controls, $P=0.021$), UCP2 (62% of controls, $P=0.024$) and of UCP3 (52% of controls, $P=0.047$) mRNA.

As mentioned, the choice of NRTI therapy was the dominant determinant of mitochondrial protein mass. In further regression analysis, the severity of mtDNA depletion was associated with mitochondrial protein levels ($P=0.04$), and after adjusting for this effect, only zidovudine remained independently significant ($P=0.007$). Similar results were obtained in regression analysis of associations with PPAR γ expression, in which greater severity of mtDNA depletion was associated with decreased PPAR γ ($P=0.005$), while use of zidovudine remained independently associated with higher PPAR γ gene expression ($P=0.049$). Thus, while the effects of stavudine on mitochondrial protein mass and PPAR γ appear to be exerted through its effects on mtDNA depletion, the effect of zidovudine on these parameters appears at least in part, to be independent of mtDNA depletion.

The only gene whose expression (mRNA) was significantly altered in the zidovudine group was UCP1; 18-fold compared to both controls ($P=0.009$) and stavudine recipients ($P=0.042$). However, as described above, the combination of non-stavudine NRTIs (zidovudine or abacavir) with PIs resulted in the highest and most significant median UCP1 mRNA expression (228-fold higher than controls, $P<0.001$). This was supported by general linear model (GLM)

Figure 3. The addition of PIs to zidovudine- or abacavir-based HAART, but not stavudine-based HAART, is associated with markedly elevated expression of UCP1



Horizontal bar indicates mean; naive, HIV-positive, treatment-naive individuals; AZT/ABC no PI, zidovudine-based, non-PI-containing HAART recipients and abacavir-based, non-PI-containing HAART recipients; AZT/ABC + PI, zidovudine-based, PI-containing HAART recipients and abacavir-based, PI-containing HAART recipients; d4T no PI, stavudine-based, non-PI-containing HAART recipients; d4T + PI, stavudine-based, PI-containing HAART recipients.

analysis, which suggested that in this system, UCP1 mRNA levels are predominantly influenced by an interaction between the use of combined non-stavudine NRTIs and PI therapy ($R^2=0.209$, $P=0.01$).

No effect of abacavir on mtDNA (101.1% of controls, $P=0.980$), mitochondrial protein mass (73% of controls, $P=0.541$), or expression of PPAR γ (100.7% of controls, $P=0.987$), UCP2 (129% of controls, $P=0.268$) or UCP3 (89.1% of controls, $P=0.812$) mRNA was detected, although the statistical power to detect an independent effect of abacavir therapy was limited by the number of samples assessed ($n=3$). While UCP1 mRNA expression was elevated 10-fold compared to controls, this elevation was statistically non-significant ($P=0.174$).

Discussion

In this cross-sectional study, stavudine and zidovudine therapy had significant effects on adipose tissue. Use of either stavudine or zidovudine was associated with mtDNA depletion and increased mitochondrial protein mass. However, only stavudine was also associated with reduced expression of PPAR γ , UCP2 and UCP3 mRNA; zidovudine did not independently influence expression of any gene examined. PI therapy, when combined with non-stavudine NRTIs (that is, zidovudine or abacavir), was associated with markedly elevated expression (mRNA) of a brown adipocyte marker, UCP1.

The significant mtDNA depletion exhibited by stavudine recipients when compared to both controls (12.8% of controls, $P<0.001$) and zidovudine recipients (37.2% of zidovudine treated values, $P=0.031$) is consistent with *in vitro* [26] and *in vivo* studies [27] that associate stavudine with significantly more severe mtDNA depletion than zidovudine.

In addition to severe mtDNA depletion, stavudine recipients also expressed significantly lower levels of PPAR γ (53.9% lower, $P=0.021$), UCP2 (62.2% lower, $P=0.024$) and UCP3 (51.8% lower, $P=0.047$) mRNA when compared with controls. PPAR γ is a transcription factor expressed predominantly in adipocytes [28], where it is upregulated early in the differentiation process [29,30] and activates transcription of many genes involved in lipid metabolism [31–33]. Similarly, expression of UCP2 mRNA has been shown to increase during differentiation of murine primary cultures and clonal lines [34], and a human brown preadipocyte cell line [30]. UCP2 expression is believed to be augmented by the PPAR γ /RXR heterodimer [35] following the identification of two functional peroxisome proliferator response elements within the proximal promoter of the human UCP2 gene [36]. Impaired adipocyte differentiation is consistent with findings from the only other study to date to evaluate adipocyte differentiation in HAART recipients conducted by Bastard and colleagues [37], who reported a 70% reduction in expression of PPAR γ protein in lipoatrophic PI-containing HAART recipients when compared to HIV-seronegative controls, as well as comparable reductions in expression of transcription factors C/EBP α , C/EBP β and SREBP1c, and differentiation markers HSL, GLUT4 and leptin. Interestingly, while they attributed their findings to a specific effect of PI therapy, 21 of the 26 patients in the study were also receiving stavudine treatment.

Stavudine could indirectly contribute to impaired adipocyte differentiation through depletion of mtDNA copy number and subsequent effects on ATP production [38]. In this context, an increase in the cytosolic AMP:ATP ratio promotes the activation of a compensatory response that seeks to restore bioenergetic equilibrium via AMP-activated kinase (AMPK). Artificial activation of AMPK using 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) in differentiating 3T3-L1 adipocytes resulted in inhibited expression of transcription factors C/EBP α and PPAR γ , and late markers of differentiation such as fatty acid synthase (FAS) and acetyl-CoA carboxylase [39]. Interestingly, this is believed to be mediated by inhibition of SREBP-1 mRNA expression [40], the proposed mechanism by which PIs impair adipocyte differentiation.

Current use of zidovudine was associated with moderate mtDNA depletion (34% of control, $P=0.031$ and 37% of stavudine, $P=0.033$) and elevated mitochondrial protein mass. Using linear regression analysis, the elevated mitochondrial protein mass in zidovudine recipients was found to be attributed, in part, to an independent effect of zidovudine, rather than through its effects on mtDNA depletion. This may be due to the immediate effects zidovudine exerts on both mitochondrial function and proliferation, in the absence of mtDNA depletion [14,41].

Initial analysis revealed zidovudine recipients expressed (mRNA) a brown adipocyte-specific marker, UCP1, at levels 18-fold higher than controls ($P=0.009$) or stavudine recipients ($P=0.042$). That zidovudine induces uncoupling of oxidative phosphorylation is contrary to indirect evidence [42,43]. A similar pattern was observed in the few abacavir recipients in our study (10-fold of controls, $P=0.174$) and on subsequent analysis we found that zidovudine- or abacavir-based, PI-containing HAART recipients expressed UCP1 mRNA 96-fold that expressed by zidovudine- or abacavir-based, non-PI containing HAART recipients ($P=0.003$). Elevated UCP1 mRNA expression in recipients of relatively non-toxic (compared to stavudine-based regimens), PI-containing HAART regimens may represent an adaptive response to the increased free fatty acid flux associated with PI therapy. As proposed previously by our group [44], by increasing UCP1 during times of increased fatty acid availability, the generation of reactive oxygen species is minimized, mitochondrial metabolic processes that may otherwise become inhibited by excessive substrate availability and/or ATP production are maintained, and fatty acids are removed from the mitochondrial matrix where they may be deleterious. The origin of the increased UCP1 expression is unclear; it may result from a proliferation and differentiation of brown preadipocytes known to exist in traditional white adipose tissue depots [45] or from a phenotypic conversion of pre-existing white adipocytes [34,46].

While measurements of mtDNA and mitochondrial protein were performed on isolated adipocytes, gene expression was assessed at the adipose tissue level and thus reflect mRNA expression of a heterogenous cell population that includes vascular cells, fibroblasts and adipocytes [47]. This limits interpretation of the gene expression data for a number of reasons. First, the use of GAPDH (a broadly expressed cellular marker) as an endogenous control allows for the possibility that reduced expression of adipocyte-specific markers such as PPAR γ may reflect either reduced adipocyte PPAR γ expression (suggestive of impaired adipocyte differentiation) or reduced adipocyte number relative to the overall cell population. In the latter scenario reduced

PPAR γ expression associated with stavudine therapy could result from selective adipocyte cell death within fat tissue, a possibility supported by studies investigating apoptosis markers in fat samples from lipotrophic patients [48,49]. Secondly, whilst these preliminary data indicate that antiretroviral therapy influences the expression of other non-adipocyte-specific markers such as UCP2, it is uncertain which cell populations within adipose tissue are involved in these effects. Whilst these limitations could be overcome through the use of collagenase digestion, we have not been able to obtain a collagenase protocol that maintained the integrity of the 18S and 28S RNA. We therefore propose to focus on protein expression in collagenase-digested samples as a more appropriate end-point in future longitudinal studies.

In conclusion, stavudine use was associated with significant mtDNA depletion, increased mitochondrial mass and reduced expression of PPAR γ , UCP2 and UCP3 mRNA. Among these gene expression markers, PPAR γ is adipocyte-specific so that decreased levels may signify either reduced adipocyte mass per adipose tissue mass or reduced adipocyte differentiation. The combination of decreased PPAR γ and UCP2 is suggestive of impaired adipocyte differentiation, with evidence that decreased gene expression is specifically associated with the severity of mtDNA depletion. Zidovudine therapy was associated with less severe mtDNA depletion and similar mitochondrial protein mass as stavudine therapy. However, the absence of a similar reduction in gene expression suggests that the less severe mtDNA depletion observed in zidovudine recipients may be below a threshold required for toxicity. Therapies that are not associated with severe mtDNA depletion, and coupled with PI therapy, are associated with statistically and biologically significant elevations in expression (mRNA) of a brown adipocyte marker, UCP1, which may represent an adaptive response to increased fatty acid flux.

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