

## Alterations in thigh subcutaneous adipose tissue gene expression in protease inhibitor-based highly active antiretroviral therapy

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### Abstract

Use of protease inhibitor (PI)-based highly active antiretroviral therapy (HAART) has been associated with altered regional fat distribution, insulin resistance, and dyslipidemias. To assess how PI-based HAART affects adipocyte gene expression in male HIV-1-infected patients, reverse transcription-polymerase chain reaction was used to quantify messenger RNA expression of adipocyte transcription factors and adipocytokines in thigh and abdominal subcutaneous adipose tissue from male (1) HIV-1 seronegative subjects (control,  $n = 9$ ), (2) asymptomatic treatment-naïve HIV-1-infected patients (naïve,  $n = 6$ ), (3) HIV-1-infected patients who were receiving antiretroviral agents but never received PIs (PI naïve,  $n = 5$ ), (4) HIV-1-infected patients who were receiving PI-based HAART (PI,  $n = 7$ ), and (5) HIV-1-infected patients who discontinued the PI component of their antiviral therapy more than 6 months before enrollment (past PI,  $n = 7$ ). In the PI group, the messenger RNA expression levels of the CCAAT/enhancer-binding protein  $\alpha$ , leptin, and adiponectin (18%,  $P < .01$ ; 23%,  $P < .05$ ; and 13%,  $P < .05$ , respectively) were significantly lower than the levels measured in the PI-naïve group. These results are consistent with previous studies on the effects of PIs on cultured adipocytes. Prospective longitudinal studies of thigh fat adipose tissue gene expression could provide further insights on the pathogenesis of metabolic complications associated with PI-based HAART.

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### 1. Introduction

The use of protease inhibitor (PI)-based highly active antiretroviral therapy (HAART) has dramatically reduced rates of AIDS-related morbidity and mortality [1]. However, PI-based HAART has been associated with serious side effects that include insulin resistance, hyperlipidemia, and body fat redistribution (commonly referred to as the “HIV lipodystrophy syndrome”) [2–7]. The changes in fat distribution observed in HIV lipodystrophy include overlapping features of loss of fat in the limbs and face (lipoatrophy) and gain of fat in the abdomen and behind the neck (lipohypertrophy). Although use of PIs has been implicated in the development of these complications, it is now clear that several factors, such as nucleoside reverse transcriptase inhibitors (NRTIs), HIV disease, age, and genetic predisposition, contribute to the pathogenesis of this syndrome.

The addition of PIs to NRTIs appears to accelerate the development of lipoatrophy compared with NRTIs alone [8]. Furthermore, withdrawal of PIs may lead to reversal of metabolic abnormalities but not reversal of lipoatrophy [8].

Previous studies demonstrated that a number of PIs directly perturbed adipocyte biology in cultured preadipocytes and in mature adipocytes. Indinavir, ritonavir, amprenavir, and nelfinavir inhibited differentiation of preadipocytes and decreased gene expression of adipocyte transcription factors such as peroxisomal proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), CCAAT/enhancer-binding protein  $\alpha$  (C/EBP $\alpha$ ), and sterol regulatory element binding protein-1c (SREBP-1c) in vitro [9–11]. Indinavir, ritonavir, and nelfinavir decreased steady-state levels of adiponectin messenger RNA (mRNA) levels in mature 3T3-L1 adipocytes [12]. Indinavir, ritonavir, and amprenavir reversibly inhibit glucose uptake mediated by the GLUT4 transporter in mature 3T3-L1 adipocytes [13]. These alterations in gene expression would be expected to inhibit adipocyte differentiation, which may underlie lipoatrophy, and reduce glucose

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transport into adipocytes and other insulin-sensitive tissues, which may contribute to impaired glucose tolerance.

Bastard et al [14] reported that patients receiving PI-based HAART had lower abdominal subcutaneous fat PPAR $\gamma$ , C/EBP $\alpha$ , SREBP-1c, and leptin mRNA expression levels compared with that measured in HIV-1 seronegative control subjects. They also found that tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) mRNA levels were higher in patients with HIV-1 lipodystrophy compared with HIV-1 seronegative individuals. To further assess the contribution of PI use to alterations in subcutaneous adipose tissue (SAT) gene expression, we compared the mRNA expression levels of 7 genes in thigh and abdominal subcutaneous fat biopsies obtained from (1) HIV-1 seronegative subjects (control), (2) asymptomatic treatment-naïve HIV-1-infected patients (naïve), (3) HIV-1-infected patients who received antiretroviral agents but never received PIs (PI naïve), (4) HIV-1-infected patients currently on PIs (PI), and (5) HIV-1-infected patients who stopped PIs for more than 6 months (past PI). We hypothesized that current PI use would be associated with lower mRNA expression for adipocyte transcription factors (PPAR $\gamma$ , C/EBP $\alpha$ , and SREBP-1c), a marker of adipocyte differentiation (lipoprotein lipase, or LPL), and adipocytokines (leptin, adiponectin); and greater mRNA expression for an inflammatory cytokine (TNF- $\alpha$ ) in thigh and abdominal SAT.

## 2. Methods

### 2.1. Experimental subjects

Twenty-seven nondiabetic nonobese (body mass index [BMI] <30) HIV-1-infected patients (25 men, 2 women) were recruited for this cross-sectional study from the AIDS Clinical Trials Unit and the Infectious Diseases Outpatient Clinic at Washington University School of Medicine (St Louis, Mo). The protocol was approved by the Human Studies Committee of the Washington University Medical Center and all subjects provided informed consent. Socio-demographic and clinic data were obtained as well as a medication history including the type and duration of all antiretroviral therapy. Anthropomorphic data included BMI and quantitative assessment of SAT and intra-abdominal adipose tissue areas using proton magnetic resonance imaging (MRI). For comparative purposes, subcutaneous abdominal and thigh fat biopsies were obtained from 12 nondiabetic nonobese HIV-1 seronegative research volunteers (9 men, 3 women).

### 2.2. Fat biopsies

The subjects were biopsied after an overnight fast and after resting for an hour. Fat biopsies were obtained by needle aspiration under local anesthesia. The samples were rinsed in cold saline. One portion was immediately fixed in formalin and another portion was immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for subsequent RNA

isolation. The formalin-fixed fat biopsies were embedded in paraffin, 5- $\mu\text{m}$  sections were cut and stained with hematoxylin and eosin, and the sections were inspected to confirm the presence of adipose tissue.

### 2.3. Reverse transcription–real-time polymerase chain reaction

Total RNA was extracted from frozen fat samples using TRIzol reagent (GIBCO-BRL, Grand Island, NY) according to the manufacturer's recommendation. Total RNA (1  $\mu\text{g}$ ) was reverse transcribed for 1 hour at  $42^{\circ}\text{C}$  in 20- $\mu\text{L}$  reaction containing  $1\times$  ImProm-II RT buffer (Promega Co, Wis), 0.5  $\mu\text{g}$  oligo-dT, 0.5 mM dNTP, and 1 unit rRNasin ribonuclease inhibitor. Oligonucleotide primers and TaqMan probes for the adipocyte genes were designed using Primer Express and sequences from the GenBank database (<http://www.ncbi.nlm.nih.gov>). Sequences of the forward and reverse primers and probe are shown in Table 1. Amplification of each target DNA was performed with TaqMan polymerase chain reaction reagent kits in the ABI 7700 sequence detection system according to the protocols provided by the manufacturer (Perkin Elmer/PE Applied Biosystems, Foster City, Calif). For each set of gene primers, complementary DNA (cDNA) from 1 of the control fat samples was serially diluted to generate a standard curve for each target gene by plotting Ct values (cycle number at which the fluorescence signal exceeds background) vs log cDNA dilution. The slopes obtained for actin and the genes examined were identical within experimental error, indicating that amplification efficiencies of the genes examined were equal. Triplicate assays were run at 2 concentrations of cDNA within the linear range of the assay. The intra-assay coefficient of variation ranged from 0.18% to 3.37% and the inter-assay coefficient of variation ranged from 1.41% to 4.87%. Because the target genes and the actin gene were amplified with equal efficiencies, the  $-\Delta\text{Ct}$  ( $-\Delta\text{Ct} = \text{Ct actin gene} - \text{Ct target gene}$ ) values were used to quantitate the logarithmic transformation of the mRNA level of the target gene relative to that of actin. The fold change in target gene expression relative to control subjects was calculated as  $2^{-\Delta\Delta\text{Ct}}$ , where  $\Delta\Delta\text{Ct} = \text{median } \Delta\text{Ct experimental group} - \text{median } \Delta\text{Ct control group}$ .

### 2.4. Magnetic resonance imaging

Proton magnetic resonance images were obtained for 2 of 9 HIV-1 seronegative subjects and all of the HIV-1-infected patients on a 1.5-T superconducting magnet (Siemens, Iselin, NJ) using a T<sub>1</sub>-weighted pulse sequence. Consistent slice localization was accomplished by using the iliac crest as a landmark to position the subject in the magnet. For abdominal scans, 18 serial cross-sectional images (10 mm thick, TR = 205 milliseconds, TE = 4.1 milliseconds) were obtained at the level of the L<sub>3</sub>–L<sub>4</sub> interspace to quantify abdominal SAT and visceral adipose tissue (VAT) areas [15]. For thigh scans, 10 serial cross-sectional images (8 mm

Table 1  
Oligonucleotide primers and TaqMan probes

	Forward primer	Reverse primer	TaqMan probe
$\beta$ -Actin	GCCCTGAGGCACTCTTCCA	GCGGATGTCCACGTCACA	CTTCCTTCCTGGGCATGGGTCCTG
PPAR $\gamma$	AAGGCGAGGGCGATCTTG	CCCATCATTAAGAATTCATGTCAA	CAGGAAAGACAACAGACAAATCAATT
C/EBP $\alpha$	CTTGGTCAAGGCCATGGGCAACT	GCTTTC GGAGGCAC CGGAATCT	CGGTCCCACAGCCGCGCGCCTCA
SREBP-1c	ACAGCCCACAACGCCATT	CCCACCACCAGATCCTTGAG	CGCACCCTCCTCCATCAATGAC
Adiponectin	GGCCGTGATGGCAGAGAT	CCCGGGTACTCCGGTTTC	AGGGTGAGAAAGGAGATCCAGGTCTTATTGG
Leptin	GGAATGAAGTCCAAACCGGTG	GTGCGGATTCTGTGGCTTT	CAATGACATTTACACACGTCAGTCTCCTCCAA
TNF- $\alpha$	AATGGCGTGGAGCTGAGAGAGATA	GGACCTGGGAGTAGATGAGGTACA	CCAGCTGGTGGGCCATCAGAGG
LPL	TGAGAGTGAAACCCA TACCAATCA	TGGAACTTCAGGCA GAGTGAAT	ACCGTGGCCGAGAGT GAGAACATCC

Table 2

Study characteristics of male control, naive, PI-naive, PI, and past-PI groups

	Control (N = 9)	Naive (N = 6)	PI naive (N = 5)	PI (N = 7)	Past PI (N = 7)	P
BMI (kg/m <sup>2</sup> )	25.1 ± 0.6	26.6 ± 0.7	24.8 ± 1.4	25.3 ± 1.3	25.8 ± 1.1	.8
Age (y)	30.8 ± 2.4	41.5 ± 3.5	32.6 ± 1.9	38.5 ± 3.5	49.1 ± 3.2	.0007
CD4 (cells/μL)	525 ± 160	550 ± 50	525 ± 50	700 ± 110	510 ± 80	.5
Viral load (copies/mL)	N/A	19000 ± 10000	<400	1200 ± 1200	<400	.03
Duration HIV (y)	N/A	3.5 ± 2.1	5.6 ± 1.2	7.7 ± 2.1	10.6 ± 2.3	.12
Duration ART (y)	N/A	N/A	3.0 ± 1.0	4.2 ± 0.8	5.2 ± 0.4	0.17
Duration PI (y)	N/A	N/A	N/A	3.2 ± 0.5	3.4 ± 0.3	.7
VAT (cm <sup>2</sup> )	190 ± 60	140 ± 20	115 ± 10	170 ± 20	220 ± 50	.2
SAT (cm <sup>2</sup> )	115 ± 5	195 ± 20	155 ± 40	145 ± 40	160 ± 30	.7
Thigh fat (cm <sup>2</sup> )	51 ± 7	54 ± 7	41 ± 7	29 ± 6	25 ± 4	.009
Triglyceride (mg/dL)	140 ± 50	90 ± 10	300 ± 110	360 ± 80	320 ± 90	.12

The mean values ± SE are shown.

thick, TR = 1500 milliseconds, TE = 12 milliseconds) were obtained from a region 10 to 25 cm above the superior border of the medial condyle on the head of the tibia to quantify thigh subcutaneous fat area [16]. Analyze Direct software (Mayo Clinic, Rochester, Minn) was used to identify pixel intensities that corresponded to adipose tissue, identify and segment the regions of interest (eg, abdominal VAT, abdominal SAT, and thigh fat), and quantify the adipose tissue areas in each cross-sectional image.

### 2.5. Statistical analysis

Statistical analyses were carried out with the aid of Graphpad Prism 3.0 on the  $-\Delta C_t$  values, which represent a logarithmic transformation of the mRNA levels. Differences in mean  $-\Delta C_t$  values between more than 2 categorical groups were calculated by the 1-way analysis of variance (ANOVA) followed by the Bonferroni posttest. The Pearson correlation analysis was used to identify associations between 2 variables in the patients with HIV-1 receiving antiretroviral therapy because normality was established for the variables by the Kolmogorov-Smirnov test. The threshold of significance was set at  $P = .05$ . All  $P$  values were 2-tailed.

## 3. Results

### 3.1. Study characteristics of male control, naive, PI-naive, PI, and past-PI groups

Because gender effects on fat distribution could potentially confound the interpretation of the data, the male

subjects were analyzed separately. As shown in Table 2, the 5 groups were matched with respect to mean BMIs. The past-PI subjects recruited were older than the control subjects ( $P < .001$ ) and the PI-naive subjects ( $P < .01$ ). All of the HIV-1-infected patients had CD4 cell counts of more than 200 cells/μL except one past-PI patient (188 cells/μL). Plasma viral RNA was less than 400 copies/mL in all the HIV-1-infected patients treated with antiretroviral agents except for one patient in the PI group (8108 copies/mL). Plasma HIV RNA in the asymptomatic treatment-naive HIV-1-infected patients (naive) ranged from less than 400 to 53 409 copies/mL.

The duration of known HIV-1 infection in the HIV-1-infected patients ranged from 0.2 to 19 years, and differences between the HIV-1-infected groups did not reach statistical significance. The mean total duration of PI treatment in the PI and past-PI groups was similar. The PIs used included indinavir (10 patients), nelfinavir (11 patients), liponavir/ritonavir (3 patients), ritonavir (7 patients), and saquinavir (7 patients). Three of five patients in the PI-naive, 5 of 7 in the PI, and 5 of 7 in the past-PI groups received stavudine (also termed *D4T*, see Table 2), which has been implicated in the development of HIV-1 lipodystrophy [17,18].

In general, thigh subcutaneous fat area was more affected by lipodystrophy than abdominal subcutaneous fat area. Differences in abdominal VAT and SAT did not reach statistical significance between the 4 HIV-1-infected groups. On the other hand, the mean thigh fat area in the past-PI group ( $25 \pm 4$  cm<sup>2</sup>) was significantly lower than the

Table 3

Adipocyte mRNA expression in thigh subcutaneous fat from male control, naive, PI-naive, PI, and past-PI patients

	Control (N = 9)	Naive (N = 6)	PI naive (N = 5)	PI (N = 7)	Past PI (N = 7)	ANOVA (P)	Control vs PI (P)	Naive vs PI (P)	PI naive vs PI (P)
PPAR $\gamma$	-8.5 ± 0.2	-7.9 ± 0.2	-9.2 ± 0.3	-9.7 ± 0.2	-9.4 ± 0.4	.0004	<.05	<.05	
C/EBP $\alpha$	-3.9 ± 0.1	-4.7 ± 0.5	-4.4 ± 0.3	-6.9 ± 0.6	-5.5 ± 0.6	.008	<.001	<.05	<.01
SREBP-1c	-9.4 ± 0.3	-10 ± 0.5	-9.1 ± 1.1	-11.0 ± 0.8	-10.3 ± 0.7	.3			
Adiponectin	-4.1 ± 0.4	-3.3 ± 0.5	-3.3 ± 0.6	-5.4 ± 0.5	-4.0 ± 0.3	.02		<.05	<.05
Leptin	-7.1 ± 0.3	-7.2 ± 0.5	-7.6 ± 0.6	-10.5 ± 0.6	-9.1 ± 0.8	.0004	<.001	<.01	<.05
TNF- $\alpha$	-12.9 ± 0.1	-11.6 ± 0.6	-10.7 ± 0.9	-10.5 ± 0.9	-9.5 ± 0.9	.002			
LPL	-2.7 ± 0.2	-2.4 ± 0.4	-2.5 ± 0.8	-3.9 ± 0.6	-3.8 ± 0.6	.14			

The mean  $-\Delta C_t$  values (threshold cycle of actin – threshold cycle for target gene) ± SE are shown.

Table 4

Adipocyte mRNA expression in abdominal subcutaneous fat from male control, naive, PI-naive, PI, and past-PI patients

	Control (N = 9)	Naive (N = 6)	PI naive (N = 5)	PI (N = 7)	Past PI (N = 7)	ANOVA (P)	Control vs PI (P)	Naive vs PI (P)	PI naive vs PI (P)
PPAR $\gamma$	-8.4 $\pm$ 0.2	-7.8 $\pm$ 0.2	-9.2 $\pm$ 0.3	-9.0 $\pm$ 0.4	-8.1 $\pm$ 0.7	0.17			
C/EBP $\alpha$	-4.6 $\pm$ 0.4	-4.5 $\pm$ 0.5	-4.7 $\pm$ 0.7	-5.9 $\pm$ 0.7	-5.3 $\pm$ 0.3	0.4			
SREBP-1c	-10.3 $\pm$ 0.5	-9.1 $\pm$ 0.3	-10.4 $\pm$ 0.8	-10.9 $\pm$ 0.6	-11.0 $\pm$ 0.3	0.1			
Adiponectin	-3.9 $\pm$ 0.3	-3.0 $\pm$ 0.4	-3.7 $\pm$ 0.6	-5.0 $\pm$ 0.4	-3.8 $\pm$ 0.5	0.05			
Leptin	-7.7 $\pm$ 0.6	-6.6 $\pm$ 0.3	-8.2 $\pm$ 0.6	-10.1 $\pm$ 0.3	-8.3 $\pm$ 0.6	0.003	<.05	<.01	
TNF- $\alpha$	-12.7 $\pm$ 0.4	-11.6 $\pm$ 0.7	-11.6 $\pm$ 0.7	-11.8 $\pm$ 1.0	-11.3 $\pm$ 0.5	0.5			
LPL	-2.1 $\pm$ 0.2	-2.4 $\pm$ 0.3	-2.7 $\pm$ 0.9	-3.5 $\pm$ 0.3	-2.8 $\pm$ 0.6	0.25			

The mean  $-\Delta\text{Ct}$  values (threshold cycle of actin – threshold cycle of target gene)  $\pm$  SE are shown.

mean thigh fat area in the naive group ( $54 \pm 7 \text{ cm}^2$ ,  $P < .05$ ). It is possible that patients were taken off PIs because they developed lipoatrophy. The thigh fat areas correlated negatively with the total duration of antiretroviral therapy (Pearson's  $r = -0.62$ ,  $P = .004$ ,  $n = 19$ ) in the treated male HIV-1-infected patients.

The thigh fat areas of the male HIV-1 seronegative patients (control) and the male asymptomatic treatment-naive patients (naive) were combined to define the range of nonlipoatrophic or “normal” thigh fat areas in this study. The lowest normal thigh fat area in the control and naive patients was  $31 \text{ cm}^2$ . For the purposes of this study, we defined thigh fat areas of less than  $31 \text{ cm}^2$  as peripheral lipoatrophy. On this basis, 2 of 5 in the PI-naive group, 4 of 7 in the PI group, and 5 of 7 in the past-PI group had peripheral lipoatrophy.

### 3.2. Thigh and abdominal subcutaneous fat mRNA expression in control, naive, PI-naive, PI, and past-PI groups

Thigh subcutaneous fat PPAR $\gamma$ , C/EBP $\alpha$ , leptin, adiponectin, and TNF- $\alpha$   $-\Delta\text{Ct}$  values (proportional to the logarithmic transformation of the mRNA levels) were significantly different between the 5 study groups (see Table 3). The C/EBP $\alpha$ , adiponectin, and leptin mRNA levels ( $2^{-\Delta\text{Ct}}$ ) in the PI group were, respectively, 18%, 23%, and 13% of the levels measured in the PI-naive group. The C/EBP $\alpha$  and leptin mRNA levels in the PI group were 13% of the levels measured in the control groups. The TNF- $\alpha$  mRNA level was approximately 10-fold higher in the past-PI group compared with the control group ( $P < .05$ ). In abdominal subcutaneous fat, only leptin mRNA expression was different among the 5 groups, but adiponectin mRNA expression tended to differ as well (see Table 4).

### 3.3. Univariate regression analyses of thigh subcutaneous fat mRNA expression

Because adipocyte transcription factors PPAR $\gamma$ , C/EBP $\alpha$ , and SREBP-1c mediate activation of target genes, including adiponectin, leptin, and LPL [19–23], univariate regression analyses were carried out to explore the relationship between thigh subcutaneous fat mRNA expression of the adipocyte transcription factors and their putative target genes in treated male HIV-1-infected patients. There was a striking correlation between C/EBP $\alpha$  and leptin mRNA

expression in thigh subcutaneous fat (Pearson  $r = 0.8$ ,  $P < .0001$ ,  $n = 19$ ), consistent with a previous report that C/EBPs up-regulated the leptin promoter [19]. There was a significant correlation between SREBP-1c and adiponectin mRNA expression in thigh subcutaneous fat (Pearson  $r = 0.56$ ,  $P = .0125$ ,  $n = 19$ ), consistent with a recent report that SREBP-1c regulated mouse adiponectin expression [22]. Lipoprotein lipase mRNA expression did not correlate significantly with mRNA expression of the 3 transcription factors, but correlated negatively with triglyceride levels (Pearson  $r = -0.59$ ,  $P = .007$ ,  $n = 19$ ). This is consistent with the observation that low post-heparin LPL activity contributed to HIV-dyslipidemia [15].

## 4. Discussion

Epidemiologic studies and studies on cultured adipocytes and animal models have implicated PIs in the pathogenesis of the syndrome of metabolic complications commonly referred to as the HIV-1 lipodystrophy syndrome. The lower thigh fat C/EBP $\alpha$ , leptin, and adiponectin mRNA levels observed in the PI group compared with the PI-naive group are consistent with previous studies on the effects of PIs on gene expression in cultured adipocytes.

Gene expression in abdominal fat was less affected than gene expression in thigh fat, perhaps because abdominal fat areas were less affected than thigh fat areas in this cohort of subjects (as above). Regardless, these observations suggest that these 2 subcutaneous fat depot sites do not exhibit equivalent gene expression levels and would not be expected to respond similarly to anti-HIV medications. A number of previous studies have observed changes in abdominal subcutaneous fat gene expression with the presence or absence of lipodystrophy [14,24–26]. In this cross-sectional study, we observed no significant difference in thigh or abdominal fat mRNA expression levels between treated HIV-1-infected patients with and without lipoatrophy as determined by magnetic resonance imaging. It is difficult to directly compare the results of this study with previous studies because clinical assessment rather than objective measurements was used to define the presence or absence of lipodystrophy. Furthermore, detailed information on PI use in the patients with and without lipodystrophy was not provided in these reports.



Inhibition of the GLUT4 glucose transporter by a number of the PIs has been implicated in the mechanism of PI-induced insulin resistance [13,27]. This raises the question as to whether PI inhibition of glucose uptake or metabolism mediates PI effects on adipocyte gene expression. Inhibition of glucose uptake or metabolism appears to down-regulate transcription of the leptin gene [28,29]. Administration of recombinant leptin and adiponectin ameliorates ritonavir-induced dyslipidemias in a rodent model, suggesting that reduction of adiponectin and leptin expression plays a role in the pathogenesis of this complication [12,30].

Whether newly developed PIs, such as atazanavir, used alone or in combinations with other PIs (eg, ritonavir), also affect gene expression in cultured adipocytes or in adipose tissue biopsies is not known [31]. Prospective longitudinal studies of thigh fat adipose tissue gene expression could provide further insights on the pathogenesis of metabolic complications and anthropomorphic changes associated with PI-based HAART.

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