Differential Subcutaneous Adipose Tissue Gene Expression Patterns in a Randomized Clinical Trial of Efavirenz or Lopinavir-Ritonavir in Antiretroviral-Naive Patients

L. Egaña-Gorroño, a E. Martínez, b P. Domingo, c M. Loncà, b T. Escribà, b J. Fontdevila, d F. Vidal, e E. Negredo, f J. M. Gatell, a,b M. Arnedo a

Group of Genomics and Pharmacogenomics in HIV, Retrovirology and Viral Immunopathogeny Laboratory, IDIBAPS, Hospital Clinic de Barcelona, Barcelona, Spain; Department of Infectious Diseases, Hospital Clinic de Barcelona, University of Barcelona, Barcelona, Spain; Department of Infectious Diseases, Hospital Sant Pau, Barcelona, Spain; Plastic Surgery Department, Hospital Clinic de Barcelona, University of Barcelona, Barcelona, Spain; Hospital Universitari de Tarragona Joan XXIII, IISPV, University of Rovira i Virgili, Tarragona, Spain; Fundació Lluita Contra la SIDA, Hospital Germans Trias i Pujol, Badalona, Spain

Gene expression studies of subcutaneous adipose tissue may help to better understand the mechanisms behind body fat changes in HIV-infected patients who initiate antiretroviral therapy (ART). Here, we evaluated early changes in adipose tissue gene expression and their relationship to fat changes in ART-naive HIV-infected patients randomly assigned to initiate therapy with emtricitabine/tenofovir plus efavirenz (EFV) or ritonavir-boosted lopinavir (LPV/r). Patients had abdominal subcutaneous adipose tissue biopsies at baseline and week 16 and dual-energy-X-ray absorptiometry at baseline and weeks 16 and 48. mRNA changes of 11 genes involved in adipogenesis, lipid and glucose metabolism, mitochondrial energy, and inflammation were assessed through reverse transcription-quantitative PCR (RT-qPCR). Additionally, correlations between gene expression changes and fat changes were evaluated. Fat increased preferentially in the trunk with EFV and in the limbs with LPV/r (P < 0.05). After 16 weeks of exposure to the drug regimen, transcripts of CEBPA, ADIPOQ, GLUT4, LPL, and COXIV were significantly down-regulated in the EFV arm compared to the LPV/r arm (P < 0.05). Significant correlations were observed between LPL expression change and trunk fat change at week 16 in both arms and between CEBPA or COXIV change and trunk fat change at the same time point only in the EFV arm and not in the LPV/r arm. When combined with emtricitabine/tenofovir as standard backbone therapy, EFV and LPV/r induced differential early expression of genes involved in adipogenesis and energy metabolism. Moreover, these mRNA expression changes correlated with trunk fat change in the EFV arm. (This was a substudy of a randomized clinical trial [LIPOTAR study] registered at ClinicalTrials.gov under identifier NCT00759070.)

Thymidine analogue reverse transcriptase inhibitors (NRTI), including stavudine and didavudine, and the first protease inhibitors (PIs) developed in the mid-1990s have been recognized as major determinants of lipodystrophy in HIV-infected patients (1–4). Both types of drugs have been associated with mitochondrial dysfunction and oxidative stress (5), altered adipogenesis and adipocyte differentiation (6, 7), impaired glucose transport (8), and altered expression of genes involved in lipid metabolism (9). Although lipodystrophy is now less of a problem, given the implementation of newer drugs with fewer adipose/metabolic effects, studies in antiretroviral-naive patients have clearly shown differential drug effects on body fat alterations (10, 11).

Studies of genetic polymorphisms may help to identify HIV-infected patients under antiretroviral therapy (ART) at higher risk for metabolic complications (12–14). Moreover, gene expression studies of subcutaneous adipose tissue may help us to better understand the mechanisms behind body fat changes in HIV-infected patients who initiate ART. Antiretroviral-drug-induced gene expression changes have been detected in adipocytes, in vitro (15, 16) and in vivo, in healthy volunteers (17) and in HIV-infected patients (18–21). In this study, we aimed to compare the effects of efavirenz (EFV) and those of ritonavir-boosted lopinavir (LPV/r), combined with emtricitabine and tenofovir, on body fat changes to evaluate the in vivo influence of each regimen on human subcutaneous adipose tissue gene expression and, additionally, to assess whether these transcript changes may be correlated with fat changes.

(These results were previously presented at the 20th Retrocon-
line and weeks 16 and 48. Abdominal subcutaneous adipose tissue biopsy specimens were obtained from each patient at baseline and week 16.

**Body fat assessment.** Total body fat and limb and trunk fat were measured by DXA as previously described (22). Briefly, patients were positioned straight on a table with all body parts in the scan field, palms down and separated from the thighs, and legs rotated inward 25°. All patients were scanned with the same Hologic (Bedford, MA, USA) X-ray equipment by the same experienced technician, who was blind to treatment assignment. Fat determinations were expressed in kilograms.

**Fat tissue biopsies and RNA isolation.** Fatty tissue samples were harvested from a periumbilical incision. Briefly, pieces of tissue measuring 0.5 cm³ were excised from the infraumbilical area. The samples were snap-frozen in liquid nitrogen. The ATB dust was then homogenized in 1 ml TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) and precipitated in ethanol and water, 1 ml of cDNA at a final concentration of 4 ng. Thermocycler conditions were as follows: 95°C hot start for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was run in triplicate, and controls lacking RNA or cDNA were included in each set of experiments.

**Statistical analysis.** Statistical analysis was performed according to the protocol using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Paired t tests or Student’s t tests were used for pairwise comparisons. The influence of nongenetic factors on fat changes (defined as any increase/decrease of fat relative to baseline) was assessed through stratified analysis, controlling for age, baseline CD4⁺ T-cell count, baseline viral load, and first-line ART (EFV or LPV/r) using univariate analyses of variance. Furthermore, all covariates were assessed in a multivariate analysis based on a logistic regression model using SPSS v15.0 (IBM Corporation, Armonk, NY, USA).

For the gene expression analysis, raw Ct (cycle threshold; the number of cycles required for the fluorescent signal to cross the threshold) values of the expression of each individual mRNA were obtained using SDS software v.2.3. The Ct values were then exported to RQ Manager v1.2 software (Applied Biosystems) for ΔCt (ΔCt = Ct target gene − Ct endogenous control gene) value determination in order to quantitate the mRNA level of the target gene relative to that of the endogenous control (18).

Finally, Spearman’s rank order correlations were calculated in order to determine the strength and direction of an association between two rank variables: gene expression changes and fat changes.

**RESULTS**

**Population characteristics.** The baseline characteristics of the study participants are shown in Table 2. Medians were used to show central tendencies, and interquartile ranges (IQR) (IQR = upper quartile [Q3] − lower quartile [Q1]) were calculated as measures of variability and statistical dispersion. A total of 19 HIV-1-infected, ART-naive men were enrolled in the study. Ten (52.6%) patients received EFV, while 9 (47.4%) patients received LPV/r as initial therapy. The plasma viral load reached undetectable levels (<50 RNA copies) in all patients by week 16. No patient was coinfected with hepatitis C or B virus. None of them was a substance abuser or under any medication that could affect metabolism and body composition. No statistically significant differences in baseline characteristics were observed between the arms.

**Body fat changes.** Globally, patients in both arms experienced fat increases at weeks 16 and 48 relative to baseline. Fat increased more in the trunk in the EFV arm and in the limbs in the LPV/r arm. A large amount of fat increase at week 48 was already apparent at week 16 in both treatment groups. Total body fat and limb and trunk fat levels were significantly higher at week 16 (total body fat, P = 0.007; limb fat, P = 0.033; trunk fat, P = 0.007) and week 48 (total body fat, P = 0.016; limb fat, P = 0.007; trunk fat, P = 0.029) than at week 0 in the EFV arm. Similarly, the LPV/r arm showed a tendency toward fat increase at the two time points, although significant differences were observed only for limb fat at week 16 compared to baseline (P = 0.036).

No significant differences were observed between the fat changes observed at week 16 and week 48 with any of the treatments. Moreover, no significant differences were observed in any

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Function</th>
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<tbody>
<tr>
<td>CEBP/A</td>
<td>CCAAT/enhancer binding protein alpha</td>
<td>Early adipocyte differentiation&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
<td>Adipocyte differentiation&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ADIPOQ</td>
<td>Adiponectin</td>
<td>Adipokine&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>LEP</td>
<td>Leptin</td>
<td>Adipokine&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pref-1</td>
<td>Delta-like 1 homolog</td>
<td>Adipogenesis inhibitor&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Insulin-responsive glucose transporter type 4</td>
<td>Glucose metabolism&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
<td>Triglyceride hydrolase&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>COXII</td>
<td>Cytochrome c oxidase subunit II</td>
<td>Respiratory chain subunit&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>COXIV</td>
<td>Cytochrome c oxidase subunit IV</td>
<td>Respiratory chain subunit&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
<td>Proinflammatory cytokine&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein 1</td>
<td>Proinflammatory cytokine&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Gene involved in adipogenesis.

<sup>b</sup> Gene involved in glucose and lipid metabolism.

<sup>c</sup> Gene involved in mitochondrial metabolism.

<sup>d</sup> Gene involved in inflammation.

<sup>e</sup> Endogenous control gene.
TABLE 2 Baseline characteristics of study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
<th>EFV arm (n = 10)</th>
<th>LPV/r arm (n = 9)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr) [median (IQR)]</td>
<td>39 (8)</td>
<td>37 (7)</td>
<td>41 (15)</td>
<td>0.7</td>
</tr>
<tr>
<td>Sex [no. of men (%)]</td>
<td>19 (100)</td>
<td>10 (100)</td>
<td>9 (100)</td>
<td></td>
</tr>
<tr>
<td>Ethnicity [n (%)]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>17 (89.5)</td>
<td>9 (90)</td>
<td>8 (88.9)</td>
<td></td>
</tr>
<tr>
<td>Latin American</td>
<td>2 (10.5)</td>
<td>1 (10)</td>
<td>1 (11.1)</td>
<td></td>
</tr>
<tr>
<td>Infection mode [no. of MSM (%)]</td>
<td>19 (100)</td>
<td>10 (100)</td>
<td>9 (100)</td>
<td></td>
</tr>
<tr>
<td>Smokers [n (%)]</td>
<td>6 (31.5)</td>
<td>3 (30)</td>
<td>3 (33)</td>
<td>0.94</td>
</tr>
<tr>
<td>Viral load (log) [median (IQR)]</td>
<td>4.6 (5.14)</td>
<td>4.4 (5.11)</td>
<td>4.72 (5.16)</td>
<td>0.59</td>
</tr>
<tr>
<td>CD4⁺ (cells/µl) [median (IQR)]</td>
<td>353 (136)</td>
<td>354 (102.5)</td>
<td>320 (195)</td>
<td>0.61</td>
</tr>
<tr>
<td>Fat determination by DXA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²) [median (IQR)]</td>
<td>24.2 (3.17)</td>
<td>24.5 (3.29)</td>
<td>23.8 (3.1)</td>
<td>0.21</td>
</tr>
<tr>
<td>Total body fat (kg) [median (IQR)]</td>
<td>16.74 (8.6)</td>
<td>14.33 (5.4)</td>
<td>18.36 (8.8)</td>
<td>0.19</td>
</tr>
<tr>
<td>Limb fat (kg) [median (IQR)]</td>
<td>5.64 (4.8)</td>
<td>4.87 (3.06)</td>
<td>6.24 (5.5)</td>
<td>0.2</td>
</tr>
<tr>
<td>Trunk fat (kg) [median (IQR)]</td>
<td>10.27 (6)</td>
<td>8.84 (3.9)</td>
<td>12.72 (5.3)</td>
<td>0.22</td>
</tr>
<tr>
<td>Fat mass ratio [median (IQR)]</td>
<td>1.3 (0.34)</td>
<td>1.29 (0.5)</td>
<td>1.32 (0.25)</td>
<td>0.59</td>
</tr>
<tr>
<td>Weight (kg) [median (IQR)]</td>
<td>75.6 (11)</td>
<td>75 (15)</td>
<td>78 (16)</td>
<td>0.22</td>
</tr>
</tbody>
</table>

* MSM, men who have sex with men; BMI, body mass index.

AART-Induced Fat and Gene Expression Changes

Univariate analyses controlling for age, baseline CD4⁺ T-cell count, baseline viral load, and first-line ART (EFV or LPV/r) showed no factors significantly associated with fat changes (see Table S2 in the supplemental material).

Adipose tissue gene expression changes. RNAs from all samples were suitable for the gene expression study (RIN > 7). The mRNA levels of the antiadipogenic marker Pref-1 were not detectable in any sample.

Overall, both treatments induced expression changes in the assessed genes. Opposite expression profiles were shown between the two regimens: the EFV arm revealed a down-regulated pattern of changes at week 16 and fat changes at weeks 16 and 48 from baseline.

mRNAs for markers of adipogenesis, including CEBP/A (P = 0.007) and ADIPOQ (P = 0.015); genes involved in glucose and lipid metabolism, including GLUT4 (P = 0.028) and LPL (P = 0.03); and the mitochondrial toxicity marker COXIV (P = 0.026) were significantly down-regulated after 16-week exposure to the EFV-containing regimen (Fig. 2A). On the other hand, the LPV/r arm showed significant up-regulated of CEBP/A (P = 0.016), GLUT4 (P = 0.035), and COXIV (P = 0.005) (Fig. 2B).

Moreover, statistically significant differences were observed between the study arms for CEBP/A (P = 0.002), ADIPOQ (P = 0.034), GLUT4 (P = 0.013), LPL (P = 0.023), and COXIV (P = 0.0174) expression changes (Fig. 2C).

Transcripts for PPARG, LEP, COXII, TNF-α, and MCP-1 were not significantly altered in either of the two treatments (see Table S3 in the supplemental material).

Correlations between ART-induced gene expression changes and fat changes. Spearman’s correlations were carried out to explore any relationship between significant mRNA expression changes at week 16 and fat changes at weeks 16 and 48 from baseline (Fig. 3A). LPL expression level changes showed a significant correlation with trunk fat changes at the same time point in the EFV (r = 0.733; P = 0.02) and LPV/r (r = 0.816; P = 0.019) arms. Furthermore, significant correlations were detected between changes of CEBP/A (r = 0.648; P = 0.04) and COXIV (r = 0.818; P = 0.005) expression at week 16 with trunk fat changes at the same time point in the EFV arm, but not in the LPV/r arm (Fig. 3B, C, D, and E).
No correlations were found between gene expression changes at week 16 and body fat changes at week 48.

**DISCUSSION**

The influence of contemporary antiretroviral drugs on body fat composition is still a matter of concern. This is the first randomized study examining the *in vivo* influence of EFV and LPV/r, in combination with emtricitabine/tenofovir, as initial therapy for HIV infection on early adipose tissue gene expression changes. Moreover, correlations between mRNA changes and body fat changes as measured by DXA were evaluated.

Overall, opposite gene expression profiles were observed between the two study arms: the EFV arm revealed a down-regulated pattern while the LPV/r arm showed an up-regulated profile at week 16 compared to baseline. Our results determine that CEBP/A, ADIPOQ, GLUT4, LPL, and COXIV were differentially expressed across the study arms after 16 weeks of exposure to therapy. The above-mentioned mRNAs and consequent protein levels are known to be decreased in HIV-infected patients with lipodystrophy (23). The adipogenesis master gene, CEBP/A, which is known to play a role in the development of adipocyte morphology and function (24, 25), is significantly down-regulated in HIV-1-infected lipodystrophic patients compared to non-lipodystrophic infected individuals (18,26,27). Our study revealed significantly lower levels of CEBP/A in the EFV arm and up-regulated levels in the LPV/r arm after 16 weeks of exposure to the respective antiretroviral drug. Along with this finding, we observed a significant reduction of the adiponectin (ADIPOQ) transcript level, a protein hormone that modulates glucose regulation and fatty acid oxidation, among other metabolic processes, in the presence of EFV. ADIPOQ is characteristic of differentiated adipocytes (28,29), and serum hormone levels are inversely correlated with body fat percentage in adults (30). Together with reduced CEBP/A levels, lower expression of ADIPOQ has been detected *in vitro* in adipocyte cultures treated with EFV (15), and additionally, lower serum adiponectin levels have been commonly observed in HIV-1-infected patients under ART with lipodystrophic symptoms (31,32). Adipokines, such as adiponectin and leptin, play an important role in adipose tissue physiology and are believed to be a link between obesity and insulin resistance (33,34). In obesity and type 2 diabetes, expression of the glucose transporter gene GLUT4 is selectively decreased in adipocytes (35,36). The inhibition of GLUT4 is a primary mechanism for inducing insulin resistance (37), and HIV-infected lipodystrophic patients show decreased GLUT4 levels compared to non-lipodystrophic patients (18,38). Regarding lipid metabolism, expression levels of the lipoprotein lipase gene (LPL) were evaluated in this study. Lower LPL levels have been observed in HIV-1-infected patients than in the noninfected population (23,39). A reduction of GLUT4 and LPL would lead to decreased uptake of fatty acid and glucose by the adipocytes. Consistent with these observations, our results show that GLUT4 and LPL mRNA levels were significantly down-regulated in patients who initiated EFV-based therapy. On the other hand, GLUT4 transcript levels were significantly regulated in the LPV/r arm. Several PIs, including LPV/r, directly reduce glucose uptake through the inhibition of GLUT4 (40,41). Our findings suggest that the transcription of GLUT4 might increase in the presence of LPV/r as a compensatory mechanism for the PI-mediated inhibition of GLUT4.

mRNA of the nuclear DNA-encoded cytochrome c oxidase subunit IV (COXIV), was not altered *in vitro* even in the presence of high concentrations of antiretroviral drugs (15). However, in this study, the transcript was significantly down-regulated and up-regulated after exposure to EFV and LPV/r, respectively. The transcript encodes the last enzyme in the respiratory electron transport chain of mitochondria, and the reduction of its levels is characteristic of differentiated adipocytes (35,36). CEBP/A is selectively decreased in adipocytes (35,36). The inhibition of GLUT4 is a primary mechanism for inducing insulin resistance (37), and HIV-infected lipodystrophic patients show decreased GLUT4 levels compared to non-lipodystrophic patients (18,38). Regarding lipid metabolism, expression levels of the lipoprotein lipase gene (LPL) were evaluated in this study. Lower LPL levels have been observed in HIV-1-infected patients than in the noninfected population (23, 39). A reduction of GLUT4 and LPL would lead to decreased uptake of fatty acid and glucose by the adipocytes. Consistent with these observations, our results show that GLUT4 and LPL mRNA levels were significantly down-regulated in patients who initiated EFV-based therapy. On the other hand, GLUT4 transcript levels were significantly regulated in the LPV/r arm. Several PIs, including LPV/r, directly reduce glucose uptake through the inhibition of GLUT4 (40,41). Our findings suggest that the transcription of GLUT4 might increase in the presence of LPV/r as a compensatory mechanism for the PI-mediated inhibition of GLUT4.

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In addition, both regimens differentially affected the fat changes, but they differed in their preferential targets, either trunk or limb fat. Therefore, these correlations seemed to be independent of the regimen used. LPL levels have been associated with body weight and fat cell size in the noninjected population (43). Furthermore, significant correlations were observed between CEBP/A or COXIV expression changes and trunk fat changes at week 16 in the EFV arm, but not in the LPV/r arm. Given the associations found between gene expression and fat changes at the same time point, our data do not allow us to conclude that early molecular changes will be predictive of subsequent alterations in body fat composition, at least until week 48. However, our results suggest a direct effect of specific transcript level changes and trunk fat changes at the same time point, an observation that should be considered for future functional studies.

The present study has several limitations. (i) Out of 50 patients enrolled in the LIPOTAR clinical trial, only 19 patients (all of them men) gave informed consent for adipose tissue extraction and gene expression study. (ii) DXA fat measurements do not distinguish between subcutaneous and intra-abdominal fat, and potential changes in the intra-abdominal compartment might not have been captured. (iii) mRNA profiles cannot be directly extrapolated to protein levels. (iv) A follow-up longer than 48 weeks might be necessary to detect any potential gene expression change that could predict body fat changes.

In summary, our data indicate that both EFV and LPV/r, combined with tenofovir/emtricitabine, increased body fat early, but they differed in their preferential targets, either trunk or limb fat. In addition, both regimens differentially affected the in vivo gene expression pattern of human adipose tissue in the short term. Gene expression patterns showed opposite behaviors between EFV and LPV/r in terms of adipogenesis and mitochondrial metabolism. Moreover, the mRNA changes observed were correlated with trunk fat changes in the EFV arm but not in the LPV/r arm. Further collaborative studies and functional analyses are needed in order to address whether differential mRNA expression changes induced by antiretroviral drugs, such as the ones observed in this study, will have differential impacts on body fat alterations.

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M.A., J.M.G., and E.M. conceived and designed the experiments. P.D., M.L., J.F., F.V., and E.N. provided samples and collected data. L.E.-G. and T.E. performed the experiments. M.A., L.E.-G., and E.M. analyzed the data. M.A., L.E.-G., and E.M. wrote the manuscript. We all critically reviewed and subsequently approved the final version.

J.M.G. and E.M. have been consultants on advisory boards, have participated in speakers’ bureaus, have received research grants, or have conducted clinical trials with Roche, Boehringer-Ingelheim, Abbott, BMS, GSK, Gilead, Janssen, Merck, and Pfizer. M.A., L.E.-G., T.E., M.L., J.F., F.V., P.D., and E.N. report no conflicts of interest relevant to this article.
REFERENCES


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