

LMNA Messenger RNA Expression in Highly Active Antiretroviral Therapy–Treated HIV-Positive Patients

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Summary: The *LMNA* gene encodes for lamins A and C as major products, which are involved in nuclear stability, chromatin structure, and gene expression. Several *LMNA* mutations cause an insulin-resistant lipodystrophy that shares features with HIV-related lipodystrophy. Some HIV-treatment agents alter lamin A/C maturation, organization, and stability in 3T3-L1. We aimed to test the hypothesis that human adipose tissue *LMNA* expression can be altered in highly active antiretroviral therapy (HAART)–treated HIV-positive patients with lipodystrophy. We have also analyzed both isoforms and explored if their expression is associated with insulin resistance or inflammation in these patients. A cross-sectional study that analyzed abdominal subcutaneous adipose tissue from 39 treated HIV-positive patients (25 of whom had lipodystrophy) and 21 uninfected control subjects was performed. We have observed lower levels of lamin A isoform but normal levels of lamin C isoform in all HIV-infected patients, irrespective of the presence or absence of lipodystrophy, which reinforces the idea that an altered lamin A/C ratio could reflect a pathogenic condition. We have also found a correlation between *LMNA* adipose expression and several cytokine and adipogenic gene markers in HIV-positive patients, regardless of the presence or absence of lipodystrophy. Hence, in the present study, the lower lamin A expression observed in HIV-positive patients is related to HIV itself or to treatments rather than to the presence of lipodystrophy.

Key Words: highly active antiretroviral therapy, HIV infection, lipodystrophy, *LMNA*

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A-type lamins are nuclear proteins that form the nuclear lamina, a filamentous network located just underneath the inner nuclear membrane. A-type lamins are the product of the *LMNA* gene, which, through alternative splicing, encodes for lamins A and C (lamin A/C) as major products.¹ They participate in structural integrity of the nucleus and in controlling gene expression.¹ A-type lamin proteins interact with several transcription factors, including sterol responsive element binding protein-1 (SREBP-1).² Many different mutations in the *LMNA* gene cause numerous human genetic disorders, termed *laminopathies*. These include Dunnigan-type familial partial lipodystrophy (FPLD), a severe monogenic form of insulin-resistant lipodystrophy that shares common features with highly active antiretroviral therapy (HAART)–related lipodystrophy in HIV-infected patients.³ Studies to date, however, have shown no mutations in *LMNA* or its promoter region in patients with HAART-associated lipodystrophy.^{4–6}

The HAART-related lipodystrophy syndrome has a prevalence of 50% to 80% in patients infected with HIV and presents with peripheral lipoatrophy, central hypertrophy, and insulin resistance.⁷ Its pathogenesis is unknown, but different mechanisms have been described as implicated: HIV infection itself, inflammation state, antiretroviral drugs, adipocyte apoptosis, and adipogenic alterations. Regarding the latter, nelfinavir and indinavir exposition over 3T3-F442A preadipocytes altered SREBP-1 localization and adipocyte differentiation by promoting perturbations in the lamin A/C maturation, organization, and stability. This suggested that these alterations in the lamin A/C network could be the responsible for the SREBP-1 nuclear mislocalization resulting in altered adipocyte differentiation.⁸ These data, however, have not been replicated subsequently.⁹

Despite the extensive work in *LMNA* genotypic variation associated with diverse conditions, little is known about differential mechanisms at the molecular level by which the lamin A/C isoforms contribute to different phenotypes. Several observations led to the proposal that lamin A/C isoforms may contribute differentially to cellular functions. Hence, some disease-associated mutations that affect only the lamin A isoform have been described,^{10,11} whereas development of lamin C–only mice, which produce lamin C but no lamin A or prelamin A, are entirely healthy.¹² Similar levels in messenger RNA (mRNA) expression of lamin A and C isoforms in human preadipocytes and adipocytes have been previously described at the mRNA and protein level.¹³ An unbalanced lamin A/C ratio could reflect a pathogenic condition, however, as previously described.^{14,15}

In light of these observations, we have tested the hypothesis that human subcutaneous adipose tissue (SAT) *LMNA* expression can be altered with HAART treatment in HIV-positive patients or with lipodystrophy development. Furthermore, we have analyzed lamin A and C isoform expression and explored their relation to adipose inflammatory environment and systemic insulin resistance in these patients.

METHODS

Participants

Design and Setting

The present work was a cross-sectional case-control study. We have studied a group of HIV-infected patients receiving HAART with and without lipodystrophy and a healthy control group, both recruited at the Hospital Universitari Joan XXIII (Tarragona, Spain).

Participants and Definitions

Fourteen HIV-positive patients receiving HAART without lipodystrophy (HIV⁺HALS⁻) and a group of 25 HIV-positive patients who developed lipodystrophy while on HAART (HIV⁺HALS⁺) were included in this study. These patients were recruited from a cohort of 400 HIV-infected patients receiving stable HAART for at least 1 year. All the HIV-infected patients who receive HAART at our HIV outpatient clinic were followed up at regular scheduled 3-month visits. At each visit, the patients were given a short unstructured questionnaire devoted to assessing the appearance of body changes suggestive of lipodystrophy and a complete physical examination to assess the type (lipoatrophy, lipohypertrophy, or mixed) and degree (slight, moderate, or severe) of Fat Redistribution Syndrome (FRS). Waist and hip diameter and their ratio (WHR), height, weight, bicipital and tricipital fold, and body mass index (BMI) were measured. The presence of FRS was defined by changes in the body fat composition that were important enough to be recognized by the patient and the attending physician(s). Criteria for lipoatrophy were 1 or more of the following: loss of fat from the face, arms, and legs; prominent veins in the arms and legs; and a thin bottom. Criteria for lipohypertrophy were an increase in abdominal perimeter and breast and/or neck fat deposition (1 or both). Mixed lipodystrophy was defined by the presence of at least 1 characteristic of lipoatrophy and 1 characteristic of lipohypertrophy being concomitantly present in 1 patient. FRS was categorized in accordance with the scale proposed by Carr et al¹⁶: nonexistent (0), slight (1), moderate (2), and severe (3). This categorization was evaluated in the face, arms, legs, buttocks, abdomen, neck, and breasts. The sum of the values corresponding to each corporal zone indicated the degree of FRS: none (0), slight (1 to 6), moderate (7 to 12), and severe (13 to 18).¹⁶ Of the 400 individuals prospectively assessed, 284 (71%) had no criteria for lipodystrophy, whereas 116 (29%) had some degree of lipodystrophy: 33 (28%) had slight lipodystrophy, 40 (35%) had moderate lipodystrophy, and 43 (37%) had severe lipodystrophy, according to the scale of Carr et al.¹⁶ Among all these individuals, 14 patients without lipodystrophy and 25

with severe lipodystrophy agreed to participate. In these patients, a CT scan was performed as an objective measurement of body composition. The control group for the HIV-positive cohort was made up of 21 healthy subjects. HIV-infected and healthy controls were of white race and reported that their body weight had been stable for at least 3 months before the study. Exclusion criteria included the following: (1) clinically significant hepatic, neurologic, or other major systemic disease, including malignancy; (2) history of drug or alcohol abuse, defined as >80 g of alcohol intake per day in men and >40 g of alcohol intake per day in women, or serum transaminase activity more than twice the normal upper limit; (3) an elevated serum creatinine concentration; (4) an acute major cardiovascular event in the previous 6 months; (5) acute illnesses and current evidence of acute or chronic inflammatory or infective diseases; and (6) personal or family history suggesting laminopathies. The hospital's ethics committee approved the study, and informed consent was obtained from all participants.

Methods

CT Scan

To assess the distribution of visceral adipose tissue (VAT) and SAT, a single-slice CT scan (ELSCINT CT Helicoidal TWIN scan (Hackensack, NJ) was performed at the level of L4 in the 39 HIV-1-infected patients (25 with FRS and 14 without FRS) in whom *LMNA* mRNA SAT levels were assessed. The volume of adipose tissue was measured in cubic centimeters.

Insulin Sensitivity Study

Insulin sensitivity (S_i) and glucose effectiveness were measured in the HIV-infected patients (n = 39) using the frequently sampled intravenous glucose tolerance test (FSIGTT), as previously described.¹⁷

Analytic Methods

Blood samples were drawn from each subject after overnight fasting. Plasma and serum samples were stored at -80°C until analytic measurements were performed, except for glucose and glycated hemoglobin A1c (HbA_{1c}), which were determined immediately after the blood was drawn.

Serum glucose was measured by the glucose oxidase method with a Hitachi autoanalyzer (Boehringer, Mannheim, Germany). A lipid profile (triglycerides, total cholesterol, and high-density and low-density lipoprotein cholesterol) was measured by usual enzymatic methods.

HbA_{1c} was measured by the high-performance liquid chromatography method (Bio-Rad, Munich, Germany), with a recommended normal range of the assay being 4.1% to 6.5%.

Plasma interleukin-6 (IL-6) levels were measured by the highly sensitive quantitative sandwich-enzyme-immunoassay technique with the Human IL-6 Quantikine HS ELISA Kit (R&D Systems, Minneapolis, MN). The mean of the minimum detectable concentration was 0.039 pg/mL. Intra- and inter-assay coefficients of variation (CVs) were <9.8% and <11.2%, respectively. Plasma high sensitive C reactive protein (hsCRP) was measured by a highly sensitive immunonephelometry kit (Dade Behring, Marburg, Germany). Plasma adiponectin concentrations were measured by radioimmunoassay (Linco

Research, St. Charles, MO). The intra- and interassay CVs were <5%. Serum insulin was measured by radioimmunoassay (Coat-A-Count Insulin; Diagnostic Products Corporation, Los Angeles, CA). The intra-assay CV was 6.6%.

Adipose Tissue Samples

Adipose tissue samples were obtained from subcutaneous abdominal depots by surgical biopsy. All patients had fasted overnight; at the beginning of surgery, 1 to 4 g of SAT was removed from each proband and was immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Total RNA Isolation and Reverse Transcription

Total RNA was extracted from 400 to 500 mg of frozen SAT using the RNeasy Lipid Tissue Midi Kit (Qiagen Science, Valencia, CA) according to the manufacturer's instructions. One microgram of RNA was retrotranscribed to complementary DNA (cDNA) using the Reverse Transcription System (Promega Corporation, Madison, WI) in a final volume of 20 μL .

Real-Time Quantitative Polymerase Chain Reaction

Primers

The following primers were used: 5'ggatgaggatggagatgacc3' and 5'gcagaagagccagaggagat3' for specific lamin A isoform, 5'ggatgaggatggagatgacc3' and 5'cacgggggagggctgggagag3' for specific lamin C isoform, 5'ctatggagttcatgcttgtg3' and 5'gtactgacattatt3' for peroxisome proliferator activated receptor gamma (PPAR γ), 5'gagcactgaaagcatgatcc3' and 5'gctggttatctctcagctcca3' for tumor necrosis factor- α (TNF α), 5'cggtacatcctcgacgg3' and 5'tgatattttcacagc3' for IL-6, and 5'tctgtgctgctgctcatag3' and 5'catgctccttgccacaat3' for monocyte chemoattractant protein-1 (MCP-1). The housekeeping genes used to normalize gene expression were: β -actin 5'ggactcgcagcaagagatgg3' and 5'agcactgtgtggcgctacag3' and cyclophilin A (CYPA) 5'caaatgctggaccaacac3' and 5'gctccacaatattatgctctt3'.

Real-Time Polymerase Chain Reaction

Gene expression analysis was performed on a Light-Cycler Instrument (Roche Diagnostics, Basel, Switzerland), using the SYBR green fluorescence method.

Results Analysis

For each sample, the derived gene quantification was calculated from an external standard curve, created with serial dilutions of a cloned polymerase chain reaction (PCR) fragment from the respective gene, using LightCycler Software version 3.5 (Roche Diagnostics).

Adipose tissue expression relative quantification levels of each gene were normalized using a weighted mean of β -actin and CYPA. Statistical analysis showed no differences in gene expression for these housekeeping genes among the studied groups.

Statistical Analysis

Statistical analysis was performed by using the SPSS/PC+ statistical package (v. 13.0 for Windows; SPSS, Chicago, IL). Data are expressed as mean value \pm SD. Variables that did

not have a Gaussian distribution were logarithmically transformed to perform statistical analysis or were analyzed by nonparametric tests. Differences in concentrations and in clinical or laboratory parameters between groups were compared by using an independent samples *t* test, ANOVA, or a nonparametric test when appropriate. Differences in gender and in retroviral treatment used between studied groups were analyzed by the Pearson/Fisher χ^2 test. The relation between variables was tested using Pearson/Spearman correlation analysis and stepwise multiple linear regression analysis. Statistical significance occurred if the computed 2-tailed probability value was <0.05.

RESULTS

Anthropometric and Biochemical Characteristics of the Study Groups

Clinical characteristics, anthropometric characteristics, CT scan measurements, and analytic characteristics of the subjects studied are shown in Table 1. All HIV-positive groups had received HAART for 51.8 ± 28.7 months, and the duration of antiretroviral combination therapy were comparable in HALS-negative and HALS-positive groups (see Table 1). All patients in HALS-negative and HALS-positive groups were currently receiving nucleoside reverse transcriptase inhibitors (NRTIs). There were no significant differences between HALS groups in the frequency of receiving nonnucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). There were no significant differences between HALS groups in the number of patients who received a specific agent, except for stavudine (see Table 1). Of the NRTI class of antiretroviral agents, lamivudine (33%) was most frequently used in the HIV-positive HALS-negative group and stavudine (26%) and lamivudine (24%) were most frequently used in the HIV-positive HALS-positive group. Of the NNRTIs, efavirenz was most frequently used in both groups (60% and 68%, respectively). The most common PI used was indinavir in both groups (37% and 32%, respectively). There were no significant differences in the length of treatment of each specific agent between groups.

HIV-positive subjects had higher plasma IL-6 levels, and HALS-positive patients showed lower S_1 than the HALS-negative group. Despite the fact that adiponectin levels were not statistically different between the studied cohorts, a positive correlation was observed with S_1 ($R = 0.616$, $P = 0.001$).

Subcutaneous Adipose Tissue Gene Expression

The mRNA expression levels of the different genes studied are shown in Figure 1. Lamin A mRNA levels were significantly lower in HIV-positive patients compared with controls. No differences between HIV-positive patients and controls in lamin C mRNA levels were observed (see Fig. 1). We found no significant differences in lamin A and C mRNA levels between different HAART-based regimens (HAART with PI [$n = 24$] vs. NRTI + NNRTI [$n = 15$]; $P = 0.155$ for lamin A and $P = 0.506$ for lamin C). We also calculated lamin A/C ratio expression (quotient between lamin A and C expression), which is thought to be close to 1 in normal adipose cells

TABLE 1. Characteristics of the Population

	HIV ⁻ (n = 21)	HIV ⁺ HALS ⁻ (n = 14)	HIV ⁺ HALS ⁺ (n = 25)
Age (y)	40.86 ± 11.20	39.79 ± 6.96	43.56 ± 8.97
Gender (women/men)	5/16	3/11	5/20
BMI (kg/m ²)	24.66 ± 2.66	23.44 ± 2.59	24.29 ± 2.79
WHR	0.90 ± 0.09	0.89 ± 0.09	0.95 ± 0.09
SAT (cm ³)†		127.4 ± 60.9	49.9 ± 30.7
VAT (cm ³)†		37.2 ± 26.9	109.5 ± 66
Fasting glucose (mM)	5.13 ± 0.56	5.00 ± 0.40	5.21 ± 0.98
Chol (mM)	5.14 ± 1.22	5.12 ± 0.69	5.58 ± 1.42
HDL-cholesterol (mM)	1.18 ± 0.38	1.31 ± 0.54	1.10 ± 0.33
LDL-cholesterol (mM)	ND	2.96 ± 0.78	3.08 ± 0.97
Triglycerides (mM)	1.88 ± 1.93	2.15 ± 1.27	3.48 ± 2.55
Insulin (μUI/mL)*	3.37 ± 2.00	7.63 ± 3.98	11.79 ± 6.95
S ₁ †	ND	3.36 ± 0.95	2.17 ± 1.58
CD4 count (cells/mm ³)		628.69 ± 331.10	732.80 ± 292.65
HAART exposure, mo		47.36 ± 29.98	54.32 ± 28.29
No. patients receiving antiretroviral agents, n (%)			
NRTI		14 (100)	25 (100)
NNRTI		6 (46)	8 (32)
PI		7 (54)	17 (71)
Plasma IL-6 (ng/L)*	0.94 ± 1.09	2.68 ± 2.35	2.01 ± 1.20
Adiponectin (μg/mL)	14.08 ± 9.46	16.12 ± 10.69	13.36 ± 14.30

*P < 0.001; †P < 0.05.

Chol indicates cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ND, not determined.

(HIV⁻: 1.01 ± 0.39, HIV⁺HALS⁻: 0.71 ± 0.60; HIV⁺HALS⁺: 0.76 ± 0.62; see Fig. 1).

PPARγ mRNA tended to be lower in the HIV-positive patients compared with HIV-negative subjects and in HALS-positive patients compared with HALS-negative patients, respectively, but differences were not statistically significant. HIV-positive subjects had significantly higher IL-6 mRNA and tended to have higher levels of MCP-1 and TNFα compared with HIV-negative controls.

Correlation and Regression Analysis

All HIV subjects (n = 39) were considered in the correlation and regression analysis, and the presence of

lipodystrophy was taken into consideration. In the bivariate correlation analysis, lamin A mRNA expression levels were found to be positively correlated with lamin C isoform (R = 0.382, P = 0.020) and age (R = 0.440, P = 0.005) and negatively correlated with PPARγ (R = -0.479, P = 0.003). The regression analysis showed that age (β = 0.561, P = 0.002; Fig. 2A) and IL-6 mRNA expression levels (β = 0.369, P = 0.030) were determinants of lamin A mRNA levels, with independence of the presence of lipodystrophy (see Fig. 2B). SAT lamin C mRNA expression was correlated positively with lamin A isoform (R = 0.382, P = 0.020) and with MCP-1 and TNFα mRNA (R = 0.431, P = 0.008; R = 0.347, P = 0.038, respectively) and correlated negatively with PPARγ mRNA (R = -0.486, P = 0.002). In the multiple regression analysis, MCP-1 mRNA (β = 0.624, P = 0.001) was the only determinant of lamin C mRNA levels (see Fig. 2C).

DISCUSSION

In this cross-sectional study, we have analyzed abdominal SAT biopsies of HIV-infected patients treated with HAART and we have observed the existence of an altered lamin A/lamin C expression ratio attributable to a decrease in lamin A levels. Interestingly, these findings were not dependent on the presence of lipodystrophy; hence, the lower lamin A expression observed in HIV-positive patients studied here seems to be related to HIV itself or to HAART rather than to the presence of HALS. In HIV-positive patients, we have also found that PPARγ mRNA levels were a positive determinant of LMNA expression and local inflammatory mediators were negative determinants.

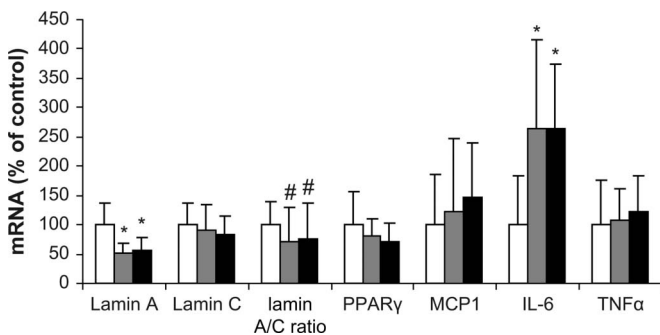


FIGURE 1. Relative quantification of gene expression levels: HIV-negative control subjects (open bars) and HIV-positive patients without (gray bars) or with (black bars) lipodystrophy. (*P < 0.001; ##P < 0.01 [HIV-positive patients compared with controls]).

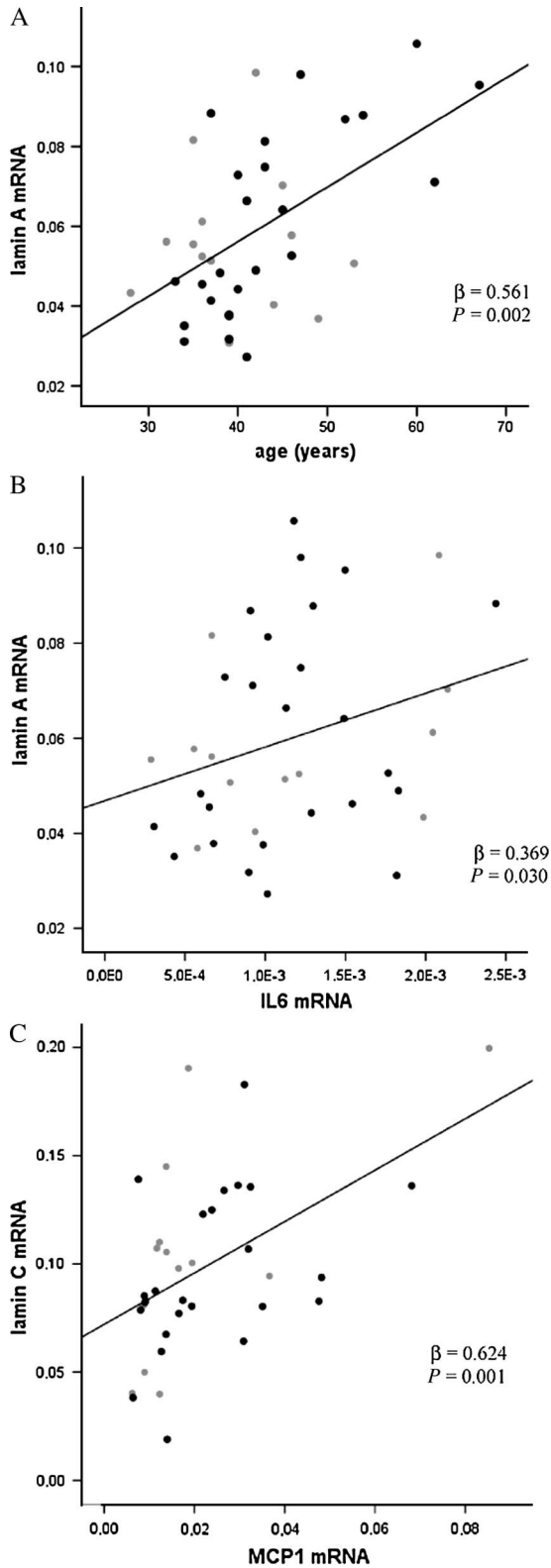


FIGURE 2. Linear correlation, confirmed by regression analysis, in HIV-positive patients. Association between lamin A isoform and age (A) or IL-6 mRNA expression (B) and between lamin C isoform and MCP-1 mRNA (C) for HIV-positive patients without (gray dots) or with (black dots) lipodystrophy.

The downregulation of lamin A in adipose tissue of our HIV cohort is in accordance with previous observations in some laminopathies, in which the prevalent alteration is accounted for by altered lamin A levels without alterations in lamin C expression. The fact that lamin A levels rather than lamin C levels are frequently found altered in diverse pathologic conditions is in line with the downregulated lamin A levels shown in our patients. In vitro studies with mice deficient cells for lamin A with normal levels of lamin C did not display full recovery of nuclear properties (nuclear shape and resistance to deformability), suggesting that lamin A might be important in the assembly of a robust lamin network.¹² In humans, the data about lamin A/C mRNA expression are scarce and reduced to patients affected with lipodystrophy secondary to laminopathies. In this sense, individuals with FPLD showed similar relative amounts of lamin A and C proteins in fibroblasts compared with healthy subjects.¹⁸ In some *LMNA* mutations causing FPLD, a loss of function affecting the nuclear lamina or its interaction with other nuclear proteins has been described,^{19,20} whereas other mutations produce proteins indistinguishable from wild-type proteins.^{21–23}

The lower lamin A levels observed in HAART-treated HIV-infected subjects compared with HIV-negative controls reinforces the idea that altered lamin A levels (or unbalanced lamin A/C ratio) could reflect a pathogenic condition, although we cannot determine whether this was an effect of antiretroviral treatment or attributable to the HIV itself. The analysis of HIV-infected subjects naive to antiretroviral drugs should aid in answering this question.

There is some controversy about *LMNA* genotypic association with insulin resistance, with some authors suggesting susceptibility to insulin resistance for some polymorphisms^{24,25} and description of a mutation causing marked insulin resistance.¹¹ Other authors have found no association with type 2 diabetes or the metabolic syndrome, however.^{26–28} We have observed an absence of correlation between *LMNA* adipose tissue expression and peripheral S_1 measured by minimal model analysis. The close relation between both isoforms and proinflammatory cytokines and the negative association with the adipogenic gene *PPAR γ* make us to speculate about the possible participation of this gene in paracrine mechanisms that contribute to insulin resistance in adipose tissue.

The *LMNA* gene is also implicated in the activation of the nuclear factor- κ B (NF- κ B) pathway,²⁹ which has an important role in HIV transactivation.³⁰ In our study, lamin A and C isoforms were dependent on the adipose tissue expression levels of IL-6 and MCP-1, respectively. This was observed in HIV-positive patients with or without lipodystrophy. HIV infection itself is characterized by the expression of several proinflammatory cytokines. Moreover, several HIV proteins (Nef, Tat, and Vpr) use proinflammatory cytokine signaling pathways, further underlining the potential importance of inflammation in HIV pathogenesis.³¹

PIs have been shown to reduce adipocyte differentiation and increase apoptosis of adipocytes in vitro. Indinavir led to altered retinoic acid signaling, mostly by activation of the RAR/RXR heterodimer.³² Because *LMNA* is regulated by

a retinoic acid–responsive element (L-RARE) in the promoter region,³³ indinavir may impair retinoic acid homeostasis and/or interact by means of the L-RARE within the *LMNA* promoter. It has been suggested that this would result in altered *LMNA* expression and subsequent impaired adipocyte differentiation, lipodystrophic body habitus, and metabolic disturbances in HIV-infected patients receiving HAART.⁵ It is possible that analyzing isolated mature adipocyte cells may help to detect if there is any alteration in *LMNA* transcriptional regulation by retinoic acid in indinavir-treated patients.

In our study, age was an important determinant variable of lamin A mRNA levels. An *LMNA* mutation leading to Hutchinson-Gilford progeria syndrome (HGPS) generates $\Delta 50$ lamin A; a truncated dominant isoform of lamin A.³⁴ Moreover, as in patients who have HGPS, cells from old healthy individuals express the $\Delta 50$ lamin A mRNA and lamin A is aberrantly localized at the nuclear periphery, implicating the *LMNA* gene in physiologic aging.³⁵ In the present study, it is worth mentioning that our selected primers for lamin A quantification do not amplify $\Delta 50$ lamin A. The analysis of the protein localization or the presence of abnormal levels of prelamin A in our cohort could help us to understand the close relation observed between age and lamin expression.

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