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Objective: HIV-infected patients receiving antiretroviral treatment (ART) often present adipose tissue accumulation and/or redistribution. adipose tissue has been shown to be an HIV/SIV reservoir and viral proteins as Tat or Nef can be released by infected immune cells and exert a bystander effect on adipocytes or precursors. Our aim was to demonstrate that SIV/HIV infection *per se* could alter adipose tissue structure and/or function.

Design: Morphological and functional alterations of subcutaneous (SCAT) and visceral adipose tissue (VAT) were studied in SIV-infected macaques and HIV-infected ART-controlled patients. To analyze the effect of Tat or Nef, we used human adipose stem cells (ASCs) issued from healthy donors, and analyzed adipogenesis and extracellular matrix component production using two dimensional (2D) and three-dimensional (3D) culture models.

Methods: Adipocyte size and index of fibrosis were determined on Sirius red-stained adipose tissue samples. Proliferating and adipocyte 2D-differentiating or 3D-differentiating ASCs were treated chronically with Tat or Nef. mRNA, protein expression and secretion were examined by RT-PCR, western-blot and ELISA.

Results: SCAT and VAT from SIV-infected macaques displayed small adipocytes, decreased adipogenesis and severe fibrosis with collagen deposition. SCAT and VAT from HIV-infected ART-controlled patients presented similar alterations. *In vitro*, Tat and/or Nef induced a profibrotic phenotype in undifferentiated ASCs and altered adipogenesis and collagen production in adipocyte-differentiating ASCs.

Conclusion: We demonstrate here a specific role for HIV/SIV infection per se on adipose tissue fibrosis and adipogenesis, probably through the release of viral proteins, which could be involved in adipose tissue dysfunction contributing to cardiometabolic alterations of HIV-infected individuals.

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Introduction

HIV-infected patients receiving current antiretroviral treatment (ART) are generally well controlled for HIV replication. However, some of them present fat accumulation and/or redistribution associated with altered adipose tissue homeostasis, differentiation, and function, which could lead to metabolic and cardiovascular comorbidities [1–5].

The pathophysiological mechanisms involved in adipose tissue dysfunction, in the context of HIV infection, are still poorly understood and probably multifactorial. Many studies have involved some ART molecules in these abnormalities [2,6–8]. However, adverse effects of ART cannot explain all aspects of the phenotype. Importantly, adipose tissue has been shown to be a HIV/simian immunodeficiency virus (SIV) reservoir [9–11], and some recent studies suggested that HIV infection *per se* could play an important role in the pathophysiology of adipose tissue alterations and associated metabolic defects. Accordingly, ART-naive HIV-infected patients can present decreased body fat [12], with sometimes fat redistribution [13], affecting both subcutaneous adipose tissue (SCAT) and visceral adipose tissue (VAT) [14]. VAT accumulation is often associated with metabolic defects, such as liver steatosis, dyslipidemia, insulin resistance and hyperglycemia [14,15]. Within adipose tissue of ART-naive patients, abnormal adipogenic gene expression and mitochondrial damage have been reported [16–19]. However, the in-vivo mechanisms whereby the virus could induce adipocyte defects in humans are still poorly understood.

Recent evidence indicates that adipose tissue inflammation and fibrosis are hallmarks of metabolically challenged adipocytes in adipose tissue disorders, contributing to the development of comorbidities [20]. In obesity, fibrotic and dysfunctional adipose tissue displays excess collagen deposition, mainly collagen 6, in humans and in rodents [21–24]. Adipocytes and adipose precursors are the main cells producing extracellular matrix (ECM) proteins [25] and, by the acquisition of a profibrotic phenotype [26,27], adipocyte precursors are key actors in the onset of fibrosis [20]. Thus, adipose tissue-resident mesenchymal stem cells (ASCs) can turn into myofibroblast-like cells expressing α-smooth muscle actin (αSMA) and transforming growth factor (TGF)-β [28–31], as a result of an unresolved chronic inflammation. Of note, in *LMNA*-linked lipodystrophy, interscapular fat pads display fibrotic changes but no evidence of ongoing inflammatory processes, suggesting that fibrosis and inflammation are not always associated [32,33]. Fibrotic lesions in adipose tissue have been observed in response to ART in people living with HIV [32,34,35], but the impact of HIV infection itself on adipose tissue fibrosis has never been studied.

As adipose tissue is an HIV reservoir [9–11], we hypothesized that HIV-infected immune cells within adipose tissue could release viral proteins, even in the presence of ART, which could exert a direct effect on bystander cells, such as adipocytes or their precursors. In fact, HIV-viral proteins like Vpr, Tat or Nef have been detected in the plasma of HIV-infected individuals receiving ART [36–38]. The pathogenic role of Vpr, which can corepress peroxisome proliferator-activated receptor γ (PPARγ), includes decreased adipose tissue depots mass and increased macrophage infiltration in a mouse transgenic model [38]. *In vitro,* several HIV proteins, including Tat or Nef, can induce inflammation and senescence that could alter adipogenesis and affect mature adipocyte function [36–42]. In addition, Tat and Nef alter osteogenesis in bone-marrow mesenchymal stem cells [40]. In this context, our aim was to characterize the impact of HIV/SIV on adipose tissue morphology and function *in vivo* together with the ability of Tat and Nef to alter ECM production by ASCs and adipocytes.

Methods

Animals, infection and adipose tissue samples

Seven cynomolgus macaques (*Macaca fascicularis*) were infected via the intravenous route with SIVmac251 as previously described [9]. They were not used for any other protocol before euthanasia. Seven non-SIV-infected animals were used as controls. Adult macaques were imported from Mauritius and housed in the animal facilities at the 'Commissariat à l'Energie Atomique et aux Energies Alternatives' (CEA, Fontenay-aux- Roses, France), under the accreditation number D92-032-02. The CEA facilities comply with the Standards for Human Care and Use of Laboratory of the Office for Laboratory Animal Welfare (OLAW, USA) under OLAW assurance number #A5826-01 and with the European Directive (2010/63, recommendation N°9). The study was approved by the 'Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche' (France) and the ethics committee 'Comité d'éthique en expérimentation animale n°44' under reference 2015102713323361.02 (APAFIS#2453). Ketamine chlorhydrate-sedated animals were euthanized by intravenous injection of sodium pentobarbital (Vetoquinol, Paris, France). SCAT and VAT samples were collected at necropsy, at the same location in each animal. Special care was taken to remove any nonadipose-associated tissues from the depot including lymph nodes. Control animals had a mean age of 7.2 ± 1.1 years and weighed 9.3 ± 5.5 kg. SIV-infected macaques had a mean age of 4.4 ± 0.3 years and weighed 5.5 ± 0.6 kg. The mean duration of SIV infection was of 342 ± 31 days, with a mean viral load of $6.8 \times 10^4 \pm 2.9 \times 10^4$ RNA copies/ml and a mean $CD4^+$ cell count of $421 \pm 174/\mu l$.

Adipose tissue samples from human HIV-infected patients and healthy donors

Human SCAT and VAT samples were obtained from HIV-infected and HIV-negative women undergoing bariatric surgery ($n = 4$ per group). Mean age was 37 ± 6.2 years, and 38.5 ± 4 years for HIV-infected and control patients, respectively. BMI was 47.7 ± 2.6 and $45.8 \pm 2.4\,kg/m^2$ in HIV-infected and control patients, respectively. HIV infection duration was of 11.8 ± 5.3 years and the mean $CD4^+$ cell count was $480 \pm 88/\mu l$. None of them had diabetes at the time of the surgery.

Otherwise, human SCAT samples used for adipose-derived mesenchymal stem cell (ASC) isolation were obtained from 14 healthy women undergoing plastic surgery (BMI $< 25\,kg/m^2$) with a mean age of 47.8 ± 3.9 years. All research patients provided informed written consent before their surgery allowing the use of their tissue specimens for research purposes. The protocols followed the Declaration of Helsinki guidelines and were approved by the local ethics committee.

Adipose stem cell isolation, culture, treatment and differentiation

ASCs were isolated using a collagenase digestion as previously described [43], seeded in αMEM (Minimum Eagle's Medium) with 10% fetal bovine serum (FBS), 2 mmol/l glutamine, penicillin/streptomycin (all from Gibco, Invitrogen Corporation, San Diego, California, USA), and 2.5 ng/ml basic fibroblast growth factor (PeproTech, Rocky Hill, New Jersey, USA). Upon confluence, adherent cells were trypsinized and seeded at 10^3 cells/cm^2. During expansion, cells were exposed or not to 40 ng/ml of the recombinant HIV-proteins Tat or Nef (Jena Bioscience, Jena, Germany) for 15 days. Differentiation of ASCs was induced by addition of proadipogenic medium (DMEM, Dulbecco Modified Eagle's Medium) with 10% FBS, 1 μmol/l dexamethasone, 500 μmol/l IBMX, 1 μmol/l rosiglitazone, 1 μmol/l insulin) for 6 days, and then maintained in DMEM (1 μmol/l insulin) for 8 days. Tat or Nef were added throughout the whole differentiation process. Cells were stained for Oil-red-O (Sigma-Aldrich, Saint-Louis, Missouri, USA) as described previously [8]. For the three-dimensional (3D) culture, Puramatrix hydrogel (Corning, Fischer Scientific, Waltham, Massachusetts, USA) was sonicated for 30 min and diluted with 20% sucrose. ASCs were embedded and differentiated in the hydrogel as previously described into round bottom wells of a 96-well plate [25].

Protein extraction and western blotting

Frozen adipose tissues biopsies were lysed with Laemmli Buffer and ground using a Precellys tissue homogenizer with CKmix ceramic beads (Ozyme, Montigny-le-Bretonneux, France). Proteins were extracted from cell monolayers as previously described [6]. Samples were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. Specific proteins were detected using antibodies directed against PPARγ, collagen1-α2, collagen6-α1 (Santa Cruz Biotechnology, Dallas, Texas, USA) and tubulin (Sigma-Aldrich). Immunoreactive complexes were detected by horseradish-peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, Massachusetts, USA) and by enhanced chemiluminescence (Amersham Biosciences GE Healthcare Europe, Velizy-Villacoublay, France).

RNA isolation and quantitative reverse transcriptase-PCR

Total RNA from human and macaque adipose tissue samples were isolated by Qiazol extraction and purification using Qiagen RNeasy mini-columns according to the manufacturer's instructions (Qiagen, Courtaboeuf, France). Total RNA was isolated from cultured cells using RNeasy mini-columns. mRNA expression was analyzed by RT-PCR as described previously [32,44]. The sequence of primers is available upon request.

Adipose tissue histology

Light microscopy and immunohistochemical studies were performed as previously described [32]. Briefly, human and macaque adipose tissue samples were collected and fixed in 4% buffered paraformaldehyde (Sigma Aldrich) for 48 h, embedded in paraffin, and sectioned at 5 μm. Adipose tissue sections were deparaffinized and stained with Sirius red for 1 h to detect collagen fibrils. The adipocyte mean areas and index of fibrosis were determined using a semiautomatic image analysis system as previously described [32] in three randomly chosen regions. Parallel sections were immunostained and quantified for collagen6-α1 expression as previously described [32,44]. The ratio of fibrosis to total adipose tissue surfaces defined the index of fibrosis. Adipocyte size distribution was determined as previously described [32,45].

TGF-β and fibronectin secretion

TGF-β and fibronectin concentrations in cell culture media from ASCs were determined after 15 days of incubation with Tat or Nef, by using Quantikine human ELISA kits for fibronectin or multianalyte cartridge ELLA immunoassay for TGF-β according to the manufacturer's instructions (Bio-Techne, San Jose, California, USA).

All experiments were performed at least three times ($n = 3–8$) on triplicate samples. Data are expressed as means ± SEM. Statistical significance between SIV-infected and noninfected macaques, or between HIV-infected and noninfected patients, was determined with nonparametric Mann–Whitney *U*-test. Relationships between index of fibrosis and adipocyte size information in VAT and SCAT were visualized by scatter plots and assessed by Pearson correlation analysis. Statistical significance between HIV-protein-treated vs. control

cells was determined with parametric (*t* test) or nonparametric tests, as appropriate.

Results

Simian immunodeficiency virus infection alters adipocyte size and induces fibrosis in macaque adipose tissue

SIV infection in macaques, which closely recapitulates the major features of HIV infection [46], allows to assess the impact of the virus on abdominal SCAT and VAT, two depots that accumulate during HIV-linked fat redistribution [2]. We first determined the impact of SIV infection on adipose tissue architecture. The repartition of adipocytes was overall homogeneous in both SCAT and VAT of control macaques with adipocytes of different sizes. In contrast, adipose tissue from SIV-infected macaques had a heterogeneous repartition with clusters of small and of medium-sized adipocytes together with fibrotic bundles (Fig. 1 a). In addition, the percentage of large adipocytes was higher in the control than in the SIV-infected group (Fig. 1b). Accordingly, the mean size of adipocytes measured in adipose tissue of control macaques was higher than that of SIV-infected animals (in SCAT, 3811 ± 1230 vs. $1866 \pm 142\,\mu m^2$ for control and SIV-infected groups, respectively; in VAT, 3505 ± 1036 vs. $1955 \pm 156\,\mu m^2$ for control and SIV-infected groups, respectively). We evaluated the level of expression of the main adipogenic transcription factor, PPARγ. We found that its expression was decreased, at the mRNA and protein level, in SIV-infected macaques as compared with controls (Fig. 1c and d). Finally, regarding ECM remodeling, marked collagen deposition was present in both depots of SIV-infected macaques with presence of fibrotic bundles and/or fibrotic thickening of fat lobules as shown by Sirius red staining and a higher index of fibrosis (3.3 and 9.9% area of fibrosis in SCAT for control and SIV-infected group, respectively; 3.2 and 8.8% area of fibrosis in VAT, for control and SIV-infected animals, respectively; Fig. 1a and e). Accordingly, an increased mRNA expression of type 1-α2 and 6-α1 collagens was observed in SCAT and to a lesser extent in VAT from infected-macaques, in association with an increased expression of fibronectin and TGF-β (Fig. 1f). We searched for relationships between adipocyte mean size and the fibrosis index in SIV-infected and control macaques. We observed a negative correlation in SCAT ($P = 0.03$, $R = -0.72$) but not VAT ($P = 0.11$, $R = -0.57$).

HIV infection alters adipocyte size and induces fibrosis in human adipose tissue

We also examined samples from HIV-infected patients. In agreement with the observations made in SIV-infected macaques, we observed a marked fibrosis in both SCAT and VAT of obese HIV-infected patients compared to sex-paired, age-paired and BMI-paired noninfected obese individuals (Fig. 2 a). First, fat architecture of HIV-infected human samples was altered, with a higher number of smaller adipocytes (Fig. 2b) and increased fibrosis characterized by compact and thick fibrils of collagen invading the intercellular area. By contrast, in noninfected patients, collagen staining was thinner and mainly located around adipocytes (Fig. 2a and c; i.e. 2.4-fold increased fibrosis in SCAT and 2.0-fold increase in VAT of HIV-infected vs. control patients, respectively). Second, we also observed a 6.4-fold induction in collagen 6-α1 expression in SCAT but not VAT of HIV-infected patients compared with control samples, as assessed using immunostaining (Fig. 2d and e). Considered together, these data suggest that HIV/SIV infection results in a marked fibrosis in adipose tissue in both depots.

HIV-proteins Tat and Nef alter extracellular matrix component production in proliferating adipose stem cells *in vitro*

To investigate the mechanisms whereby SIV/HIV infection induced fibrosis in adipose tissue and to identify the cellular and viral actors responsible for ECM remodeling, we studied the impact of two HIV proteins, either on proliferating adipocyte precursors or when these cells were differentiated into adipocytes, by using human ASCs from lean healthy donors. Proliferating ASCs, chronically treated for 15 days with recombinant HIV proteins Tat or Nef, displayed a marked increase in the mRNA and protein levels of collagen 1-α2 (Fig. 3a) and collagen 6-α1 (Fig. 3b). Both HIV-proteins induced a profibrotic phenotype in proliferating ASCs, as shown by the increased secretion of fibronectin and TGF-β1 (Fig. 3c and d) and expression of the myofibroblast marker, αSMA (*ACTA2*) (Fig. 3e). These results strongly suggest that HIV/SIV could induce fibrosis in adipose tissue through the release of Tat and Nef and interaction with ASCs.

Nef alters adipogenesis and induces extracellular matrix component production in differentiating adipocytes

We differentiated ASCs into adipocytes for 15 days in 2D culture conditions, in order to search whether HIV proteins could also impact ECM component production during the course of adipogenesis. First, Nef, but not Tat, decreased cellular lipid accumulation (Oil-red-O staining; Fig. 4a and b) and the protein and/or mRNA expression of the adipogenic markers *PPARG* and *FABP4* (Fig. 4c and d). These results were in accordance with our in-vivo data, showing a decreased adipocyte size and PPARγ expression in SIV-infected macaques. However, in the 2D-model, Nef or Tat had no significant effect on collagen production (Fig. 4e). In order to unmask an effect of HIV-proteins on ECM composition, we used a 3D system, where ASCs were embedded and differentiated into a peptide hydrogel (Puramatrix). The

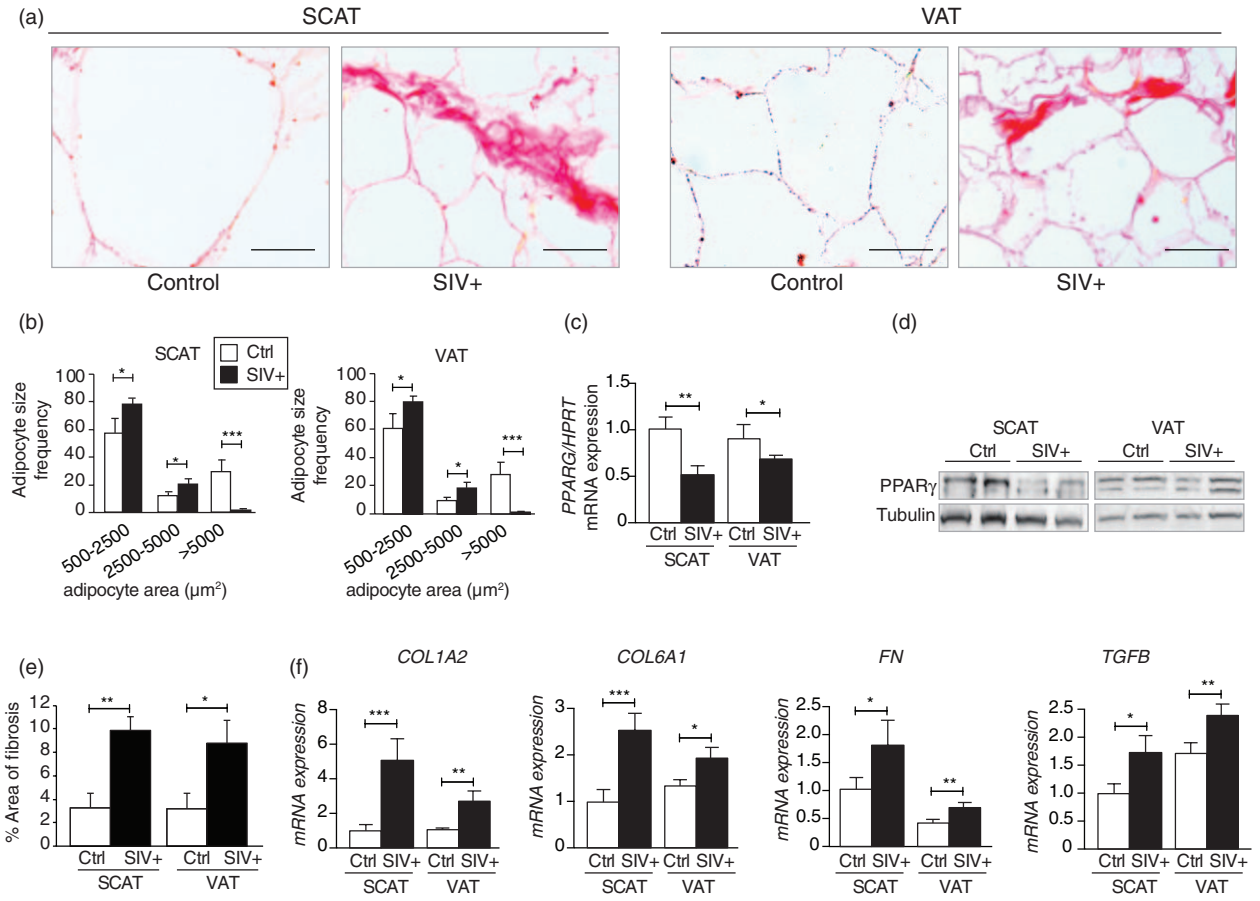

Fig. 1. Subcutaneous and visceral adipose tissue from simian immunodeficiency virus-infected cynomolgus macaques shows reduced adipocyte size and increased fibrosis. (a) Light microscopy analysis of adipose tissue depots, stained with Sirius red to detect collagen fibers. Representative photographs are shown (magnification X40) (scale bar, 50 µm). (b) Frequency distribution of adipocyte size in SCAT and VAT depots in control and SIV-infected macaques. (c) *PPARG* mRNA levels were measured using real-time PCR. The relative mRNA expression levels were normalized to *HPRT* mRNA. (d) PPARγ and tubulin (loading control) protein levels were measured. Whole-adipose tissue lysates were extracted from fat depots and analyzed by immunoblotting. Representative immunoblots are shown. (e) Index of fibrosis in VAT and SCAT measured as described in 'Material and methods' section. (f) *COL1A2*, *COL6A1*, *FN* and *TGFB* mRNA levels were measured using real-time PCR. The relative mRNA expression levels were normalized to *HPRT* mRNA. All results were obtained from triplicate measurements and are expressed as mean ± SEM for control and SIV-infected group ($n = 4–7$ animals per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control animals. SIV, simian immunodeficiency virus.

effect of HIV proteins on adipogenesis was similar to that observed in the 2D culture system, as shown by Oil-red-O staining and PPARγ protein expression (Fig. 5a and b). Moreover, Nef induced collagen 1-α2 expression and both HIV-proteins induced collagen 6-α1 expression (Fig. 5c).

Altogether, these data demonstrate that HIV proteins can have a direct impact on adipogenesis and ECM component production, in particular when differentiating adipocytes are cultured into a 3D more physiological system, thus recapitulating the observations made in SCAT from SIV-infected macaques and HIV-infected individuals. They also demonstrate that both precursor cells and adipocytes can participate to the onset of fibrosis within adipose tissue.

Discussion

We show here that the morphological and functional analysis of SCAT and VAT from SIV-infected macaques and HIV-infected individuals revealed fibrosis, with increased deposition of collagens, together with a decreased adipocyte size. To go further, we addressed the specific relationship between ASCs, adipocytes and HIV/SIV infection. We show here, for the first time, that HIV proteins, Tat and Nef, promoted the acquisition of a profibrotic phenotype of human ASCs and to a lesser extent of adipocytes.

HIV-infected patients receiving current ART, and well controlled for HIV replication, often present trunk fat accumulation associated with metabolic and

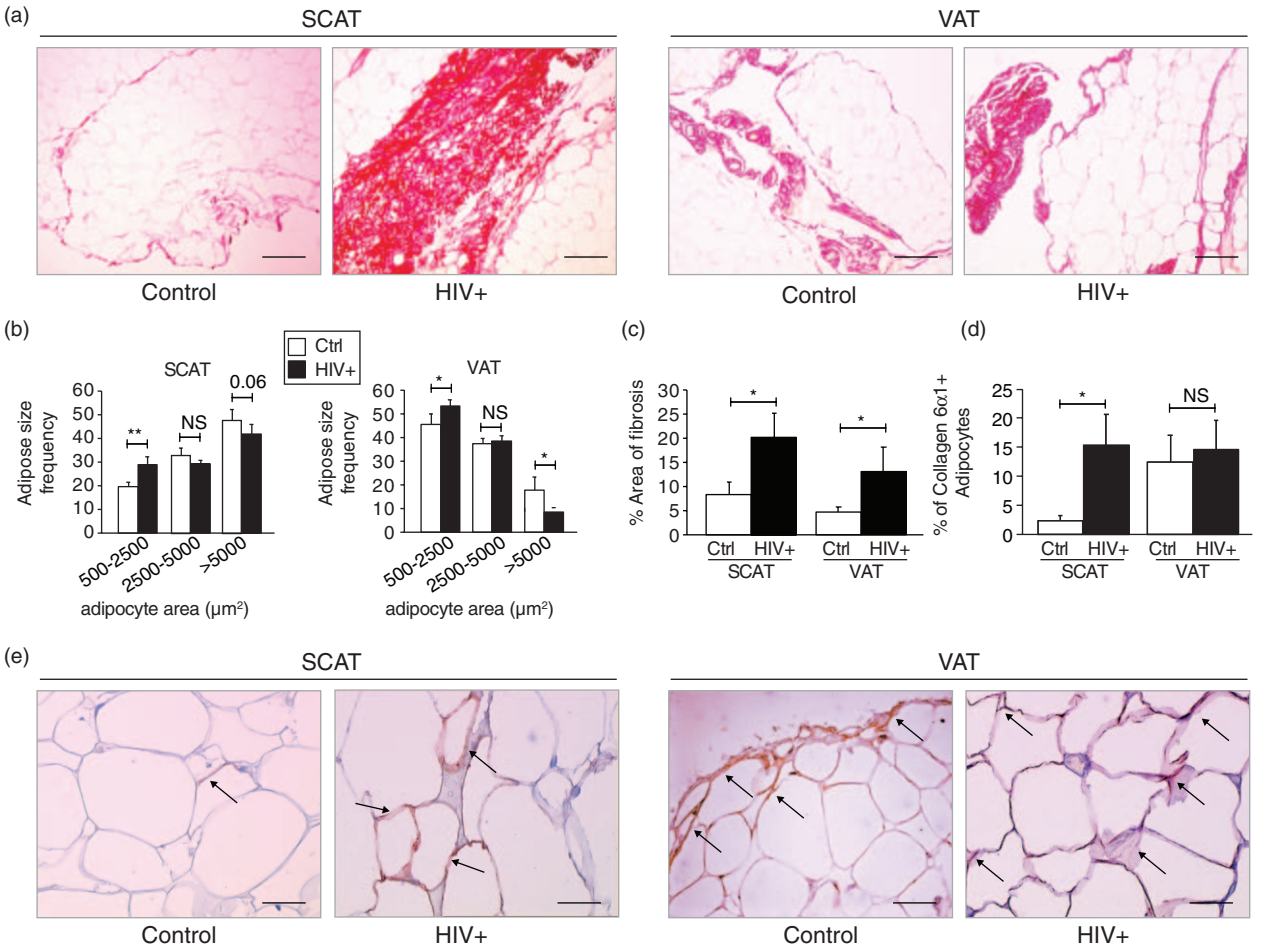

Fig. 2. Subcutaneous and visceral adipose tissue from HIV-infected obese patients shows reduced adipocyte size and increased fibrosis. (a) Light microscopy analysis of adipose tissue depots, stained with Sirius red to detect collagen fibers. Representative photographs are shown (magnification ×10) (scale bar, 100 µm). (b) Frequency distribution of adipocyte size in SCAT and VAT depots in control and HIV-infected treated patients. (c) Index of fibrosis measured in VAT and SCAT as described in the 'Material and methods' section. (d) Quantification and (e) representative photographs of collagen 6α1 immunostaining (magnification ×40; scale bar, 25 µm). Arrows indicate Collagen 6α1 positive cells. All results were obtained from triplicate measurements and are expressed as mean ± SEM for control and HIV-infected group ($n = 4$ individuals per group). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. non-HIV-infected obese individuals. NS, nonsignificant; SCAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

cardiovascular comorbidities [1]. Nonetheless, adverse effects of ART cannot explain all these metabolic alterations, and growing evidence suggest that HIV-infection per se could play a role. Importantly, adipose tissue has been shown to be an HIV/SIV reservoir, the virus being located in immune cells [9,10], suggesting that within adipose tissue, infected cells could release viral proteins, such as Tat and Nef [37,47], which in turn could represent key effectors of adipose tissue remodeling.

We observed that fibrosis was up to three-fold higher in SCAT and VAT from SIV-infected animals when compared with uninfected macaques [22]. Indeed, SIV infection was associated with fibrotic bundles and increased collagen 1 expression in both depots. These results were also confirmed in adipose tissue from obese HIV-infected patients treated with ART. In SIV-infected macaques, we observed an increased expression of collagen 6, fibronectin and of the profibrotic factor TGF-β in SCAT and to a lesser extent in VAT. These results were also confirmed in adipose tissues from obese HIV-infected patients treated with ART. An increased level of collagen 6 is often associated with pericellular fibrosis, whereas collagen 1 and collagen 3 are usually found in bundles of fibrosis [48]. Our results, regarding increased collagen 6 mainly in SCAT, may highlight different types of fibrosis in VAT or SCAT in both humans and macaques. The differences regarding collagen 6 in VAT between macaques and patients could result from species or sex differences and/or from the metabolic state and could also be related partly to ART in HIV-infected patients. Nonetheless, both our in-vitro results and the presence of an adipose tissue fibrosis in untreated

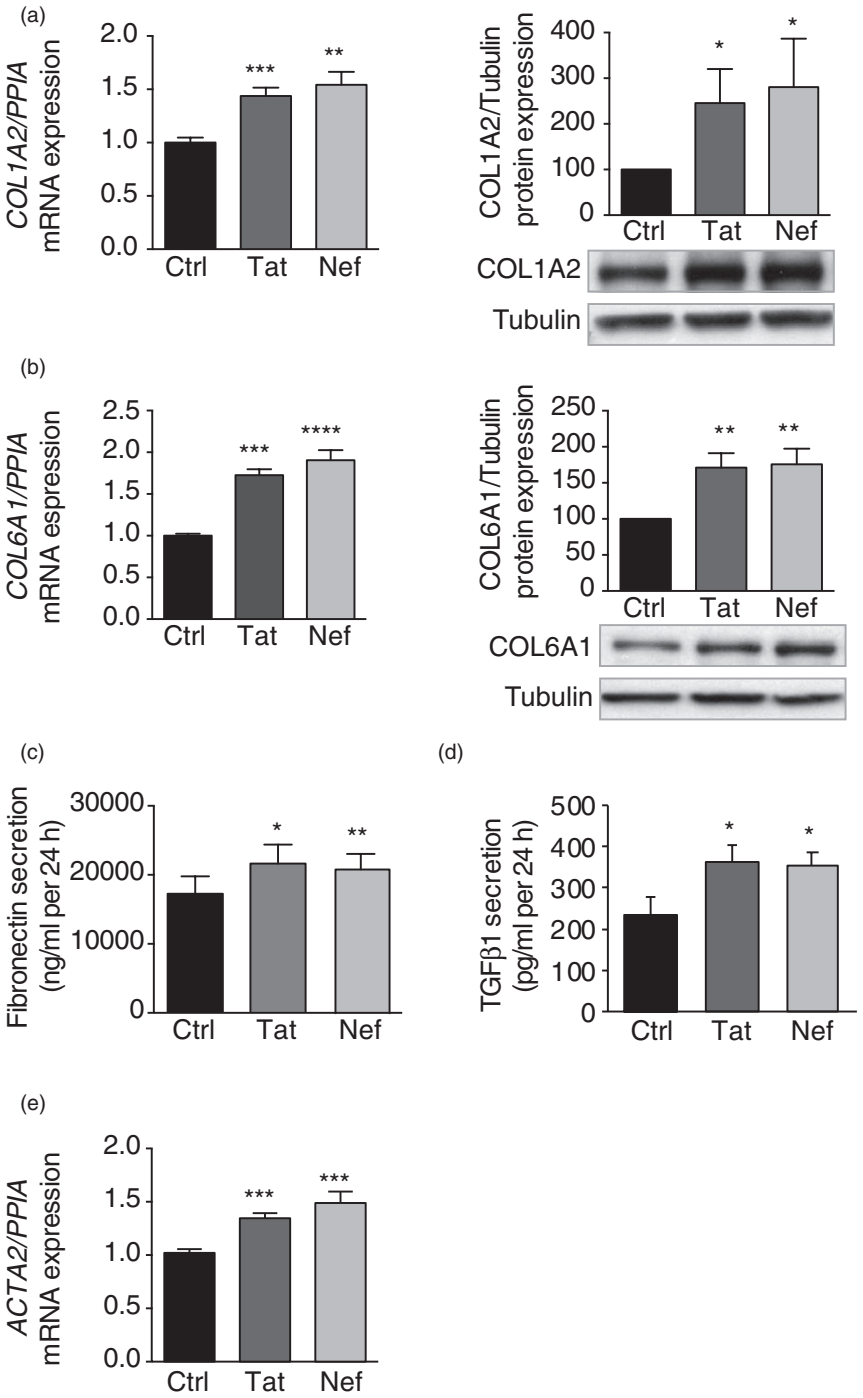

Fig. 3. Long-term exposure to Tat or Nef of proliferating adipose stem cells increases extracellular matrix component production. ASCs were maintained in a proliferating and undifferentiated state for 15 days. Total mRNA and whole-cell lysates were extracted from ASCs, treated or not with HIV-proteins Tat or Nef, and analyzed by RT-PCR and immunoblotting, respectively. (a) *COL1A2* and (b) *COL6A1* mRNA levels normalized to *PPIA* mRNA and representative immunoblots and quantification normalized to tubulin (loading control) are shown. Fibronectin (c) and TGFβ1 (d) levels in the cell culture media were determined with ELISA. (e) *ACTA2* mRNA levels normalized to *PPIA* mRNA. Results are expressed as ng/ml per 24 h for fibronectin and as pg/ml per 24 h for TGFβ1. Results are presented as mean ± SEM. All experiments were performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control cells. ASCs, adipose stem cells.

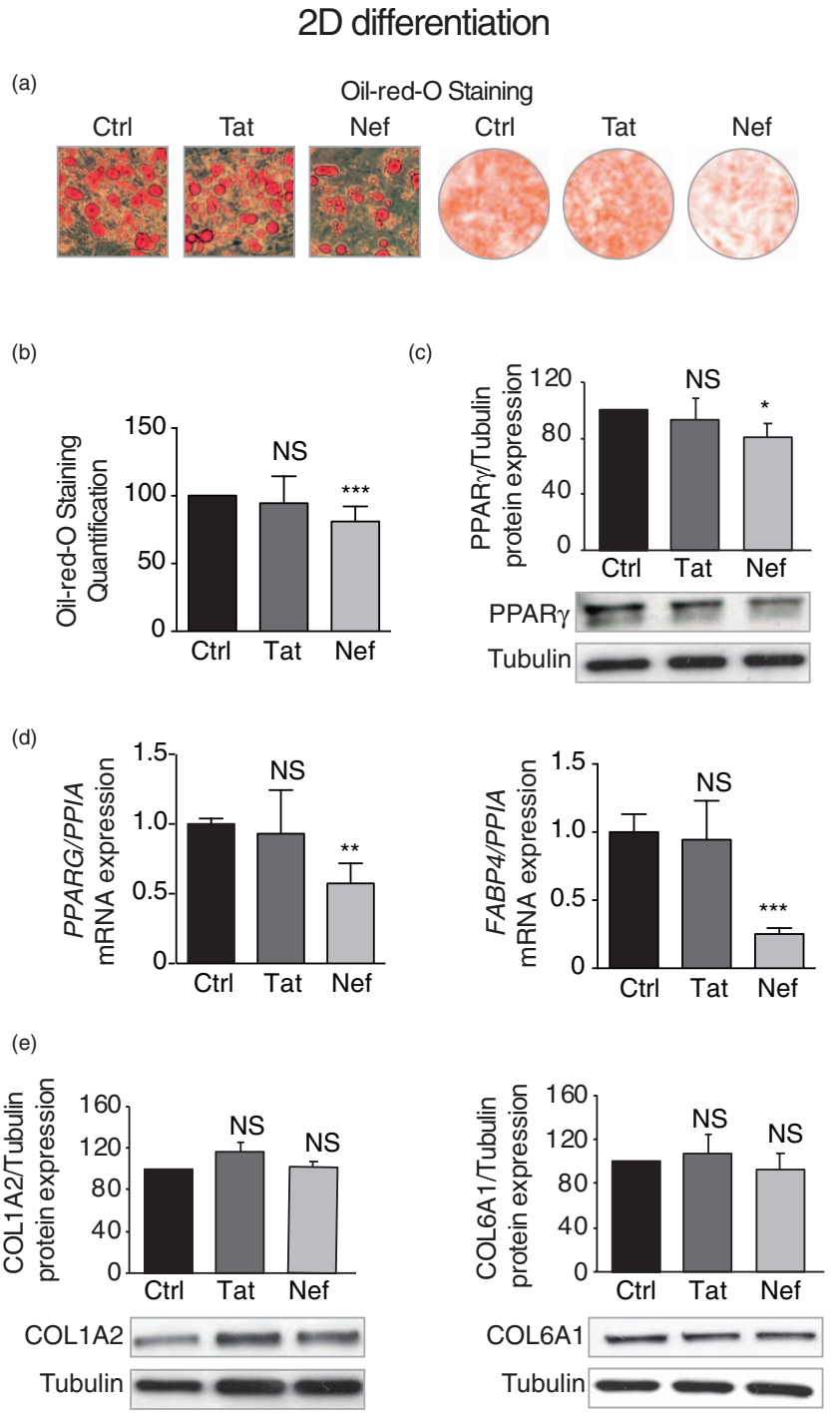

Fig. 4. Effect of a chronic treatment with Tat or Nef on adipogenesis and extracellular matrix component production of adipocyte-differentiating adipose stem cells grown in 2D culture. ASCs were differentiated into adipocytes in the presence of Tat or Nef. To evaluate the adipogenic potential of ASCs, cells were stained with Oil-Red-O 14 days postinduction of differentiation. (a) Representative micrographs of cells (left, magnification ×10) and scans of culture dishes (right) are shown. (b) quantification of Oil-Red-O staining expressed as percent of control cells. Whole-cell lysates were extracted at day 14 postinduction of differentiation from ASCs and analyzed by immunoblotting for PPARγ (c) and tubulin (loading control) protein expression. Representative immunoblots are shown. (d) *PPARG* and *FABP4* mRNA levels measured using real time RT-PCR. The relative mRNA expression levels were normalized to *PPIA*. (e) Representative immunoblots of Collagen 1A2, Collagen 6A1 and tubulin (loading control) are shown. Results are presented as mean ± SEM. All experiments were performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control cells. ASCs, adipose stem cells; NS, nonsignificant.

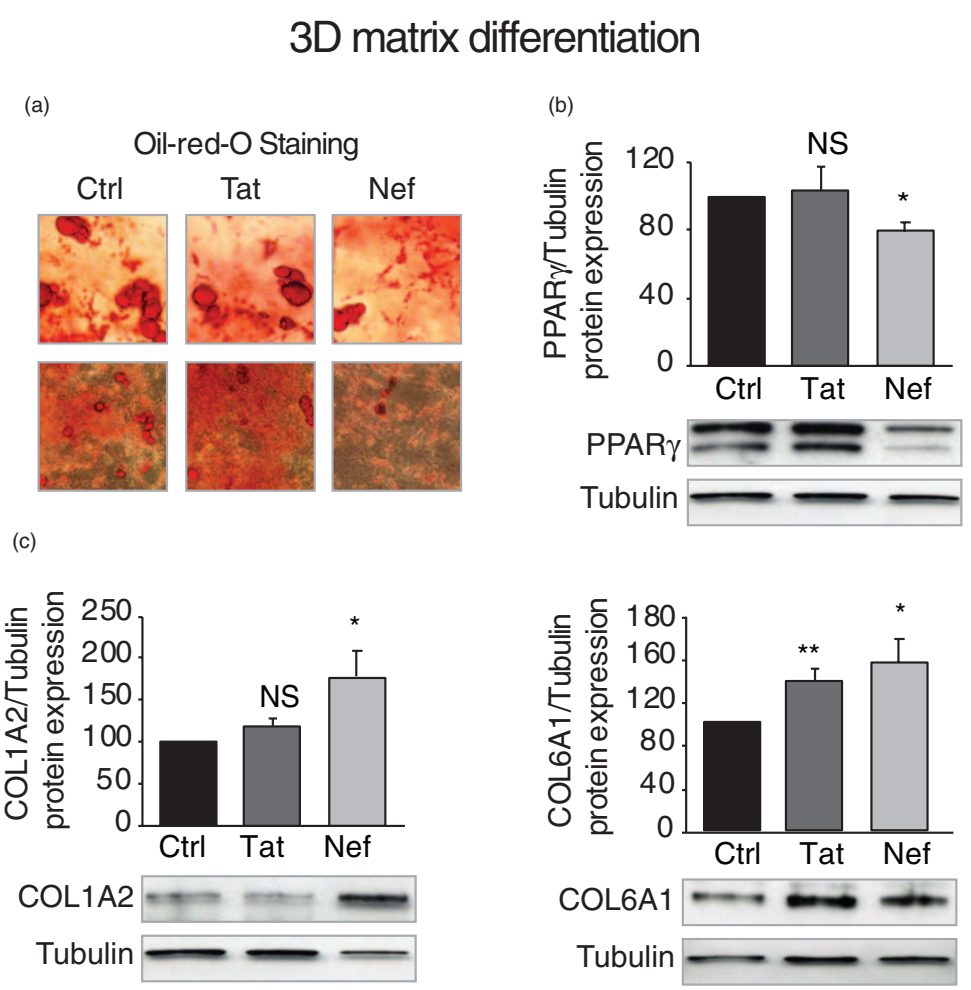

Fig. 5. Chronic treatment with Tat or Nef alters adipogenesis and extracellular matrix component production of adipocyte-differentiating adipose stem cells grown in 3D culture. Adipogenesis and ECM remodeling of ASCs differentiated for 15 days in the 3D culture model using puramatrix were evaluated. (a) Representative micrographs of Oil-Red-stained adipocytes (top, magnification ×40 and bottom: magnification ×10). (b) Representative immunoblots of PPARγ (c) Collagen 1A2, Collagen 6A1and tubulin (loading control) are shown. Whole-cell lysates were extracted at day 15 postinduction of differentiation and analyzed by immunoblotting. Results are presented as mean ± SEM. All experiments were performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, vs. control cells. ASCs, adipose stem cells; ECM, extracellular matrix; NS, nonsignificant.

SIV-infected macaques indicate a key role for SIV/HIV infection in collagen deposition and fibrosis.

Adipose precursors and other stroma–vascular cells of adipose tissue have been implicated in the onset of fibrosis [23–24]. Here, we showed that, in proliferating ASC, Tat and Nef altered ECM component production as shown by increased pro-fibrotic markers (TGF-β, fibronectin, and α-SMA). These results are in accordance with a myofibroblast-like phenotype of ASCs [27,29,30]. Similar patterns were observed in mesenchymal precursors in the case of muscle fibrosis [49]. This phenotype could result from interactions between ASCs and macrophages, the latter being a major regulator of fibrosis [20,27,31,50]. Furthermore, the impact of Tat and Nef in 3D-differentiated ASC are modest and suggest that mainly adipose precursor, and to a lesser extent adipocyte, participate to the onset of HIV-induced fibrosis. Of note, it would be interesting to study the impact on ECM in adipose tissue of other secreted HIV proteins as Vpr. It should be noted that ASCs were isolated from SCAT in accordance with HIV/SIV-induced collagen 6 production in SCAT rather than VAT.

In obese individuals, fibrosis negatively correlates with adipocyte size and has been suggested to limit adipocyte plasticity, therefore, contributing to the onset of metabolic alterations [20]. Accordingly, in our study, HIV/SIV infection was associated with smaller adipocytes in both SCAT and VAT and adipocyte mean size was negatively related to the size of fibrosis in SCAT of SIV-infected and control macaques. As previously described by the group of Villarroya [51], we observed a dramatic decrease in the adipogenic effector PPARγ suggesting

that HIV infection itself could contribute to adipose tissue failure to expand, leading to fat redistribution. In line with this observation, we found that Nef can alter adipogenesis of ASCs as shown by reduced lipid accumulation and expression of adipogenic markers in both 2D and 3D models of differentiation. Nef has been shown to interact with PPARγ in hematopoietic progenitors, suggesting a possible mechanism whereby Nef alters adipogenesis [52]. Moreover, fibrosis could also have a negative effect on adipogenesis, through the action of the profibrotic factor TGF-β [53]. Hypoxia can also participate to the onset of fibrosis and altered adipocyte differentiation and represents a possible pathway, which remains to be investigated [54]. Finally, adipose tissue inflammation can lead to excessive synthesis of ECM proteins. HIV infection is associated with low-grade inflammation in adipose tissue [19], characterized by immune cells recruitment [55,56], which can directly participate to the onset of fibrosis in SCAT and VAT [27,50]. However, several studies on chronically SIV-infected macaques showed no increased inflammatory cytokine expression in adipose tissue [9,11] supporting the idea that HIV infection can be directly responsible for fibrosis in adipose tissue.

We acknowledge that our study has some limitations. First, our in-vivo data provide association and not causality. We have evaluated Tat and Nef but not the other HIV proteins that could also induce fibrosis. Several reasons could potentially explain the differences between human and macaques regarding collagen 6, including differences in species, sex (female patients vs. male macaques), metabolic state (obese vs. lean) and/or ART. Otherwise, results obtained in human adipose tissue samples were limited to women presenting morbid obesity and undergoing bariatric surgery and can neither be directly extrapolated to all people living with HIV, nor to men. The number of patients was low, nonetheless, obese HIV-infected patients were paired for sex, age and BMI to noninfected obese individuals, and all samples were withdrawn by surgical biopsies at similar locations. As well, a similar necropsy procedure was performed for adipose tissue samples from macaques.

Altogether, these results allow a better understanding of adipose tissue alteration in SIV-infected macaques and HIV-infected patients. We show here for the first time that HIV/SIV infection per se is associated with adipose tissue fibrosis and dysfunction that could, in turn, participate to the onset of cardiovascular and metabolic disorders commonly observed in ART-controlled HIV-infected patients.

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Conflicts of interest

There are no conflicts of interest.

References

1. Lake JE, Stanley TL, Apovian CM, Bhasin S, Brown TT, Capeau J, *et al*. **Practical review of recognition and management of obesity and lipohypertrophy in human immunodeficiency virus infection.** *Clin Infect Dis* 2017; **64**:1422–1429.
2. McComsey GA, Moser C, Currier J, Ribaudo HJ, Paczuski P, Dube MP, *et al*. **Body composition changes after initiation of raltegravir or protease inhibitors: ACTG A5260s.** *Clin Infect Dis* 2016; **62**:853–862.
3. Beraldo RA, Meliscki GC, Silva BR, Navarro AM, Bollela VR, Schmidt A, *et al*. **Anthropometric measures of central adiposity are highly concordant with predictors of cardiovascular disease risk in HIV patients.** *Am J Clin Nutr* 2018; **107**:883–893.
4. Bakal DR, Coelho LE, Luz PM, Clark JL, De Boni RB, Cardoso SW, *et al*. **Obesity following ART initiation is common and influenced by both traditional and HIV/ART-specific risk factors.** *J Antimicrob Chemother* 2018; **73**:2177–2185.
5. Srinivasa S, Fitch KV, Torriani M, Zanni MV, Defilippi C, Christenson R, *et al*. **Relationship of visceral and subcutaneous adipose depots to markers of arterial injury and inflammation among individuals with HIV.** *AIDS* 2018; **33**:229–236.
6. Caron M, Auclairt M, Vissian A, Vigouroux C, Capeau J. **Contribution of mitochondrial dysfunction and oxidative stress to cellular premature senescence induced by antiretroviral thymidine analogues.** *Antivir Ther* 2008; **13**:27–38.
7. Lagathu C, Kim M, Maachi M, Vigouroux C, Cervera P, Capeau J, *et al*. **HIV antiretroviral treatment alters adipokine expression and insulin sensitivity of adipose tissue in vitro and in vivo.** *Biochimie* 2005; **87**:65–71.

8. Hernandez-Vallejo SJ, Beaupere C, Larghero J, Capeau J, Lagathu C. **HIV protease inhibitors induce senescence and alter osteoblastic potential of human bone marrow mesenchymal stem cells: beneficial effect of pravastatin.** *Aging Cell* 2013; **12**:955–965.
9. Damouche A, Lazure T, Avettand-Fenoel V, Huot N, Dejucq-Rainsford N, Satie AP, *et al*. **Adipose tissue is a neglected viral reservoir and an inflammatory site during chronic HIV and SIV infection.** *PLoS Pathog* 2015; **11**:e1005153.
10. Couturier J, Suliburk JW, Brown JM, Luke DJ, Agarwal N, Yu X, *et al*. **Human adipose tissue as a reservoir for memory CD4+ T cells and HIV.** *AIDS* 2015; **29**:667–674.
11. Couturier J, Agarwal N, Nehete PN, Baze WB, Barry MA, Jagannadha Sastry K, *et al*. **Infectious SIV resides in adipose tissue and induces metabolic defects in chronically infected rhesus macaques.** *Retrovirology* 2016; **13**:30.
12. Visnegarwala F, Raghavan SS, Mullin CM, Bartsch G, Wang J, Kotler D, *et al*. **Sex differences in the associations of HIV disease characteristics and body composition in antiretroviral-naive persons.** *Am J Clin Nutr* 2005; **82**:850–856.
13. Madge S, Kinloch-de-Loes S, Mercey D, Johnson MA, Weller IV. **Lipodystrophy in patients naive to HIV protease inhibitors.** *AIDS* 1999; **13**:735–737.
14. Agarwal N, Balasubramanyam A. **Viral mechanisms of adipose dysfunction: lessons from HIV-1 Vpr.** *Adipocyte* 2015; **4**:55–59.
15. Balasubramanyam A, Sekhar RV, Jahoor F, Jones PH, Pownall HJ. **Pathophysiology of dyslipidemia and increased cardiovascular risk in HIV lipodystrophy: a model of 'systemic steatosis'.** *Curr Opin Lipidol* 2004; **15**:59–67.
16. Garrabou G, Lopez S, Moren C, Martinez E, Fontdevila J, Cardellach F, *et al*. **Mitochondrial damage in adipose tissue of untreated HIV-infected patients.** *AIDS* 2011; **25**:165–170.
17. Rozzi SJ, Avdoshina V, Fields JA, Trejo M, Ton HT, Ahern GP, *et al*. **Human immunodeficiency virus promotes mitochondrial toxicity.** *Neurotox Res* 2017; **32**:723–733.
18. Gallego-Escuredo JM, Villarroya J, Domingo P, Targarona EM, Alegre M, Domingo JC, *et al*. **Differentially altered molecular signature of visceral adipose tissue in HIV-1-associated lipodystrophy.** *J Acquir Immune Defic Syndr* 19992013; **64**:142–148.
19. Vidal F, Domingo P, Villarroya F, Giralt M, Lopez-Dupla M, Gutierrez M, *et al*. **Adipogenic/lipid, inflammatory, and mitochondrial parameters in subcutaneous adipose tissue of untreated HIV-1-infected long-term nonprogressors: significant alterations despite low viral burden.** *J Acquir Immune Defic Syndr* 2012; **61**:131–137.
20. Sun K, Tordjman J, Clement K, Scherer PE. **Fibrosis and adipose tissue dysfunction.** *Cell Metab* 2013; **18**:470–477.
21. Henegar C, Tordjman J, Achard V, Lacasa D, Cremer I, Guerre-Millo M, *et al*. **Adipose tissue transcriptomic signature highlights the pathological relevance of extracellular matrix in human obesity.** *Genome Biol* 2008; **9**:R14.
22. Divoux A, Tordjman J, Lacasa D, Veyrie N, Hugol D, Aissat A, *et al*. **Fibrosis in human adipose tissue: composition, distribution, and link with lipid metabolism and fat mass loss.** *Diabetes* 2010; **59**:2817–2825.
23. Pasarica M, Gowronska-Kozak B, Burk D, Remedios I, Hymel D, Gimble J, *et al*. **Adipose tissue collagen VI in obesity.** *J Clin Endocrinol Metab* 2009; **94**:5155–5162.
24. Khan T, Muise ES, Iyengar P, Wang ZV, Chandalia M, Abate N, *et al*. **Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI.** *Mol Cell Biol* 2009; **29**:1575–1591.
25. Pellegrinelli V, Heuvingh J, du Roure O, Rouault C, Devulder A, Klein C, *et al*. **Human adipocyte function is impacted by mechanical cues.** *J Pathol* 2014; **233**:183–195.
26. Lacasa D, Taleb S, Keophiphath M, Miranville A, Clement K. **Macrophage-secreted factors impair human adipogenesis: involvement of proinflammatory state in preadipocytes.** *Endocrinology* 2007; **148**:868–877.
27. Bourlier V, Sengenes C, Zakaroff-Girard A, Decaunes P, Wdziekonski B, Galitzky J, *et al*. **TGFbeta family members are key mediators in the induction of myofibroblast phenotype of human adipose tissue progenitor cells by macrophages.** *PLoS One* 2012; **7**:e31274.
28. Marcelin G, Ferreira A, Liu Y, Atlan M, Aron-Wisnewsky J, Pelloux V, *et al*. **A PDGFRalpha-mediated switch toward CD9 (high) adipocyte progenitors controls obesity-induced adipose tissue fibrosis.** *Cell Metab* 2017; **25**:673–685.
29. Desai VD, Hsia HC, Schwarzbauer JE. **Reversible modulation of myofibroblast differentiation in adipose-derived mesenchymal stem cells.** *PLoS One* 2014; **9**:e86865.
30. Diaz-Flores L, Gutierrez R, Lizartza K, Gomez MG, Garcia Mdel P, Saez FJ, *et al*., Anat Rec (Hoboken). **Behavior of in situ human native adipose tissue CD34+ stromal/progenitor cells during different stages of repair. Tissue-resident CD34+ stromal cells as a source of myofibroblasts.** *Anatomical record* 2015; **298**:917–930.
31. Wynn TA. **Cellular and molecular mechanisms of fibrosis.** *J Pathol* 2008; **214**:199–210.
32. Bereziat V, Cervera P, Le Dour C, Verpont MC, Dumont S, Vantyghem MC, *et al*., Lipodystrophy Study Group. **LMNA mutations induce a non-inflammatory fibrosis and a brown fat-like dystrophy of enlarged cervical adipose tissue.** *Am J Pathol* 2011; **179**:2443–2453.
33. Le Dour C, Wu W, Bereziat V, Capeau J, Vigouroux C, Worman HJ. **Extracellular matrix remodeling and transforming growth factor-beta signaling abnormalities induced by lamin A/C variants that cause lipodystrophy.** *J Lipid Res* 2017; **58**:151–163.
34. Bastard JP, Caron M, Vidal H, Jan V, Auclair M, Vigouroux C, *et al*. **Association between altered expression of adipogenic factor SREBP1 in lipoatrophic adipose tissue from HIV-1-infected patients and abnormal adipocyte differentiation and insulin resistance.** *Lancet* 2002; **359**:1026–1031.
35. Utay NS, Kitch DW, Yeh E, Fichtenbaum CJ, Lederman MM, Estes JD, *et al*., A5317 AIDS Clinical Trials Group Team. **Telmisartan therapy does not improve lymph node or adipose tissue fibrosis more than continued antiretroviral therapy alone.** *J Infect Dis* 2018; **217**:1770–1781.
36. Dupin N, Buffet M, Marcelin AG, Lamotte C, Gorin I, Ait-Arkoub Z, *et al*. **HIV and antiretroviral drug distribution in plasma and fat tissue of HIV-infected patients with lipodystrophy.** *AIDS* 2002; **16**:2419–2424.
37. Raymond AD, Campbell-Sims TC, Khan M, Lang M, Huang MB, Bond VC, *et al*. **HIV Type 1 Nef is released from infected cells in CD45(+) microvesicles and is present in the plasma of HIV-infected individuals.** *AIDS Res Hum Retroviruses* 2011; **27**:167–178.
38. Agarwal N, Iyer D, Patel SG, Sekhar RV, Phillips TM, Schubert U, *et al*. **HIV-1 Vpr induces adipose dysfunction in vivo through reciprocal effects on PPAR/GR co-regulation.** *Sci Transl Med* 2013; **5**:213ra164.
39. Diaz-Delfin J, Domingo P, Wabitsch M, Giralt M, Villarroya F. **HIV-1 Tat protein impairs adipogenesis and induces the expression and secretion of proinflammatory cytokines in human SGBS adipocytes.** *Antivir Ther* 2012; **17**:529–540.
40. Beaupere C, Garcia M, Larghero J, Feve B, Capeau J, Lagathu C. **The HIV proteins Tat and Nef promote human bone marrow mesenchymal stem cell senescence and alter osteoblastic differentiation.** *Aging Cell* 2015; **14**:534–546.
41. Otake K, Omoto S, Yamamoto T, Okuyama H, Okada H, Okada N, *et al*. **HIV-1 Nef protein in the nucleus influences adipogenesis as well as viral transcription through the peroxisome proliferator-activated receptors.** *AIDS* 2004; **18**:189–198.
42. Cotter EJ, Chew N, Powderly WG, Doran PP. **HIV type 1 alters mesenchymal stem cell differentiation potential and cell phenotype ex vivo.** *AIDS Res Hum Retroviruses* 2011; **27**:187–199.
43. Zhu M, Heydarkhan-Hagvall S, Hedrick M, Benhaim P, Zuk P. **Manual isolation of adipose-derived stem cells from human lipoaspirates.** *J Vis Exp* 2013; **79**:e50585.
44. Lagathu C, Eustace B, Prot M, Frantz D, Gu Y, Bastard JP, *et al*. **Some HIV antiretrovirals increase oxidative stress and alter chemokine, cytokine or adiponectin production in human adipocytes and macrophages.** *Antivir Ther* 2007; **12**:489–500.
45. Dalle H, Garcia M, Antoine B, Boehm V, Huong Do TT, Buyse M, *et al*. **Adipocyte glucocorticoid receptor deficiency promotes adipose tissue expandability and improves the metabolic profile under corticosterone exposure.** *Diabetes* 2019; **68**:305–317.
46. Reimann KA, Parker RA, Seaman MS, Beaudry K, Beddall M, Peterson L, *et al*. **Pathogenicity of simian-human immunodeficiency virus SHIV-89.6P and SIVmac is attenuated in cynomolgus macaques and associated with early T-lymphocyte responses.** *J Virol* 2005; **79**:8878–8885.
47. Debaisieux S, Rayne F, Yezid H, Beaumelle B. **The ins and outs of HIV-1 Tat.** *Traffic* 2012; **13**:355–363.
48. Divoux A, Clement K. **Architecture and the extracellular matrix: the still unappreciated components of the adipose tissue.** *Obes Rev* 2011; **12**:e494–e503.

49. Uezumi A, Ito T, Morikawa D, Shimizu N, Yoneda T, Segawa M, *et al*. **Fibrosis and adipogenesis originate from a common mesenchymal progenitor in skeletal muscle.** *J Cell Sci* 2011; **124 (Pt 21)**:3654–3664.
50. Vila IK, Badin PM, Marques MA, Monbrun L, Lefort C, Mir L, *et al*. **Immune cell Toll-like receptor 4 mediates the development of obesity- and endotoxemia-associated adipose tissue fibrosis.** *Cell Rep* 2014; **7**:1116–1129.
51. Giralt M, Domingo P, Villarroya F. **HIV-1 infection and the PPARgamma-dependent control of adipose tissue physiology.** *PPAR Res* 2009; **2009**:607902.
52. Prost S, Le Dantec M, Auge S, Le Grand R, Derdouch S, Auregan G, *et al*. **Human and simian immunodeficiency viruses deregulate early hematopoiesis through a Nef/PPARgamma/STAT5 signaling pathway in macaques.** *J Clin Invest* 2008; **118**:1765–1775.
53. Pellegrinelli V, Carobbio S, Vidal-Puig A. **Adipose tissue plasticity: how fat depots respond differently to pathophysiological cues.** *Diabetologia* 2016; **59**:1075–1088.
54. Halberg N, Khan T, Trujillo ME, Wernstedt-Asterholm I, Attie AD, Sherwani S, *et al*. **Hypoxia-inducible factor 1alpha induces fibrosis and insulin resistance in white adipose tissue.** *Mol Cell Biol* 2009; **29**:4467–4483.
55. Kaminski DA, Randall TD. **Adaptive immunity and adipose tissue biology.** *Trends Immunol* 2010; **31**:384–390.
56. d'Ettorre G, Paiardini M, Zaffiri L, Andreotti M, Ceccarelli G, Rizza C, *et al*. **HIV persistence in the gut mucosa of HIV-infected subjects undergoing antiretroviral therapy correlates with immune activation and increased levels of LPS.** *Curr HIV Res* 2011; **9**:148–153.