Mitochondrial damage in adipose tissue of untreated HIV-infected patients

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Objective: Antiretrovirals, especially thymidine-analogue nucleoside reverse transcriptase inhibitors (tNRTIs), may cause the mitochondrial damage in adipose tissue that has been associated with lipodystrophy development. HIV itself may damage blood cell mitochondria. However, the viral capacity to induce adipose tissue mitochondrial lesion is still a matter of doubt. We aimed to assess whether untreated HIV infection was associated with adipose tissue mitochondrial abnormalities.

Design: Single-site, cross-sectional, controlled observational and exploratory study without intervention.

Methods: We included 24 uninfected controls and 18 HIV-infected patients with undetectable viral load and no clinical signs of lipodystrophy stratified as antiretroviral naive ($n = 11$) or at least 6-month antiviral-treated with a double NRTI combination, including lamivudine plus one tNRTI ($n = 7$). Subcutaneous adipose tissue was homogenated to determine mtDNA content by rtPCR and mitochondrial function per mitochondria through the spectrophotometric measurement of cytochrome *c* oxidase activity normalized by citrate synthase amount (COX/citrate synthase). Differences in mitochondrial parameters among groups were sought to determine the contribution of HIV and antiretrovirals to mitochondrial alterations.

Results: Compared with uninfected controls (arbitrarily assigned 100%), naive individuals presented a marked decrease in adipose tissue mtDNA content and COX/citrate synthase function (62 and 75% remaining content/activity, $P < 0.001$ and $P < 0.05$). Antiretrovirals did not increase this impairment (69 and 70% remaining content/activity, $P < 0.05$ compared to controls and $P =$ not significant compared to naives). Additionally, molecular and functional mitochondrial parameters were positively correlated ($P < 0.05$).

Conclusion: In nonlipodystrophic HIV-infected naive patients, viral infection is associated with adipose tissue mtDNA decrease and mitochondrial dysfunction independently of antiretroviral treatment.

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Introduction

Although clinical evidence of lipodystrophy is currently decreasing due to safer antiretroviral treatment (ART), lipodystrophy is still one of the most frequent adverse effects in HIV-infected patients on therapy.

Lipodystrophy etiology has not been completely elucidated, although ART is classically considered to play a main role. Some protease inhibitors and nonnucleoside reverse transcriptase inhibitors (non-NRTI) can trigger apoptosis that could underlie loss of peripheral adipose tissue [1,2]. Drugs belonging to protease inhibitor, NRTI, and non-NRTIs families could alter adipogenesis and adipocyte differentiation, which may contribute to lipodystrophy development [2–4]. Additionally, NRTIs could inhibit mitochondrial DNA (mtDNA) polymerase γ, the enzyme responsible for mtDNA synthesis and repair, thereby causing mtDNA depletion, which can lead to mitochondrial dysfunction and cell death [5]. This latter toxic antiretroviral effect, particularly attributed to thymidine analogues (tNRTIs), has been suggested to play a key role in lipodystrophy development [6].

Nonetheless, HIV and host-dependent factors (genetics and environment) are also thought to be involved [7]. Although adipocytes may be potentially infected by HIV, there is controversial evidence [8,9]. However, indirect effects of HIV infection, even under effective ART, have been reported in lipodystrophy adipose tissue to contribute to lipodystrophy [10,11]. These indirect effects include premature aging, immune activation, and inflammation through macrophage infiltration across adipose tissue and pro-inflammatory adipokine and cytokine secretion [11,12]. Additionally, HIV-infected and activated adipose tissue-resident macrophages could release viral proteins, which may contribute to adipocyte lesion [11].

Additionally, mtDNA depletion and mitochondrial impairment have been found in peripheral blood monocellular cells (PBMCs) of ART-naive patients, suggesting that HIV itself could cause mitochondrial lesion [13–15].

Classical studies have blamed NRTI therapies of mtDNA depletion and mitochondrial dysfunction found in adipose tissue of lipodystrophy [16–21] or nonlipodystrophy HIV patients [22–26]. However, the role of HIV in mitochondrial adipose tissue lesion is still a matter of doubt [10].

We hypothesized that adipose tissue mtDNA depletion and consequent dysfunction might be detected in antiretroviral-naive patients and that this impairment could be compensated in patients under effective ART without lipodystrophy.

Methods

Patients

We performed a single-site, cross-sectional, controlled observational exploratory study without intervention. Patients were consecutively included on their routine clinical visits in the Hospital Clinic of Barcelona (Barcelona, Spain), after signing the informed consent approved by the Ethical Committee of our hospital. We included 24 healthy volunteers and 18 asymptomatic HIV-infected adults matched by age and sex. Clinical data were recruited on admission. The inclusion criteria for HIV patients were diagnosis of HIV infection, age at least 18 years, and no clinical evidence of lipodystrophy [21]. HIV patients were stratified into two subgroups: 11 antiretroviral-naive and seven on ART, to assess HIV and antiretroviral isolated or combined effects on mitochondrial adipose tissue lesion in non-lipodystrophy patients. Inclusion criteria for treated patients were undetectable viral load (< 50 copies/ml) and receipt of stable double NRTI ART for at least 6 consecutive months prior to study entry consisting of lamivudine (3TC) and one tNRTI [stavudine (d4T) or zidovudine (ZDV)]. Double NRTI-treated patients with thymidine analogues were included to assess mitochondrial effects of ART classically considered harmful for mitochondria. Exclusion criteria were familial history of mitochondrial disease, treatment with mitochondrial toxic drugs (including tobacco and alcohol), opportunistic infections, or neoplasia.

Sample

Approximately, 80 mm^3 (50 mg) of subcutaneous adipose tissue (SAT) was obtained by punch biopsy from the periumbilical region under local anesthesia. When it was not possible, SAT was obtained from the arm, respecting patient and clinician advice. Obtention of white adipose tissue was always confirmed by anatomopathological means. All biopsies were immediately frozen at −80°C until analysis.

Mitochondrial parameters

Mitochondrial parameters were measured in 10% (w/v) adipose tissue homogenates after skin, connective tissue, and blood removal from the biopsy.

Mitochondrial DNA was measured in 300–600 ng of total DNA extracted by phenol-chloroform. Only those samples showing a purity absorbance cut-off between 1.7 and 1.9 (260/280 nm ratio) were analyzed in triplicates. Quantitative rtPCR (efficiency replication 2^n between 1.8 and 2) allowed mtDNA quantification by expressing the highly conserved mitochondrially encoded ND2 gene with respect to the nuclear-encoded housekeeping 18SrRNA gene (mtDNA/nuclear DNA; ND2/18SrRNA) [14]. In order to minimize interassay and intra-assay variability, samples from the three different kinds of patients were set together in the same rtPCR cycle and analyzed using the same internal curve of standard. PCR sensibility yielded between 3 and 30 000 ng for the amplified product. Positive and negative controls were systematically used and melting temperature test allowed specificity analysis for the amplified product of each sample.

Mitochondrial function was measured in duplicate by spectrophotometric quantification of cytochrome *c* oxidase activity (COX or mitochondrial complex IV; EC1.9.3.1) normalized by mitochondrial mass through citrate synthase measurement (citrate synthase; EC4.1.3.7, widely considered a reliable mitochondrial mass marker) and expressed as the COX/citrate synthase ratio [14]. Specific absorbance of spectrophotometric analyses was systematically monitored and, in order to minimize interassay and intra-assay variability, enzymatic activities of the three different kinds of patients were set together in the same cycle of analysis and performed using identical reagents.

Statistical analysis

Clinical and epidemiological parameters are expressed as median values and 95% confidence interval of the mean and experimental results as percentages compared to median values of uninfected individuals (arbitrarily assigned 100%).

The minimum sample size to detect mitochondrial differences was estimated based on previous experimental PBMC results obtained in similar population groups [14].

Differences in mitochondrial parameters between groups and correlation between quantitative parameters were analyzed using nonparametric statistical tests, with level of significance set at 0.05.

Results

Clinical and epidemiological characteristics of participants are summarized in Table 1. They were white, predominantly men (84–100%, depending on the group), with median age ranging from 37 to 47 years, depending on group assignment. Naive individuals had been recently HIV-diagnosed (median time 18 months after first seropositive HIV testing). Treated individuals, who had been HIV-infected and HAART-treated for a median times of 120 and 40 months, respectively, received at least 6 months of ART consisting of 3TC and one tNRTI (d4T/ZDV) prior to study entry. As expected, $CD4^+$ T-cell count and viral load were significantly increased and reduced, respectively, in HIV-treated patients compared to naive individuals because of therapeutic ART activity.

Compared with uninfected controls (assigned 100%), naive patients showed a marked decrease in SAT mtDNA content (62% remaining mtDNA, $P < 0.001$, Fig. 1a) and COX function (75% remaining COX/citrate synthase, $P < 0.05$, Fig. 1b). With respect to uninfected controls, HIV-treated individuals also showed a remarkable reduction in SAT mtDNA content (69% remaining mtDNA, $P < 0.05$, Fig. 1a) and COX function (70% remaining COX/citrate synthase, $P < 0.05$, Fig. 1b). However, mitochondrial parameters between both groups of infected individuals were similar irrespective of ART administration.

Table 1. Clinical and epidemiological characteristics of individuals included in the study.

	Uninfected ($n = 24$)	HIV-Naive ($n = 11$)	HIV-Treated ($n = 7$)	*P* value[a]
Age (years) (median ± 95% CI of the mean)	47 [33–56]	37 [32–43]	44 [25–55]	NS
Male sex (%)	84	100	86	NS
Time on HIV (mon) (median ± 95% CI of the mean)[b]	–	18 [12–69]	120 [36–173]	NS
ART duration (mon) (median ± 95% CI of the mean)	–	–	40 [24–47]	–
2 NRTI regimen: 3TC+T-analogue (d4T/ZDV) (*n*)[c]	–	–	7	–
$CD4^+$ T-cell count (cells/μl) (median ± 95% CI of the mean)	–	178 [79–435]	491 [242–810]	$P < 0.05$
Viral load (Log_{10}copies RNA/μl) (median ± 95% CI of the mean)[d]	–	5.2 [3.7–5.9]	1.7 [1.1–3.4]	$P < 0.001$

95% CI of the mean, 95% confidence interval of the mean; mon, months; NS, nonsignificant; y, years.
[a]$P < 0.05$ was considered to be statistically significant.
[b]Determined since the time after first seropositive HIV testing.
[c]NRTI, nucleoside analogue reverse transcriptase inhibitor; 3TC, lamivudine; T-analogue, thymidine analogue (d4T, stavudine or ZDV, zidovudine).
[d]Viral load < 50 copies/ml was considered to be 49 copies/ml for statistical analysis.

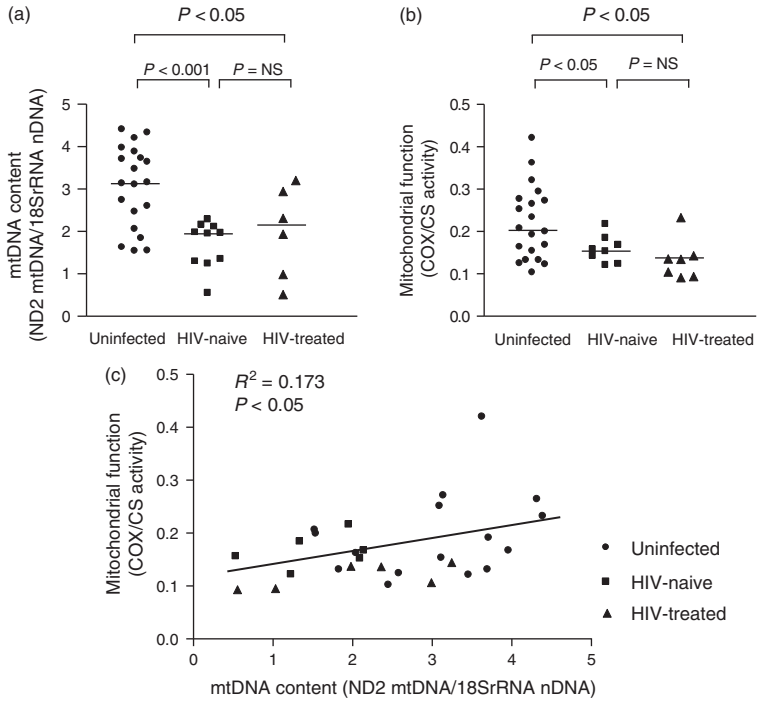

Fig. 1. Mitochondrial parameters of patients included in the study. Adipose tissue mitochondrial DNA content (a); mitochondrial function measured as cytochrome *c* oxidase activity related to mitochondrial mass (COX/citrate synthase relative units) (b); and correlation between both mitochondrial parameters (c). In (a) and (b), horizontal lines represent median values for each parameter and study group. NS, nonsignificant.

In HIV-infected patients, viral load negatively correlated with the levels of mtDNA and COX/citrate synthase function, being exclusively significant for COX/citrate synthase activity ($P < 0.05$).

Additionally, both molecular and functional mitochondrial parameters (mtDNA content and COX/citrate synthase activity) were positively correlated ($P < 0.05$, Fig. 1c).

Discussion

As an exploratory study, the number of included patients was small. The reduced sample size was due to the invasiveness of the approach, which required adipose tissue biopsy collection. Unfortunately, this is a common characteristic of many other studies in the literature [17–20,25,26]. However, despite the reduced number of cases, we were able to obtain conclusive results for the testing of our hypothesis. Other limitation of our study could be the different location of SAT. We did not use visceral adipose tissue or SAT susceptible to containing brown adipocytes (present on the buffalo hump depots of lipodystrophy patients). We analyzed periumbilical SAT of nonlipodystrophy individuals, unless clinician or patient advice asked for an alternative location. In those few cases, SAT was obtained from the arm. Therefore, we think that dual SAT location should not affect our findings because both kinds of samples are constituted by white adipocytes and were always present in all the groups of patients analyzed.

Our results indicate that mtDNA depletion and associated mitochondrial dysfunction are present in SAT of naive patients, even just after 18 months of infection. Such finding corroborates our hypothesis that HIV itself may play a role in adipose tissue mitochondrial lesion, classically considered a characteristic feature of lipodystrophy. However, most of the studies performed on adipose tissue did not find mitochondrial differences between seronegative and naive [18,20,24,25] or treated HIV patients [25,26], except when tNRTI-treated individuals were individually compared. These studies mainly concluded that ART led to adipose tissue mitochondrial impairment [18–20,22–26] and demonstrated mitochondrial benefits of tNRTI interruption or switching to less mitotoxic schedules [7,17,27].

Based on our findings, mitochondrial damage in fat of naive individuals suggests that the virus itself may contribute to the mitochondrial dysfunction present in the target tissue of lipodystrophy, which is indeed confirmed by the negative correlation found between viral load and SAT mitochondrial function. These results corroborate previous findings of altered mitochondrial function in adipocytes [10,11] and PBMC [13,14] of naive patients. According to the present

findings, HIV could be contributing to the mitochondrial impairment found on adipose tissue of lipodystrophy patients, together with ART. That fact could explain rare cases of lipodystrophy in naive individuals and, above all, would explain reported association between decreased number of $CD4^+$ T lymphocytes and higher risk of lipodystrophy [28]. Such finding abrogates for the current established guidelines of early treatment management of HIV infection to avoid deleterious effects triggered by the virus and to prevent reservoirs constitution.

We did not observe additive mitochondrial toxicity when considering dual presence of HIV and ART, though considering tNRTI drugs with well known mitochondrial toxicity. This may be explained because ART-negative effects exerted against mitochondria may be balanced by ART-positive mitochondrial effects decreasing viral load and its derived mitotoxic capacity, thereby leading to similar levels of mitochondrial lesion in naive and treated patients. Such finding is in agreement with results of clinical trials reporting SAT increase in HIV individuals after tNRTI introduction [29,30]. However, we do not know future consequences of HIV-induced mitochondrial dysfunction compensated by ART into lipodystrophy development.

Both molecular and functional mitochondrial parameters were positively correlated confirming the strong dependence of mitochondrial function on mitochondrial genome [5,14]. A similar correlation was observed by Hammond *et al.* [24] in adipose tissue of HIV-treated patients concomitant with evidence of cellular toxicity. Homeostatic transcriptional and translational upregulatory mechanisms can compensate HIV and ART-induced mtDNA depletion in PBMCs [31]. However, observed adipose tissue mitochondrial dysfunction in naive and treated patients suggests that such upregulatory mechanism designed to preserve mitochondrial function may be weaker in adipocytes. This would explain adipocyte susceptibility to become damaged by mitotoxic agents and physiologic vulnerability of individuals to lipodystrophy. Additionally, the idea of a complex scenario involving other modulating factors (inflammation, oxidative stress, apoptosis, alteration of adipogenesis and adipocyte differentiation or adipokine and cytokine levels) is gaining in strength and may help to explain lipodystrophy and different regional fat behavior (visceral versus subcutaneous) [1–4,10–12]. Probably, all these factors condition lipodystrophy. Nonetheless, without HIV or ART mitotoxic activity, lipodystrophy would not probably be developed.

In summary, we have demonstrated that uncontrolled HIV infection is associated with mitochondrial abnormalities in SAT. These effects are partially compensated by ART, even when such treatment contains tNRTIs. Clinical implications of these findings in the context of currently administered ART are unknown and would deserve prospective evaluation in longitudinal studies to assess mitochondrial effects of ART introduction in adipose tissue of HIV individuals.

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