BRIEF REPORT

No Longitudinal Mitochondrial DNA Sequence Changes in HIV-infected Individuals With and Without Lipoatrophy

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The potential for mitochondrial (mt) DNA mutation accumulation during antiretroviral therapy (ART), and preferential accumulation in patients with lipoatrophy compared with control participants, remains controversial. We sequenced the entire mitochondrial genome, both before ART and after ART exposure, in 29 human immunodeficiency virus (HIV)–infected Swiss HIV Cohort Study participants initiating a first-line thymidine analogue–containing ART regimen. No accumulation of mtDNA mutations or deletions was detected in 13 participants who developed lipoatrophy or in 16 control participants after significant and comparable ART exposure (median duration, 3.3 and 3.7 years, respectively). In HIV-infected persons, the development of lipoatrophy is unlikely to be associated with accumulation of mtDNA mutations detectable in peripheral blood.

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Lipoatrophy is an important and poorly reversible complication associated with human immunodeficiency virus (HIV) infection and antiretroviral therapy (ART), particularly the thymidine-analogue nucleoside reverse-transcriptase inhibitors, stavudine and zidovudine. The pathogenesis of lipoatrophy is incompletely understood. A widely implicated mechanism is mitochondrial dysfunction, which is associated with HIV infection and thymidine-analogue treatment [1].

Several studies now suggest a genetic predisposition to lipoatrophy, but results have not been uniform regarding, for example, an association with certain mitochondrial DNA (mtDNA) haplogroups [2–4]. A related question is the potential for mtDNA genomic damage, possibly induced by thymidine analogues, to contribute to lipoatrophy [5, 6]. The accumulation of mtDNA point mutations has been experimentally linked to premature aging [7], and advancing age is an unexplained risk factor for lipoatrophy in HIV-infected persons [8]. Martin et al [5] recorded the accumulation during ART of multiple mutations in mtDNA extracted from the peripheral blood. The mtDNA mutations were preferentially seen in patients who developed lipoatrophy compared with control participants without lipoatrophy. However, the lipoatrophy case patients had more intensive ART exposure than the control patients; therefore, it remained possible that lipoatrophy or mtDNA mutations might have developed with prolonged ART exposure in the participants classified as controls [5]. In a second study with longitudinal mtDNA samples, McComsey et al [6] observed no accumulation of mtDNA mutations in peripheral blood samples, but the median duration of follow-up was only 13 months.

This study was therefore conducted to reassess the value of mitochondrial genome sequencing in HIV-infected participants with and without lipoatrophy, and with significant and comparable ART exposure. We assessed a potential correlation between the development of lipoatrophy and the accumulation of mtDNA mutations in peripheral blood.

METHODS

Study participants were enrolled in the Swiss HIV Cohort Study (SHCS; http://www.shcs.ch), which involves a standardized clinical evaluation of fat loss and fat accumulation every 6 months. Participants gave written, informed consent for genetic testing. The SHCS Genetics Project was approved by the ethics committees of participating centers. Participants were male and self-identified as white, to minimize the influence

of sex and of population structure on mtDNA sequences. Each participant started a first-line zidovudine- or stavudine-containing combination ART regimen after 1 April 1998, had a cell sample stored prior to ART initiation, and had no fat loss recorded prior to ART. Case patients developed significant fat loss (patient/physician agreement; ⩾2 body sites), consistently recorded at ⩾4 subsequent, biannual SHCS visits, without reversion to no fat loss. To avoid excluding participants who rapidly developed lipoatrophy, we required no minimum duration of ART. Controls had no fat loss for ⩾3 years after ART initiation.

We permitted modifications of ART components other than zidovudine or stavudine. We excluded participants with ART interruption for ⩾30 days as well as those with initial immune reconstitution followed by a CD4 cell decline to <200 cells/μL, to exclude significant complications (eg, tuberculosis or lymphoma) as confounding factors for fat loss. To exclude ART nonadherence or active viral replication as a confounder for the presence or absence of lipoatrophy, all participants had HIV RNA counts of <400 copies/mL starting ⩾1 year after ART initiation.

The treating HIV physician confirmed in writing the presence or absence of lipoatrophy and interviewed each participant regarding his ancestry. We assessed the value of clinical lipoatrophy classification by quantitative assessment of total and regional fat mass by dual-energy x-ray absorptiometry (DEXA; Hologic QDR-4500W) in a subset of study participants. DEXA provided quantitative assessments of total body mass, peripheral (sum of arm and leg fat) and total fat mass, and the ratio between peripheral and total fat mass to adjust for differences in body weight [9].

Sequencing and Mutational Screening of mtDNA

We extracted mtDNA from frozen leukocyte pellets. We sequenced the entire genomic (16569 bp) mtDNA at 2 time points: prior to any ART exposure, and after lipoatrophy diagnosis (cases) or after ⩾3 years of ART (controls). Polymerase chain reaction (PCR) amplification and direct forward and reverse sequencing was performed by Macrogen, using BigDye Terminator sequencing chemistry on a 3730XL DNA Sequencer (Applied Biosystems). Primers and PCR conditions were as published elsewhere [10]. ABI PRISM SeqScape software (version 2.6; Applied Biosystems) was used to analyze the electropherogram files. All sequences were analyzed without knowledge of sample classification as case or control. We performed quality checks of mtDNA sequences as described in the supplementary material. We aligned mtDNA sequences and compared them with the revised Cambridge reference sequence (available at http://jid.oxfordjournals.org/) using SeqScape and further manual check. We classified variant positions relative to this reference sequence either as phylogenetically informative (useful to assign a particular sequence to a mtDNA haplogroup) or uninformative [11]. Among the latter class, we distinguished mutations occurring in the mtDNA coding region, which have the potential to be nonsynonymous, from those occurring in the noncoding control region. We used 3 large databases to identify known sequence variants in humans: PhyloTree (http://www.phylotree.org), MITOMAP (http://www.mitomap.org), and mtDB (http://www.genpat.uu.se/mtDB/). We made classifications into haplogroups using the phylogenetic tree of global human mtDNA variation by van Oven and Kayser [12], available at PhyloTree.

RESULTS

We included 13 case patients with lipoatrophy and 16 control patients without lipoatrophy, whose characteristics are shown in Table 1. Lipoatrophy was first noted in case patients after a median ART duration of 2 years (interquartile range [IQR], 1.5–3.3 years). Of the 6 case patients and 4 control patients that underwent DEXA, the cases had higher body weight, total mass, and total fat, and slightly more peripheral fat than the controls, but despite this, their peripheral to total fat ratio was decreased, consistent with lipoatrophy [9]. However, these differences were not statistically significant in this small study population (Table 1).

The entire mtDNA was sequenced from samples taken before ART and after a median ART duration of 3.3 years in cases and 3.7 years in controls. Comparison of each participant's mtDNA sequences before and during ART revealed no point mutations, no insertions or deletions, and no evidence of point heteroplasmy in any samples (Supplementary Table 1).

Participants belonged to the main mtDNA haplogroups H, U, K, J, and L (13, 7, 2, 6, and 1 participant, respectively). Thus, H and U were most frequent haplogroups, followed by J and K, findings that are consistent with the European origin of the participants (see Supplementary Results), whereas 1 participant carried an L haplogroup (L0a2a2a). By pooling certain haplogroups (ie, U and K; J and L), a formal test of homogeneity showed that this simplified haplotype distribution did not deviate from 3 previously published western European regional samples ($P = .55$, $P = .40$, and $P = .14$, respectively; see Supplementary Results). A slight excess of haplogroup J (21%) was observed in the study population, compared with 10%, 9%, and 6%, respectively, in the regional European samples.

Case participants who belonged to mtDNA haplogroups H, U, K, J, and L numbered 6, 4, 1, 2, and 0, respectively, and control participants numbered 7, 3, 1, 4, and 1. No formal comparison of the frequency distribution of haplogroups between cases and controls was feasible because of limited sample size. Single haplogroup frequency comparison indicated that the frequency of haplogroup H did not differ between cases and controls ($P > .05$), and haplogroup J was more frequent among

Table 1. Clinical Characteristics of 13 Case Patients With Lipoatrophy and 16 Control Patients Without Lipoatrophy Who Had Previously Initiated a First-line thymidine analogue–containing Antiretroviral Therapy Regimen

	Lipoatrophy cases (*n*= 13)	Controls without lipoatrophy (*n* = 16)	*P*[1]
Age at pre-ART mtDNA sample, median (IQR), years	51.8 (46.4–62.8)	45.1 (39.4–55)	.16
Age at ART initiation, median (IQR), years	53.2 (46.4–62.8)	45.2 (39.6–55.1)	.12
CD4 cell count at ART initiation, median (IQR), cells/μL	206 (142–224)	178 (117–238)	.90
HIV RNA at ART initiation, median (IQR), copies/mL	77,000 (32,000–93,000)	176,000 (97,000–218,000)	.03
Body weight at ART initiation, median (IQR), kg	72 (67–78)	70 (64–74)	.41
Body mass index at ART initiation, median (IQR), kg/m^2	23 (21.7–26.1)	22.5 (21.8–25.2)	.46
Initial ART regimen	NRTI components: ZDV+3TC (*n* = 11), d4T+DDI (*n* = 1), d4T+3TC (*n* = 1); Third agent: efavirenz (*n* = 6), nelfinavir (*n* = 4), lopinavir/ritonavir (*n* = 2), indinavir (*n* = 1)	NRTI components: ZDV+3TC (*n* = 12), ZDV+3TC+ABC (*n* = 1), d4T+DDI (*n* = 2), d4T+3TC (*n* = 1); Third agent: efavirenz (*n* = 5), nelfinavir (*n* = 4), lopinavir/ritonavir (*n* = 4), indinavir (*n*=2), saquinavir/ritonavir (*n* = 1)	.81 .50
Age at during-ART mtDNA sample, median (IQR), years	56 (49.8–66.1)	48.8 (43.6–58.3)	.18
Cumulative ART exposure at during-ART mtDNA sample, median (IQR), years	3.3 (2.5–4.1)	3.7 (3.4–3.8)	.16
Body site(s) of fat loss	Face (*n* = 9), arms (*n* = 12), legs (*n* = 11), buttocks (*n*= 3)	No fat loss	
Fat accumulation	*n*= 9	*n*= 5	
Hyperlactatemia, peak level, symptoms/acidosis	*n*= 1, 2.6 mmol/L, asymptomatic	*n* = 1, 5.9 mmol/L, asymptomatic	
Peripheral neuropathy	*n*= 2 (attributed to vitamin B12 deficiency in 1 participant)	*n*= 1 (attributed to diabetic neuropathy)	
Pancreatitis	None	None	
DEXA, number of participants	6	4	
• Age at DEXA, median (IQR), years	59.3 (53.7–66.9)	51.4 (44.8–59.9)	.41
• Total mass, median (IQR), kg	72 (60.8–72.3)	67.4 (63.4–68.3)	.21
• Total fat, median (IQR), kg	15.3 (11.6–18.9)	10.3 (9.5–11.9)	.41
• Peripheral fat, median (IQR), kg	4.9 (4.3–6.7)	4.6 (4–5.2)	.54
• Peripheral/total fat ratio, mean %	37.9	41.4	.54

NOTE. Abbreviations: ART, antiretroviral therapy; mtDNA, mitochondrial DNA; IQR, interquartile range; DEXA, dual-energy x-ray absorptiometry

[1] *P* values of tests for equality of distribution in lipoatrophy cases versus controls without lipoatrophy. All comparisons performed applying the Mann-Whitney U test except for initial ART regimens, which were compared with the χ^2 homogeneity test (collapsed categories: zidovudine vs stavudine, and efavirenz vs nelfinavir vs other).

controls, whereas haplogroup U was slightly more common among cases (both $P > .05$).

We also compared mtDNA sequences with the revised Cambridge reference sequence. As expected, numerous variant mtDNA positions or small insertions or deletions were indentified. Among 90 phylogenetically uninformative variant positions (43 in the coding region, 47 in the noncoding control region), 86 are known to be polymorphic in the human population, thus providing no signal of a possible association with lipoatrophy (see Supplementary Results). Among the 4 variants not previously recorded as polymorphic, only 1 (a single transition at mtDNA position 7962 in a lipoatrophy case bearing haplogroup H) was nonsynonymous.

DISCUSSION

Using whole mitochondrial genome amplification and direct sequencing, we observed no accumulation of mtDNA mutations or deletions in a comprehensive mtDNA sequence analysis of an HIV-infected study population that included 13 case participants with lipoatrophy and 16 control participants without lipoatrophy. In both cases and controls, mtDNA was sequenced twice: first while participants were ART-naïve and again after substantial (median, ⩾3.3 years) and comparably extensive exposure to thymidine analogue–containing ART. This finding argues against the accumulation of mtDNA mutations as a major driver behind the development of lipoatrophy. In addition, no signal was detected when comparing mtDNA haplogroups in cases and controls, or when comparing mtDNA sequences with the revised Cambridge reference mtDNA sequence.

The accumulation of multiple mtDNA mutations over time is the central tenet of the mitochondrial hypothesis of aging that has been experimentally validated in the mtDNA ''mutator mouse'' model [7]. However, the link between mtDNA damage and metabolic complications in HIV-infected individuals remains circumstantial. Previously, 2 groups have assessed the mitochondrial genome for longitudinal mtDNA changes during ART. In the study by Martin et al [5], mtDNA mutations accumulated during follow-up in 1 of 11 patients without lipoatrophy and in 4 of 5 patients with lipoatrophy. However, patients without lipoatrophy had a shorter mean ART duration (1.9 years) than the that of the lipoatrophy case patients (3.5 years). McComsey et al [6] recorded the longitudinal *reversion* of heteroplasmic mtDNA sequence variants to homoplasmic ones in no participant with lipoatrophy and 2 participants without lipoatrophy. However, this occurred in the hypervariable mtDNA control region, the median duration of follow-up in this study was only 13 months, and the authors expressed concern that the method used to detect any mtDNA mutations (temporal temperature gradient gel electrophoresis [TTGE] of the PCR products) may have been insufficiently sensitive.

Moreover, in both studies [5, 6], all recorded mtDNA mutations were heteroplasmic. He et al [13] recently reported the use of massively parallel sequencing-by-synthesis approaches to statistically quantify heteroplasmy. A high level of heteroplasmy was seen in normal participants, with variable frequencies among tissues. Thus, mtDNA heteroplasmy may not represent an important end point in lipoatrophy genetic studies, unless a significant heteroplasmy increase were detected preferentially after ART exposure, and in lipoatrophy participants compared with patients without lipoatrophy.

Our study is limited by sample size to detect mtDNA haplogroup associations and by clinical diagnosis of lipoatrophy. However, we included participants only if significant lipoatrophy was consistently present during follow-up, case or control status was confirmed in writing by the treating HIV physician, and by DEXA assessment of the study criteria in a subset of participants. Another limitation of studies of the mtDNA genome in HIV-infected persons is the source of mtDNA, which is extracted from the most accessible tissue (ie, peripheral blood). As with mtDNA depletion, if and to what extent mtDNA sequences in blood correlate with mtDNA sequences in affected tissue (ie, subcutaneous fat) is unclear [14]. The mitochondrial toxicity hypothesis of lipoatrophy [1] has evolved from a hypothesis centered on polymerase-γ to a more complex picture including the differential effects of ART in different tissue compartments. For example, recently, Payne et al [15] identified mtDNA variants in muscle biopsies, but point mutations were only found in cytochrome c oxidase–deficient fibers (ie, cells with mitochondrial dysfunction).

In conclusion, our findings, in conjunction with the reports by Martin et al [5] and McComsey et al [6], suggest that lipoatrophy genetic studies based on peripheral blood mtDNA specimens may not permit the detection of possible mtDNA changes in particular tissue types or cell populations. Subcutaneous fat biopsy samples seem best suited to evaluate a possible role for mtDNA genomic variation in the pathogenesis of lipoatrophy. Patient acceptance limits the feasibility of fat biopsies, particularly in the setting of a longitudinal study, and the paucity or lack of fat tissue in lipoatrophy patients may be a problem. In contrast, peripheral blood samples are sufficient to identify potentially lipoatrophy-predisposing single nucleotide polymorphisms in nuclear encoded genes, including *TNF* -238G > A, *APOC3*, hemochromatosis gene variants, *FAS*, *AR Beta-2*, *HLA-B*4001*, and potentially lipoatrophy-predisposing mtDNA haplogroups [2–4].

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