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Prospective evaluation of blood concentration of mitochondrial DNA as a marker of toxicity in 157 consecutively recruited untreated or HAART-treated HIV-positive patients

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Highly active antiretroviral therapy (HAART) can cause mitochondrial toxicity. The concentration of mitochondrial DNA (mtDNA) in peripheral blood cells has been reported to be a marker of this toxicity. However, these observations are controversial and were drawn from small series. Thus, we analysed the value of blood mtDNA as a marker of mitochondrial toxicity in a large cohort of human immunodeficiency virus (HIV)-infected out-patients during routine clinical evaluations. Real-time quantitative PCR was used to determine the mtDNA to nuclear DNA (nDNA) ratio in peripheral blood mononuclear cells from 157 consecutive HIV-1-infected patients (13 naive, 144 receiving HAART) and 30 HIV-1-uninfected patients. The mtDNA to nDNA ratio was significantly lower in both groups of HIV-infected patients than in the control group. No significant difference was observed between treated and naive HIV-infected patients. Lactataemia was significantly lower in controls than in the group of HIV-treated patients. None of the treated patients had lactataemia >5 mmol/l or bicarbonates <20 mmol/l. Triglyceride levels were significantly higher in the HAART-treated patients than in the nontreated patients. Clinical symptoms of lipodystrophy were observed in 62 HAART-treated patients. These symptoms were not associated with an abnormal mtDNA to nDNA ratio or plasma triglyceride concentration. The mtDNA to nDNA ratio was lower in DDI/D4T-treated patients than in AZT/3TC-treated patients. In conclusion, there are no obvious links between the mtDNA to nDNA ratio in peripheral mononuclear cells and any clinical symptoms or lactate level. Thus, the mtDNA to nDNA ratio in leukocytes does not seem to be an accurate marker of mild and/or long-term mitochondrial toxicity.
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Highly active antiretroviral therapy (HAART) has substantially reduced morbidity and mortality due to HIV-1 (human immunodeficiency virus type 1). However, long-term treatment with these drugs is sometimes associated with adverse effects, especially the development of lipodystrophy syndrome and hyperlipidaemia. Both of these side effects appear to be linked to the use of HIV protease inhibitors.[1–4] However, the broad spectrum of toxicities associated with nucleoside analogue reverse transcriptase inhibitors (NRTIs), which result in mitochondrial dysfunction, suggests that NRTIs can also cause lipodystrophy syndrome.[5]

Mitochondrial toxicity is related to the inhibition of DNA polymerase-γ, an essential enzyme for the replication of mitochondrial DNA (mtDNA), by NRTIs. This is at least partly responsible for the adverse effects of NRTIs, which include lactic acidosis, hepatic steatosis, myopathy, cardiomyopathy, peripheral neuropathy, pancreatitis and possibly the lipodystrophy syndrome.[6–9] The clinical toxicity has been attributed to mtDNA depletion associated with mitochondrial dysfunction that

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affects the respiratory chain and fatty acid beta oxidation. Mitochondrial alterations induce the release of lactates into the bloodstream.

The most dramatic presentation of mitochondrial toxicity is the development of acute lactic acidosis, which is fatal in most cases.[10,11] Prolonged high blood lactate concentrations are associated with a poor prognosis.[12,13] Venous blood lactate concentrations have been used to monitor for mitochondrial damage, but the clinical value of this method remains unclear. This method is not ideal because it is not specific and because it is subject to technical and physiological variability.[14,15] Moreover, severe mitochondrial defects may coexist in one specific organ with normal lactate levels in the blood.[16] The concentration of lactate in the blood may also increase as a result of decreased clearance in the liver due to the presence of steatosis rather than due to increased lactate production.[17] The gold standard would be a liver or a muscle biospy to evaluate the level of mitochondrial toxicity (liver steatosis or steatohepatitis), notably in HIV/HCV (hepatitis C virus) coinfected patients. However, biopsy is not practical for routine monitoring.

The depletion of mtDNA was recently reported to be an early and direct marker of NRTI toxicity.[18] It is possible that the quantification of mtDNA in peripheral blood cells could be a more sensitive and earlier indicator of the mitochondrial toxicity of drugs than cell viability.[19,20] However, other studies gave controversial results.[21,22] All of these studies were performed on small series of patients. We assessed the long-term toxicity of HAART in 157 consecutive out-patients who had been receiving HAART for a mean period of 66 months, to determine whether the mtDNA to nuclear DNA (nDNA) ratio in peripheral blood mononuclear cells can be used to screen for mitochondrial toxicity in routine practice.

Materials and methods

Patients

All the HIV patients aged between 20 and 50 years who attended the Infectiology Department of Paul Brousse Hospital (Villejuif, France) as an out-patient between May 3rd and 28th 2002 were included in this study, regardless of whether they were treated by HAART. In all, 13 asymptomatic HIV patients had never been treated with antiretroviral drugs (two females and 11 males) and 144 HIV patients had been continuously treated with antiretroviral drugs for between 1 and 168 months (mean = 66.3 months). All 144 patients had received AZT (zidovudine) and 3TC (lamivudine) and some were switched to ABC (abacavir), TDF (tenofovir), and the others to DDI (didanosine) and/or D4T (stavudine). A control group of 30 HIV-1-negative patients was also analysed. The patients gave a blood sample after a 3-h period of rest. All patients gave informed consent for inclusion, according to the rules at our institution.

Age, sex, CD4 count, HIV-1 viral load, and nature and duration of treatments with HAART were recorded at the time of inclusion (Table 1).

Blood samples taken during out-patient visits after 12 h-overnight fast period. Moreover, all the patients had a 3-h period of rest before subjecting to routine tests including lactates, creatine phosphokinase, amylase, triglyceride and cholesterol. We also collected a 7-ml venous blood sample on EDTA for peripheral blood mononuclear cells (PBMC) isolation and DNA preparation.

Biochemical Data

Venous blood collected in sodium-fluoride-potassium oxalate tubes and stored in ice until analysis was used to measure fasting blood lactate in resting patients. Cholesterol, triglycerides, HDL cholesterol, amylase, creatine phosphokinase, and bicarbonates in blood according to routine procedures using a Synchron LX20 Clinical System (Beckman Coulter, Villepinte, France).

DNA Extraction

After lysing red blood cells in Tris-HCl (20 mmol/l) and EDTA (5 mmol/l) buffer (Sigma, L'Isle D'Abeau Chesnes, France), total DNA was isolated from PBMC with a Wizard Genomic DNA purification Kit (Promega, Charbonnières, France). Total DNA was stored at −80°C.

Table 1 Age, sex ratio, duration of treatment, CD4 count, HIV-RNA load and duration of HIV-seropositivity for each group of patients

Group of patients	N=	*Sex ratio(F/M)*	*Age (mean±s.d. years)*	*Duration of treatment (mean± s.d. months)*	*CD4-count (mean± s.d. number giga/l)*	*HIV RNA (mean±s.d. log copy)*	*Duration of HIV-seropositivity (mean±s.d. months)*
Control (HIV negative)	30	9/21	37.4±9.2	—	—	—	—
Untreated HIV patients	13	2/11	36.0±3.7	—	0.484±0.159	3.71±1.22	69.1±72.0 [0.4–180]
Treated HIV patients	144	29/115	40.1±5.5	66.3±42.0	0.440±0.276	2.95±1.95	118.4±62.0* [1.5–252]

*$P = 0.06$ compared to the untreated HIV patients.

MtDNA Quantification

We quantified mtDNA in the total DNA extracted from each patient. For this, we used the method described by Gahan *et al*[23] and modified by Cote *et al*.[18] In brief, the nuclear gene (β-actin) and the mitochondrial gene (cytochrome *b* or cyt *b*) were quantified separately by real-time quantitative PCR (polymerase chain reaction). We amplified a part of the cyt *b* mitochondrial gene by use of specific primers, Mito-CytB-F CAACATCTCCGCATGAT GAAA and Mito-CytB-R CCATAATTTACGTCTC GAGTGATGTG and a specific probe 5′-6-Fam-CCATGCACTACTCACCAGACGCCTCAA-3′-Tamra. The β-actin gene was amplified by the Taqman β-Actin® mix (Roche, Meylan, France). The PCR mixtures contained 7 μl of water, 12.5 μl of QPCRmastermix® (Eurogentec, Angers, France), 0.4 pmol/μl of each primer and 0.2 pmol/μl of each probe. Each sample was subjected to real-time PCRs in triplicate. The amplification conditions consisted of an enzyme activation of 2 min at 50°C, followed by a single denaturation step of 10 min at 95°C, and 50 cycles of 15 s at 95°C and 1 min at 60°C. Fluorescence was measured at the end of each annealing step. A standard curve of 10, 100, 1000, 10 000 and 100 000 nuclear-genome equivalents was included in each run, and the same nuclear-genome equivalent values were used to quantify both the nuclear (β-actin) and mitochondrial (cyt *b*) genes. The data are expressed as the ratio of the mean mitochondrial DNA value of the triplicate measurements to the mean nuclear DNA value (β-actin) of the triplicate measurements for a given extract (mtDNA/β-actin).

Statistical Analysis

Results are expressed as means (*m*) and standard deviations (s.d.). MtDNA to nDNA ratio and concentrations were compared by use of Fisher's test. Means were compared by the Student's *t*-test after normalisation of variables. Correlations were analysed by Pearson's test. Type one error was set at 5%. Data were analysed using the R software version 1.5.1.[24]

Results

The Concentration of mtDNA in the Blood of HIV Patients

Real-time PCR was used to determine the mtDNA to nDNA ratio in mononuclear peripheral cells. The mean value in the HIV-1-negative control group was 0.38 ± 0.28 (Figure 1). This value was considered to be 100%. In untreated HIV-1 patients, the mtDNA to nDNA ratio was 63% of the value in the controls (0.14 ± 0.11, $P = 0.001$). This ratio was even lower (71% of the control value) in the treated HIV patients (0.11 ± 0.09, $P = 0.003$). There was no significant difference in the mtDNA to nDNA ratio between the treated and the untreated HIV-1 patients (Figure 1). Because the mean duration of seropositivity was shorter in the HIV-untreated group compared to the treated patients, although not significant, we have analysed the mtDNA to nDNA ratio in 13 treated HIV-1 patients matched for the seropositivity duration to untreated HIV-1 patients. The mean mtDNA to nDNA ratio in the latter patients ($n = 13$) was 0.11 ± 0.09. The difference was not significant.

Lactataemia in HIV Patients

The mean fasting venous blood lactate concentration in the control group was 1.2 ± 0.43 mmol/l (range 0.4–2.5 mmol/l). This value was similar in the untreated HIV-1 patients (1.4 ± 0.8 mmol/l, Figure 2). In the treated patients, the lactate concentration was between 0.5 and 4.0 mmol/l ($m = 1.5 \pm 0.6$ mmol/l) and was significantly higher than in controls ($P = 0.01$). None of the treated patients had a lactate concentration of above 5 mmol/l or acidosis; the lowest bicarbonate concentration was 22 mmol/l. No correlation was found between the mtDNA to nDNA ratio in the peripheral blood mononuclear cells and the fasting venous blood lactate concentration in any of the groups.

Patients with Mitochondrial Toxicity

Fatigue, muscle weakness, shortness of breath, nausea, vomiting, abdominal pain and peripheral neuropathy were evaluated during the clinical evaluation. None of these signs were associated with a change in the mtDNA to nDNA ratio or lactataemia, may be because they were mostly 'subjective'. Lipodystrophy is considered to be a symptom of long-term toxicity associated with

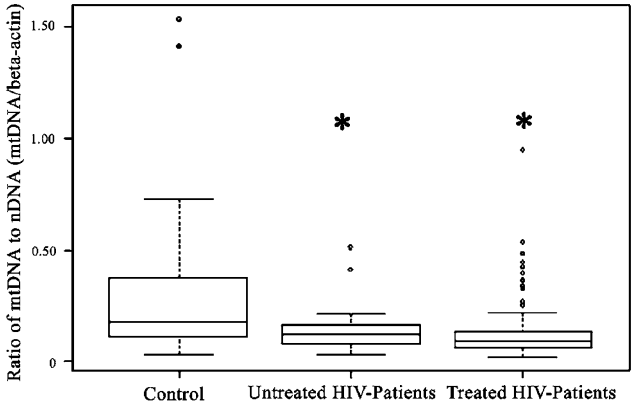

Figure 1 mtDNA to nDNA ratio from PBMC in the different group of patients. *The difference between control and untreated and treated HIV patients is significant. Fisher and Student's test, $P < 0.05$.

protease inhibitors or NRTIs. The features used to assess lipodystrophy were (1) Body mass index, (2) blood parameters and (3) lipodystrophy-specific physical traits (change in central adiposity including breast enlargement, dorsal fat accumulation and increased abdominal girth; evaluation of peripheral lipoatrophic features including decreased fat tissue in the face, buttocks and extremities leading to prominent veins). These data were compared to those independently recorded by the patients in the self-assessment questionnaire. These clinical data led to the establishment of a classification system based on the absence or presence of changes (mild or severe).

None of the HIV-negative patients had change in signs of lipodystrophy, whereas 10% of the naive HIV patients and 45% of the HAART-treated patients did ($P<0.001$). The mean mtDNA to nDNA ratio of the 62 patients who exhibited morphological manifestations of lipodystrophy was 0.16 ± 0.14; this mean value was not significantly different from that found in the HIV-1-infected patients who did not exhibit signs of lipodystrophy syndrome (0.13 ± 0.14).

The mean triglyceride level was significantly higher in the HAART-treated patients than in the nontreated patients (2.4 ± 1.0 *vs* 1.2 ± 1.7 mmol/l, respectively, $P=0.001$).The mean total cholesterol concentration was similar in the HAART-treated patients (4.8 ± 1.3 mmol/l) and in the untreated patients (4.7 ± 1.4 mmol/l) as well as the mean HDL cholesterol levels (controls: 1.45 ± 0.36 mmol/l; untreated HIV patients: 1.13 ± 0.51 mmol/l; treated HIV patients: 1.17 ± 0.40 mmol/l; no significant differences were observed). No difference was also observed in creatine phospokinase levels (104 ± 81 *vs* 104 ± 63 UI/l) or amylasaemia (67 ± 33 *vs* 82 ± 31 UI/l) between the treated and nontreated HIV patients, respectively. None of these concentrations were correlated with the mtDNA to nDNA ratio.

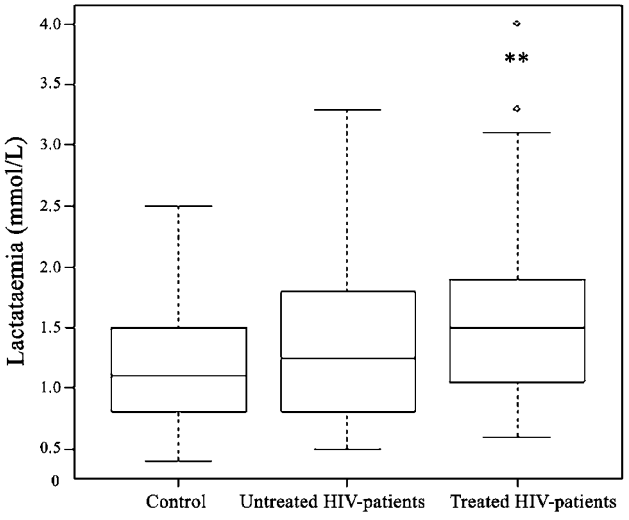

Figure 2 Lactataemia (mmol/l) in the different group of patients. **The difference between control and treated HIV patients is signicant. Fisher's and Student's test, $P<0.001$.

Effect of Treatment

We analysed the mtDNA to nDNA ratio in the HAART-treated patients according to treatment duration and type. All the patients had received AZT and 3TC and then were switched to either ABC/TDF or DDI/D4T. The mtDNA to nDNA ratio was lower in patients receiving DDI/D4T than in patients receiving AZT/3TC ($P<0.05$). When DDI/D4T was associated with either AZT/3TC or ABC/TDF, no significant decrease in mtDNA to nDNA ratio was observed (Table 2). When the treatments were considered altogether, the mtDNA to nDNA ratio was lower in patients receiving HAART for longer than 5 years than in control patients ($P<0.05$).

No difference was observed in the mtDNA to nDNA ratio between patients who had received protease inhibitors and those who had not (respectively 0.11 ± 0.07 and 0.09 ± 0.06).

Table 2 Current treatment at time of mtDNA blood levels evaluation

Current treatment	*Number of patients*	*Sex F/M*	*Age (mean±s.d. years)*	*CD4-count (mean±s.d. number/mm³)*	*Duration of the current treatment (mean±s.d. months)*	*Cumulative duration of treatment (mean±s.d. months)*	*Ratio mtDNA/β-actin (mean±s.d.)*
AZT+3TC	31	4/27	46.4±8.9	460±342	17.5±16.9	24.6±18.1	0.12±0.11
AZT+3TC+ABC+TDF	16	3/13	41.7±9.3	332±210	10.1±9.0	11.6±6.2	0.09±0.05
DDI+D4T	12	1/11	44.2±7.0	388±203	19.0±10.2	65.0±45.2	0.08±0.04
DDI+D4T+AZT+3TC	42	11/31	44.0±9.8	485±248	23.2±17.3	33.2±21.0	0.10±0.09
DDI+D4T+ABC+TDF	10	3/7	46.9±10.5	303±197	12.7±8.6	28.4±20.5	0.15±0.11
Other treatments	33	7/26	43.2±9.6	320±213	17.4±17.1	68.3±52.8	0.13±0.12

There are classified into six groups according to the combination of drugs. The duration of treatment in each group was not significantly different. The mtDNA to nDNA ratio was lower in patients receiving DDI+D4T than in patients receiving AZT+3TC ($P<0.05$). AZT = zidovudine, 3TC = lamivudine, ABC = abacavir, TDF = tenofovir, DDI = didanosine and D4T = stavudine.

Discussion

It is difficult to diagnose mitochondrial toxicity and no metabolic or serological diagnostic assays are currently able to predict which patients will be affected. However, most patients treated with NRTI do not develop mitochondrial toxicity or only have mild and/or long-term toxicity that is more difficult to evaluate during regular follow-up. Thus, we chose to assess out-patients, to mimic the basic situation of the physician faced with patients who exhibit moderate side effects compared to during their previous visit.

Acute lactate acidosis, the most dramatic presentation of mitochondrial toxicity, which is unfortunately fatal in most cases, is rare with about 1.3 cases per 1000 person-years of nucleoside exposure.[13,17] We observed the same incidence in our cohort of 1000 patients, but none of the patients in our series had an episode of lactic acidosis during the evaluation period (bicarbonate levels were above 22 mmol/l and lactate levels were below 5 mmol/l for all the patients). One patient had a severe episode of lactic acidosis 1 year before the study period, but the acid–base equilibrium was normal at the time of evaluation and this patient's mtDNA to nDNA ratio was 32% of the mean control value.

In our study, the mtDNA to nDNA ratios were lower in the HIV patients than in controls. However, this difference was observed both in naive patients and in HAART-treated patients. The decrease in the mtDNA to nDNA ratios in untreated HIV-1 patients are in agreement with those reported by Cote *et al*[18] and Montaner *et al*[19] but not with those of Petit *et al*.[25] The decrease in mtDNA to nDNA ratio in the HIV patients suggests a direct mitochondrial toxicity of the virus. These observations are supported by *in vitro* studies in which HIV-infected cells showed signs of mitochondrial necrosis.[26] A mitochondrion-controlled mechanism of cell death may occur in HIV infection.[27–29] The lack of a significant difference in the mtDNA to nDNA ratio between HIV-nontreated and HIV-treated patients may be due to the absence of acute mitochondrial toxicity in our cohort of patients. Similar results have previously been shown in two studies in which the authors failed to demonstrate mtDNA depletion in asymptomatic patients on different HAART schedules compared with HIV-negative patients.[21,22] Thus, we can also hypothesize, as the latter authors, that mitochondrial toxicity can lead to a decrease in mitochondrial DNA content in the tissue concerned by the toxicity (ie liver or skeletal muscle) and not in peripheral leukocytes. This conclusion can be compared to a case report of one patient with fatal lactic acidosis; the mitochondrial DNA content in the liver of this patient was found to be abnormally low.[10]

We found that 10% of untreated HIV-1 patients and 45% of HAART-treated patients had lipodystrophy syndrome, as previously described.[30] These values are in agreement with previous findings, showing that about 50% of patients had at least one physical abnormality characteristic of lipodystrophy after 12–18 months of treatment.[31–34] Moreover, the frequency of the lipodystrophy syndrome increased with the duration of antiretroviral treatment. However, the value of mtDNA to nDNA ratio was not correlated with the presence of fat redistribution. This may be due to the fact that the mtDNA concentration is already decreased in the mononuclear cells of patients that have never received any antiviral treatment. Only triglyceride levels were significantly higher in treated HIV patients than in naive HIV patients as recently shown by Belloso *et al*.[35]

Stavudine and didanosine are the two drugs that have most frequently been reported to be associated with hyperlactataemia and thus mitochondrial toxicity.[13,36–39] This finding is in agreement with *in vitro* studies demonstrating that these two drugs have a high capacity to inhibit gamma polymerase[40] and that the triphosphorylated form of stavudine is incorporated into replicating DNA more readily than other NRTIs.[41,42] We also found that patients treated with stavudine had lower mtDNA to nDNA ratios than those who had never received this drug. However, this difference can be observed in a cohort of patients treated for a long period with HAART but not between two routine visits.

In conclusion, although mtDNA depletion has recently been proposed to be a marker of clinically evident mitochondria-related adverse effects, it can probably not be used as a routine marker to predict who will develop mitochondrial toxicity in a cohort of patients with moderate side effects.

Duality of interest

None declared.

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