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Mitochondrial DNA and RNA Increase in Peripheral Blood Mononuclear Cells from HIV-1–Infected Patients Randomized to Receive Stavudine-Containing or Stavudine-Sparing Combination Therapy

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Background. Mitochondrial DNA (mtDNA) in peripheral blood mononuclear cells (PBMCs) has been suggested as a potential marker of mitochondrial toxicity associated with nucleoside analogue reverse-transcriptase inhibitor–containing therapy.

Methods. We quantified mtDNA and mitochondrial RNA (mtRNA) in PBMCs over the course of 48 weeks in 78 patients infected with human immunodeficiency virus type 1 (HIV-1) who were randomly assigned to receive ritonavir-boosted indinavir and efavirenz with or without stavudine. Furthermore, we analyzed the association of mtDNA and mtRNA with clinical signs and symptoms and/or abnormalities in laboratory markers attributed to mitochondrial toxicity.

Results. No statistically significant difference was found in mtDNA and mtRNA content over time between the 2 treatment arms. When arms were combined, both median mtDNA and mtRNA content showed statistically significant increases over the course of 48 weeks, from 206 to 278 copies/cell ($P < .001$) and from 154 to 288 copies/cell ($P = .003$), respectively. No statistically significant difference in mtDNA and mtRNA content was found between patients with and those without adverse events attributed to mitochondrial toxicity.

Conclusions. The observed increases in mtDNA and mtRNA content during the first year of treatment may represent a restorative trend resulting from suppression of HIV-1 infection, independent of the treatment used. Future studies should focus on well-defined mitochondrial toxicities and changes in these markers within the corresponding affected tissues simultaneously with those in PBMCs. Furthermore, with respect to studies of peripheral blood, mtDNA and mtRNA content in individual cell subtypes rather than in PBMCs may be better markers of toxicity and deserve further investigation.

The introduction of highly active antiretroviral therapy has resulted in a significant reduction in HIV-1–related

morbidity and mortality. Treatment, however, is often associated with the occurrence of adverse events, including peripheral neuropathy [1, 2], myopathy, lactic acidosis, hyperlactatemia, and lipoatrophy [3, 4]. Mitochondrial toxicity due to the inhibition of the mitochondrial DNA polymerase-γ by nucleoside analogue reverse-transcriptase inhibitors (NRTIs) has been suggested as a possible causal mechanism for the development of a number of these treatment-related adverse events. According to previous studies, use of NRTIs could have a serious impact on mitochondrial function by interfering with the process of mitochondrial DNA (mtDNA) replication [5, 6]. A decrease in mtDNA

would eventually lead to diminished cellular energy production. Tissues and cells would be affected to various extents according to relative energy requirements [7], mitochondrial biochemical threshold levels [8], differential effects of specific drugs, and their penetration within the organelle. Therefore, mtDNA has been studied as a marker, and its content has been assayed as a possible reflection of mitochondrial disruption and related pathological abnormalities [1, 9–11]. The role of mitochondrial RNA (mtRNA) content has not received much attention in this context.

To try to obtain more insight into the effect of NRTIs on mitochondria during treatment, we compared the course of mtDNA and mtRNA content in peripheral blood mononuclear cells (PBMCs) from HIV-1–infected subjects randomly assigned to receive 48 weeks of treatment with ritonavir-boosted indinavir and efavirenz in the presence or absence of the NRTI stavudine. Previous in vitro studies in T lymphoblastoid cell lines have shown that stavudine is associated, in a concentration-dependent manner, with depletion of mtDNA [12, 13]. Furthermore, patients exposed to stavudine are known to be at increased risk of developing peripheral neuropathy, symptomatic hyperlactatemia, lactic acidosis, and peripheral lipoatrophy, each of which has been suggested to reflect mitochondrial toxicity [14, 15]. Hence, we also performed a retrospective analysis to assess possible correlations between adverse events reported in the course of the trial that could possibly be attributed to mitochondrial toxicity and changes in mtDNA and mtRNA content in PBMCs over time.

PATIENTS, MATERIALS, AND METHODS

Patient characteristics. Available PBMCs from 78 of the 93 HIV-1–infected patients enrolled in the European and South American Study of Indinavir, Efavirenz, and Ritonavir (EASIER) [16] trial were obtained from a central laboratory where they had been viably cryopreserved. Blood samples from these patients had been collected at week −2 (before start of treatment) and after 12, 24, 36, and 48 weeks of treatment. The EASIER trial was a multicenter, open-label, randomized 48-week study to evaluate the efficacy, safety, and tolerability of an stavudine-sparing regimen of ritonavir-boosted indinavir (100–800 mg twice daily) plus efavirenz (600 mg once daily) with or without stavudine (40 mg twice daily for patients weighing ≥60 kg or 30 mg twice daily for patients weighing <60 kg). Participants were ≥18 years of age, had $CD4^+$ T cell counts ≥100 cells/mm³ and plasma HIV-1 RNA levels ≥5000 copies/mL, and were protease inhibitor, stavudine, and nonnucleoside reverse-transcriptase inhibitor naive. Patients, after having provided written, informed consent, were randomly assigned either to the stavudine-sparing arm or to the stavudine-containing arm.

Assessment of mitochondrial nucleic acids. Aliquots of PBMCs had been obtained from each blood sample by means of density gradient separation in Vacutainer cell-preparation tubes with Sodium Heparin (Becton Dickinson) followed by 2 additional washing and centrifugation steps, to avoid any significant degree of platelet contamination. PBMCs were subsequently counted and viably cryopreserved by means of an automatic temperature decline and storage in liquid nitrogen. Before total nucleic acid isolation, the PBMC samples were thawed by immersion of the ampule in a water bath at 37°C, microscopically checked for platelet amount, and eventually washed to limit the contamination with platelets to a level that did not alter the results of mtDNA quantification [17, 18]. After lysis of an amount equivalent to 3×10^5 PBMCs by means of chaotropic guanidinium thiocyanate buffer, total nucleic acids were extracted by a silica-based method [19].

The calibration line consisted of a dilution series of plasmid mixes containing both mtDNA and nuclear DNA (nDNA) or both mtRNA and nDNA. An equivalent of 3×10^3 cells was used as assay input for each sample, which was then assayed in duplicate by means of real-time duplex nucleic acid sequence–based amplification (Retina Mitox; Primagen). The mtRNA measured by our assay is the heavy-chain transcript of the gene coding for cytochrome-*c* oxidase subunit I. The relative mean of each mtDNA-to-nDNA and mtRNA-to-nDNA duplicate ratio was subsequently extrapolated from the calibration line to determine numbers of mtDNA and mtRNA copies per cell.

Analysis of adverse events. The trial database was retrospectively queried for any severe clinical or laboratory adverse event (AIDS Clinical Trials Group toxicity grade 3 or 4) [20] or a combination of both that occurred during treatment. Subsequently, the following clinical and laboratory adverse events were analyzed for possible relation to changes in mtDNA and mtRNA content in PBMCs over the course of 48 weeks of therapy: paresthesia, peripheral neuropathy, lipodystrophy, hepatic steatosis, lactic acidosis, and grade 3 or 4 change in amylase, aspartate and alanine aminotransferase, and hemoglobin levels. Furthermore, patients for whom a combination of ≥2 of the following signs and symptoms had been reported—anorexia, asthenia, abdominal pain, malaise, and unexplained weight loss—were also classified as possibly having experienced mitochondrial toxicity–related clinical adverse events.

Statistical methods. The primary study outcome was the change from baseline over the course of 48 weeks in the absolute number of mtDNA and mtRNA copies per cell, with comparison of patients in the stavudine-sparing arm with those in the stavudine-containing arm. The primary analysis was conducted by intention to treat, irrespective of treatment interruption or modification. A during-treatment analysis, including only data from patients undergoing randomly allocated treatment, was also performed.

The changes from baseline in numbers of mtDNA and mtRNA copies per cell were analyzed by a repeated-measure-

ments procedure using a generalized linear model (PROC MIXED; SAS software, version 8.02; SAS Institute). Such an analysis assumes that serial measurements of the outcome variable for any specific patient are correlated and provides a valid statistical estimate of the main effect. Furthermore, correlation coefficients were calculated to assess any relation between mtDNA and mtRNA content in PBMCs with HIV-1 RNA level and CD4$^+$ T cell counts at weeks -2 and 48.

For the analysis of the association between the above-mentioned selected clinical and laboratory adverse events and changes in mtDNA and mtRNA content in PBMCs, a repeated-measurement analysis was used to compare patients with and without such adverse events. The level of significance was set at 5% for all analyses. All reported P values are 2-sided.

RESULTS

Patient disposition. Of 93 HIV-1–infected patients enrolled in the EASIER trial, 78 (84%) were selected for this substudy because samples were available. This subgroup was representative of the total trial population, with similar CD4$^+$ T cell counts and HIV-1 RNA levels at baseline (mean CD4$^+$ T cell count, 365 cell/mm^3 in the main study and 394 cell/mm^3 in our substudy [SD, 209 cells/mm^3]; mean HIV-1 RNA level, 4.7 log$_{10}$ copies/mL in the main study and 4.8 log$_{10}$copies/mL in our substudy [SD, 0.6 log$_{10}$ copies/mL]).

Of the 78 patients from whom PBMC samples were available for analysis, 73 were treatment naive at trial entry and only 5 had been previously exposed to combinations of nucleoside analogues for an average duration of 3 years, including zidovudine with lamivudine ($n = 2$), zidovudine with didanosine ($n = 1$), and zidovudine with zalcitabine ($n = 2$). Patients had been randomly allocated either to the stavudine-containing regimen ($n = 40$) or to the stavudine-sparing regimen ($n = 38$) (table 1). Of the 78 patients analyzed, 21 experienced clinical and/or laboratory adverse events possibly attributable to mitochondrial toxicity (table 2).

mtDNA and mtRNA copies per cell. An analysis of the 5 patients allocated to the stavudine arm who had been exposed to antiretroviral therapy before study enrollment (2 patients who received and 3 who did not receive stavudine) did not yield any statistically significant difference in changes in mtDNA or mtRNA content, compared with those in the 73 treatment-naive patients. Hence, data from all 78 patients were included in the subsequent analyses.

The change in mtDNA content over the course of 48 weeks was not statistically significantly different between the 2 treatment arms ($P = .23$) (figure 1A). Similarly, no significant difference in the course of mtRNA content was detected between arms ($P = .68$) (figure 1B). The during-treatment analysis, limited to data from patients undergoing allocated therapy (86% at week 36 and 42% at week 48), also did not reveal any sta-

Table 1. Baseline demographic characteristics, immunologic and virological status, exposure to antiretroviral therapy, and mitochondrial DNA (mtDNA) and mitochondrial RNA (mtRNA) content of peripheral blood mononuclear cells for patients receiving ritonavir-boosted indinavir and efavirenz with or without stavudine in a study of the mitochondrial toxicity of antiretroviral therapy.

Characteristic	Indinavir/ ritonavir/ efavirenz ($n = 38$)	Indinavir/ ritonavir/ efavirenz plus stavudine ($n = 40$)
Antiretroviral naive, no. of patients	38	35
Male, no. (%) of patients	30 (79%)	30 (75%)
Age, years	36 (30–46)	34 (31–38)
White race, no. of patients	35	38
CD4$^+$ T cell count, cells/mm^3	374 (315–535)	336 (198–413)
HIV-1 RNA level, log$_{10}$ copies/mL	4.8 (4.3–5.1)	4.8 (4.2–5.3)
mtDNA content, log$_{10}$ copies/cell	2.34 (2.27–2.44)	2.28 (2.19–2.42)
mtRNA content, log$_{10}$ copies/cell	2.03 (1.84–2.33)	2.20 (1.86–2.53)

NOTE. Unless indicated otherwise, data are median (interquartile range). No comparison was significant.

tistically significant differences between the 2 regimens (mtDNA, $P = .14$; mtRNA, $P = .69$). Analysis of the change in CD4$^+$ T cell count and HIV-1 RNA level from baseline to 48 weeks of therapy did not yield any statistically significant difference between regimens ($P = .13$ and $P = .43$, respectively). Furthermore, results of correlation analyses between HIV-1 RNA level and CD4$^+$ T cell count relative to both mtDNA and mtRNA content of the 2 treatment arms at week -2 and at week 48 were not statistically significant (data not shown).

Because therapy allocation appeared to have no effect, both therapy arms were combined, and an analysis on the combined data set of the 78 patients was performed. This demonstrated a statistically significant increase over the course of 48 weeks in mtDNA content in PBMCs, from a median of 206 to 278 copies/cell ($P < .001$); there was also a significant increase over the course of 48 weeks in mtRNA content in PBMCs, from a median of 154 to 288 copies/cell ($P = .003$).

Finally, analysis of the change in mtDNA and mtRNA content in PBMCs from patients with either clinical ($n = 15$) or both clinical and laboratory ($n = 21$) adverse events possibly attributable to mitochondrial toxicity, compared with patients without such adverse events, did not yield a statistically significant difference ($P = .81$ and $P = .79$ for mtDNA and mtRNA, respectively, for patients with clinical adverse events vs. $P = .75$ and $P = .45$ for mtDNA and mtRNA, respectively, for patients with both clinical and laboratory adverse events) (figure 2). Both of these arms likewise showed a statistically significant increase in mtDNA and mtRNA content over time.

DISCUSSION

Several studies have reported changes in mtDNA content with-

Table 2. Selected grade 3 or 4 adverse clinical and laboratory events reported in patients receiving ritonavir-boosted indinavir and efavirenz with or without stavudine over the course of 48 weeks of therapy, by treatment arm.

Adverse event	Patients, no.	Indinavir/ ritonavir/ efavirenz	Indinavir/ ritonavir/ efavirenz plus stavudine
Clinical entities	9	1	8
Paresthesia	3	0	3
Peripheral neuropathy	2	0	2
Lipodystrophy	1	0	1
Hepatic steatosis	1	0	1
Lactic acidosis	2	1	1
Symptoms (combination of ≥2)	6	2	4
Abdominal pain	8	4	4
Anorexia	5	3	2
Asthenia	8	5	3
Weight loss	3	0	3
Laboratory parameters[a]	7	1	6
Amylase increase	2	0	2
Hemoglobin decrease	0	0	0
Alanine aminotransferase increase	8	3	5
Aspartate aminotransferase increase	4	1	3

[a] AIDS Clinical Trials Group toxicity grade 3 or 4.

in a number of tissues as a result of inhibition of mtDNA replication by NRTIs [9–11, 21] and have attempted to relate these to the presence of certain NRTI-associated adverse events. Blood being the most easily obtainable tissue, this has also led to attempts to relate changes in mtDNA content in PBMCs or cell fractions thereof to the occurrence of (assumed) mitochondrial toxicities originating in tissues other than blood, such as peripheral lipoatrophy, symptomatic hyperlactatemia, and lactic acidosis. Apart from the varying degree to which different NRTIs have been demonstrated to deplete mtDNA content of blood cells by inhibition of DNA polymerase-γ, a number of studies have also demonstrated that the presence of HIV-1 infection itself, in the absence of antiretroviral therapy, may also be associated with reduced mtDNA content of blood cells [9, 22–26]. Thus, net mtDNA content within tissues, including blood cells, has been suggested to be influenced by both the type and the duration of NRTI therapy [23, 27], the presence and degree of suppression of HIV-1 infection, and the differential energy needs of specific tissues [28].

To try to improve our understanding of mtDNA dynamics in blood during treatment of HIV-1 infection, we performed a longitudinal 48-week investigation of mtDNA content in PBMCs within the context of a randomized clinical trial in which participants, >90% of whom were treatment naive, began treatment with either an stavudine-sparing treatment regimen or this same regimen including stavudine. In addition, mtRNA content was assessed in the same samples on the hypothesis that, because mtRNA reflects mitochondrial transcrip-

tional activity, this could potentially be a closer marker of mitochondrial functional capacity than is mtDNA. The main finding from these 78 HIV-1–positive patients, 73 of whom started HIV therapy for the first time, was a significant increase in mtDNA content in PBMCs after the start of therapy, irrespective of the inclusion of stavudine in the regimen. This suggests that antiretroviral therapy, by suppressing viral replication, may, at least over the short term, correct any mtDNA-depleting effect of HIV-1 infection, resulting in a recovery of mtDNA content within blood cells. Within the setting of a controlled randomized clinical trial, our results confirm the findings of Miura et al. [23] in an observational cohort of patients. Our results suggest that, during an initial 48-week exposure, the inclusion of stavudine in the regimen did not hamper recovery of mtDNA. This does not rule out the possibility that, with more-prolonged exposure to stavudine, its inhibition of DNA polymerase-γ may become the prevailing effect, resulting in a longer-term reduction in mtDNA content in PBMCs, as has been demonstrated by other studies [29]. In contrast, inclusion of NRTIs in the treatment regimen, which in vitro are known to be more toxic to mtDNA (e.g., zalcitabine [30]), albeit in the presence of suboptimal suppression of HIV-1, have been shown to result in depletion of mtDNA within PBMCs, even after only short-term exposure [1]. Thus, the net effect of antiretroviral therapy on mtDNA content in PBMCs may well rely on the composite of several factors, including antiviral potency of the regimen, intrinsic capacity to decrease mtDNA of particular components of the regimen, duration of exposure, and individual suscep-
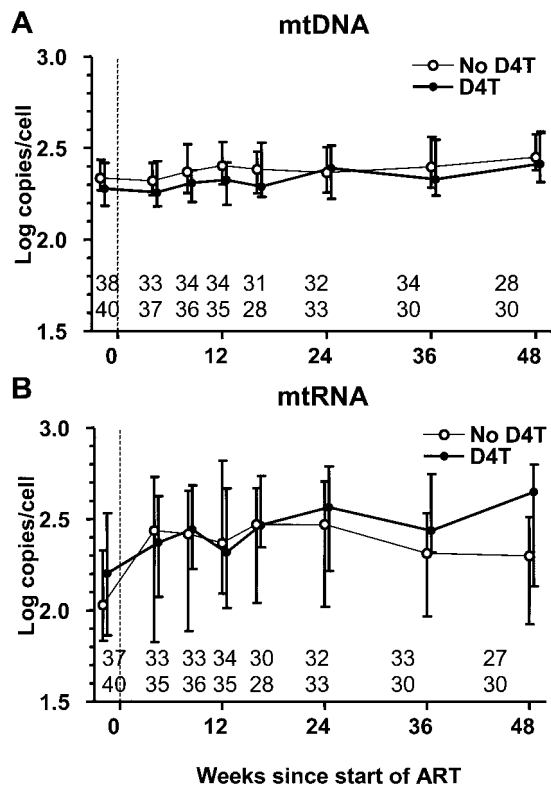
Figure 1. Comparison between stavudine (d4T)–containing and –sparing regimens. The plots illustrate the median absolute values and interquartile ranges of mitochondrial DNA (mtDNA) *(A)* and mitochondrial RNA (mtRNA) *(B)* content for each therapy arm over time. The nos. at the bottom indicate the no. of available samples at each time point.

tibility. Exactly how suppression of HIV-1 infection by antiretroviral therapy might counteract the deleterious effects of infection on mitochondria, thereby evoking recovery of mtDNA content in PBMCs, is as yet unknown [31]. One possibility is that therapy potentially counteracts generation of oxidative stress by HIV-1 infection. Oxidative stress results from a perturbation of the balance between the formation of reactive oxygen species and the antioxidant pool [32]. Previous studies reported that lymphocytes of HIV-1–infected patients presented increased levels of reactive oxygen species [33]. Increased levels of reactive oxygen species may lead to a decrease in mtDNA content [34, 35]. The simultaneous effect of antiretroviral therapy on HIV-1, leading to its suppression, together with the endogenous antioxidant pool, could alleviate the burden of reactive oxygen species by reducing them to nonlethal levels. Nonlethal concentrations of reactive oxygen species have been reported to act as secondary messengers and induce nuclear respiratory factor−1 and −2, which have been associated with the transcriptional control of nuclear-encoded mitochondrial proteins involved in the maintenance and replication of mtDNA, as well as electron transport chain proteins [36, 37]. We hypothesize an initial restorative effect, mediated by the

balance between the formation of reactive oxygen species and the antioxidant pool, on mtDNA that results from antiretroviral therapy, which apparently outweighs the inhibiting effects of NRTIs on polymerase-γ [9, 23, 38].

In the present study, we also aimed to address the course of mtRNA in PBMCs during treatment as possibly a more direct marker of mitochondrial gene expression and activity than is mtDNA. Very similar to what was seen with mtDNA, mtRNA content showed a statistically significant increase, again irrespective of randomly allocated treatment. Because mitochondrial replication and transcription are functionally linked [39], our results may indicate a recovery of nucleic acids required for adequate mitochondrial function—that is, the expression of mitochondrial DNA-encoded proteins—directly involved in the oxidative phosphorylation system and ultimately in the production of ATP.

In addition to our analysis of the influence of therapy on mtDNA and mtRNA content, we sought to assess the correlation between either of these markers and the occurrence dur-
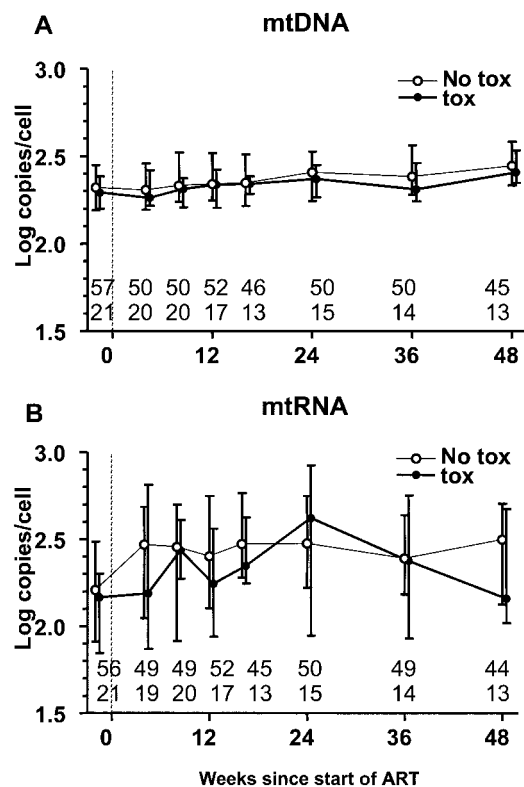

Figure 2. Comparison of patients with and without adverse events possibly attributable to mitochondrial toxicity. Mitochondrial DNA (mtDNA) *(A)* and mitochondrial RNA (mtRNA) *(B)* content are expressed as median absolute values and interquartile ranges, for both therapy arms combined, distinguishing available samples from patients with (tox; *n* = 21) and without (no tox) clinical and laboratory adverse events attributed to mitochondrial toxicity. The nos. at the bottom indicate the no. of available samples at each time point.

ing therapy of a set of adverse events selected from the trial database that could potentially be attributed to mitochondrial toxicity. No statistically significant correlation was found between either mtDNA or mtRNA content in PBMCs and the presence or absence of such adverse events. With consideration of the declining trend of mtRNA content in PBMCs from week 24 onwards for the group of patients in whom adverse events were reported, it may be interesting to further investigate mtRNA content in PBMCs as a marker of dysfunction of mitochondrial gene expression and well-defined adverse events related to mitochondrial toxicity over an extended period of follow-up. In addition, the part of the analysis regarding the utility of measuring mtDNA or mtRNA content in PBMCs as a predictive marker of NRTI-related adverse events needs to be interpreted with caution, in view of both the uncertainty with which adverse events were attributed to possible mitochondrial toxicity and the limited incidence of each particular event.

For example, changes in laboratory values, such as aspartate and alanine aminotransferase levels, as well as symptoms and signs compatible with symptomatic hyperlactatemia in the absence of information on serum lactate, could only arbitrarily be attributed to mitochondrial toxicity, and this may well have resulted in an overestimation of the number of events truly representing mitochondrial toxicity. In view of the limited number of specific events in each category, there unfortunately was insufficient statistical power to perform separate analyses for each of these. On the other hand, the lack of correlation with changes in mtDNA or mtRNA content in PBMCs may be understood in view of expected differences in energy requirement, mitochondrial threshold level [28], and drug exposure in mitochondria within specific tissues directly responsible for a certain adverse event as opposed to those in PBMCs [40]. Furthermore, it is possible that, during HIV-1 infection, factors such as cell proliferation and persistent immune activation might influence the different cell subtypes within PBMCs differently, with consequences for the relative mtDNA and mtRNA content in each cell subtype [41]. Therefore, future experiments involving peripheral blood may well yield more insight by assessing mitochondrial toxicity markers in individual cell subtypes when sampling PBMCs.

In conclusion, first-time initiation of potent combination antiretroviral therapy over the course of 48 weeks was associated with an increase in both mtDNA and mtRNA content in PBMCs, irrespective of the inclusion or lack of inclusion of stavudine. This may represent a recovery of mtDNA depletion induced by HIV-1 infection per se, which, for this particular regimen, at least over the short term, is not negated by drug-induced mitochondrial toxicity. These counteracting mechanisms should be taken into account when assessing the effect of other initial treatment regimens on mitochondria. No statistically significant relation was found between the presence or absence of a selected set of adverse events potentially attributable to mitochondrial toxicity and the change in mtDNA and mtRNA content in PBMCs. However, this result needs to be interpreted with caution, in view of limitations concerning both the number of particular events and the reliability with which some of these were attributed to mitochondrial toxicity, as well as the limited study duration. Nevertheless, it will be important and informative for future studies addressing potential relationships between changes in mitochondrial markers and certain toxicities to try to simultaneously sample both blood and the particular tissue that is relevant to the toxicity being studied.

Acknowledgments

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