Mitochondrial toxicity in HIV-infected patients both off and on antiretroviral treatment: a continuum or distinct underlying mechanisms?

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Mitochondrial toxicity contributes to serious adverse effects observed in HIV-infected individuals treated with nucleoside reverse transcriptase inhibitors (NRTIs). However, similar mitochondrial abnormalities have recently been found even in treatment-naïve patients, suggesting that chronic HIV per se could contribute to the toxicity observed in NRTI-exposed individuals. This review gives a current status of the field, with particular focus on recent observations suggesting that distinct mechanisms might cause such toxicity in both NRTI-exposed individuals and those naïve to antiretroviral treatment.

Keywords: HIV-1, mitochondria, DNA polymerase gamma, nucleoside reverse transcriptase inhibitors, persistent immune activation

Introduction

Combination antiretroviral therapy (ART) has over the last decade proven its excellent clinical efficacy.1 Increasing attention has therefore been focused on long-term adverse effects of ART, including the devastating fat distribution changes observed in the lipodystrophy syndrome and the increased risk for cardiovascular disease.2–5 However, although much effort has been made in understanding the possible underlying mechanisms for such adverse effects, our insight is still incomplete and reliable surrogate markers for ART-related toxicity are not accessible. It should be noted, though, that some nucleoside reverse transcriptase inhibitors (NRTIs; Table 1) have been noticeably associated with many of these long-term adverse effects.6 The triphosphate forms of NRTIs compete with endogenous nucleotides for HIV DNA synthesis by the HIV enzyme reverse transcriptase (RT), thus acting as chain terminators. Unfortunately, NRTIs can to a variable extent also be substrates for mitochondrial DNA (mtDNA) polymerase γ and, as a result, interrupt replication even of mtDNA. It was hypothesized early on that mitochondrial toxicity caused by inhibition of DNA polymerase γ (DNA pol γ) was responsible for NRTI-associated adverse effects.6 First, zidovudine-associated skeletal myopathy was observed early after the introduction of the drug in 1987, and subsequently ragged-red fibres and reduction of mtDNA content as well as morphological changes in the mitochondria were demonstrated in skeletal muscle.7,8 Second, mitochondrial changes were also observed in HIV-infected patients with peripheral neuropathy, insulin resistance, hyperlactataemia and lactic acidosis, the latter being a life-threatening condition often associated with hepatosteatosis and/or pancreatitis.9–13 In 1999, it was postulated that NRTI-associated mitochondrial toxicity might play an essential role also in the lipodystrophy syndrome14,15 and was particularly associated with exposure to the thymidine analogue stavudine.16 Against this background, assessments of mitochondrial toxicity in patients on ART therefore became a relevant area for clinical research using peripheral blood mononuclear cells (PBMC) as a readily available cellular material. Later, it was learned that mtDNA content may be quite unaffected in PBMC, while at the same time it is decreased in specific tissues.7,10,17 Another complicating factor was the observation that mtDNA was reduced in PBMC not only in patients treated with certain NRTIs, but also in ART-naïve patients.18–22 Thus, HIV infection per se appears to be associated with mitochondrial toxicity in HIV-infected individuals, at least in PBMC, and this phenomenon complicated the data interpretation of NRTI toxicity in ART-exposed patients.

The objective of this review is to discuss the possible mechanisms underlying mitochondrial toxicity in relation to treatment with NRTIs as well as to untreated HIV infection. Enhanced understanding of the mechanisms behind these effects is relevant for future management of long-term ART and adverse effects in HIV-infected patients.
Mitochondria: structure and function in human cells

Mitochondria are small intracellular organelles (diameter of 0.5–1 μm) located in the cellular cytoplasm and were first discovered by Altman in 1890. In the early 1960s it was found that mitochondria contain their own, almost exclusively maternally inherited, DNA and in 1981, the mitochondrial genome, consisting of 16569 bp, was sequenced. All cells contain mitochondria, with the exception of erythrocytes. A single cell may contain hundreds to thousands of mitochondria, particularly cells with a high energy demand, and each mitochondrion contains 2–10 copies of mtDNA. Mitochondria are highly motile and were recently shown to undergo both fusion and fission through complex mechanisms. Because mitochondria lack several enzymes for DNA repair, they are particularly susceptible to mutation. The mutation rate of mtDNA is 10 times higher than that of nuclear DNA. However, mitochondrial dysfunction probably requires a minimum threshold level of either mutant mtDNA or mtDNA mass to become clinically relevant.

Mitochondria have a double lipid membrane, with an inner membrane that is folded into numerous cristae surrounding the matrix space. This matrix contains copies of the mtDNA genome, which encodes subunits of four of the five complexes of the oxidative phosphorylation (OXPHOS) system located in the inner membrane of the mitochondrion. The OXPHOS system is responsible for providing most of the energy to cells; however, mitochondria also play a key role in apoptosis, β-oxidation of free fatty acids and calcium homeostasis.

Table 1. Nucleoside and nucleotide reverse transcriptase inhibitors and their relation to mitochondrial toxicity and clinically adverse effects as well as evidence of reversibility of mitochondrial toxicity

<table>
<thead>
<tr>
<th>Nucleoside or nucleotide analogues</th>
<th>Mitochondrial toxicity</th>
<th>Clinically adverse effects related to mitochondrial toxicity</th>
<th>Evidence of reversibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine analogues</td>
<td>yes</td>
<td>myopathy, lactic acidosis, peripheral neupathy, nephropathy</td>
<td>dose reduction, oxidative phosphorylation, switch-studies</td>
</tr>
<tr>
<td>zidovudine (AZT/ZDV)</td>
<td>yes</td>
<td>lactic acidosis, peripheral neuopathy, nephropathy</td>
<td>dose reduction, insulin resistance, switch-studies</td>
</tr>
<tr>
<td>stavudine (d4T)</td>
<td>yes</td>
<td>lactic acidosis, peripheral neuopathy, nephropathy</td>
<td>dose reduction, insulin resistance, switch-studies</td>
</tr>
<tr>
<td>didanosine (ddI)</td>
<td>yes</td>
<td>peripheral neuopathy, nephropathy</td>
<td>dose reduction, insulin resistance, switch-studies</td>
</tr>
<tr>
<td>zalcitabine (ddC)</td>
<td>yes</td>
<td>peripheral neuopathy, nephropathy</td>
<td>dose reduction, insulin resistance, switch-studies</td>
</tr>
<tr>
<td>lamivudine (3TC)</td>
<td>no</td>
<td></td>
<td></td>
</tr>
<tr>
<td>emtricitabine (FTC)</td>
<td>no</td>
<td></td>
<td></td>
</tr>
<tr>
<td>abacavir (ABC)</td>
<td>no</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tenofovir disoproxil fumarate (TDF)</td>
<td>no?</td>
<td>nephropathy?</td>
<td>switch-studies</td>
</tr>
</tbody>
</table>

NRTI-related effects on mitochondria: differences between drugs and tissue-specific susceptibilities

Effects on DNA pol γ

The nuclear-encoded mitochondrial DNA pol γ is the only DNA polymerase found in mitochondria and is therefore crucial for mtDNA replication as well as for mtDNA repair capacity. DNA pol γ consists of a catalytic subunit of both polymerase and 3′–5′ exonuclease activity, which is associated with the mitochondrial DNA pol γ accessory subunit that stimulates the catalytic and exonuclease activity of the larger catalytic subunit. Inhibition of mtDNA replication subsequently leads to a reduction of mtDNA content, and thereby decreased synthesis of the mtDNA-encoded protein subunits of the OXPHOS system and reduced ATP production, as well as enhanced generation of reactive oxygen species. Quantification of the mtDNA content has therefore frequently been used as a tool for determining mitochondrial toxicity in clinical research. However, reduced mtDNA content along with normal mtRNA levels encoded by mitochondrial genes has also been found, suggesting possible up-regulating mechanisms for mitochondrial transcription.

Nevertheless, many NRTIs do hamper mtDNA synthesis in vitro, by acting as competitive substrates to endogenous nucleotides and chain terminators at the nucleotide-binding site of DNA pol γ, because NRTIs lack the 3′-hydroxyl group necessary for further elongation of the mtDNA strand. NRTIs might also persist in the mtDNA strand because of inefficient DNA pol γ exonuclease activity. Generally, both...
endogenous nucleosides and NRTIs must become phosphorylated to triphosphate forms to be substrates for DNA polymerases in DNA synthesis. The triphosphate forms of zalcitabine, didanosine, stavudine and lamivudine were all well incorporated into the elongating mtDNA strand in vitro, but less so for tenofovir, zidovudine and abacavir. One factor that may be important for a lower clinical toxicity profile of lamivudine is that this NRTI appears to be more efficiently removed by DNA pol γ exonuclease activity than other NRTIs.42

The less effective incorporation of zidovudine triphosphate into mtDNA may be outweighed by a number of other mechanisms that might explain the toxicities of zidovudine. First, even zidovudine monophosphate might act as a substrate for mtDNA synthesis and may also inhibit DNA pol γ exonuclease activity.42 Second, zidovudine has also been related to decreased levels of complex IV of the OXPHOS system, even in absence of mtDNA depletion, impairment of the ADP/ATP translocase as well as inhibition of adenylate kinase, an enzyme involved in ATP formation.39-45 In a recent review, Lund and Wallace proposed that zidovudine might also compete with NADH directly at complex I.46 Decreased OXPHOS activity may also lead to increased electron leakage into the matrix and generation of reactive oxygen species, which in turn initiate a cascade of further oxidative damage.47 In fact, zidovudine exposure was also associated with oxidative stress and reduced ATP production in vitro.48

**Effects on thymidine kinases (TKs)**

Endogenous thymidine and cytidine, as well as the thymidine analogues zidovudine and stavudine, are phosphorylated to monophosphates by the nucleotide kinases TK1, which is localized in the cellular cytoplasm, or TK2 in the mitochondria. Zidovudine may even inhibit TK2, and cause reduced levels of endogenous nucleotides and thereby decreased synthesis of endogenous nucleotides and mtDNA.49 Reduction of endogenous nucleotides could also be caused by favouring the transport of phosphorylated NRTIs into the mitochondria compared with endogenous nucleotides.50

TK1 is expressed only during the S phase in mitotic dividing cells, while TK2 is expressed in post-mitotic tissue like skeletal muscle, adipocytes and neurons.51 Whereas mtDNA changes in these tissues have particularly been associated with exposure to didanosine and zalcitabine, treatment with stavudine has been especially associated with mtDNA changes in peripheral fat.52-54 In addition, zidovudine may induce mtDNA depletion in skeletal muscle and, although to a lesser extent, has also been associated with lipatrophy.55,56 It might therefore be speculated that the susceptibility to mitochondrial changes mainly depends on TK2 in resting tissues like fat, muscle and neurons with low TK1, despite the greater affinity for TK1 than TK2 of thymidine analogues. Because PBMC probably lack TK1,33 this could possibly explain the observation that exposure to didanosine, which is not a substrate for thymidine kinases, seems to be more likely associated with mtDNA depletion in PBMC compared with thymidine analogues.20,61-63 In keeping with this notion, exposure to didanosine and tenofovir was recently associated with a depleted CD4+ T cell count, despite virological suppression.54 This could possibly be linked to apoptosis caused by mitochondrial toxicity.

On the other hand, increased total TK activity has recently been associated with cellular activation in HIV infection and it might be speculated that even TK1 activity could be increased in PBMC in HIV-infected individuals.65,66 In keeping with this, increased intracellular concentrations of NRTIs were found in PBMC from patients with low CD4+ T cell counts, and the concentrations in fact decreased when the CD4+ T cell count and immune activation improved.67,68 As reviewed by Anderson et al.,69 intracellular NRTI triphosphate concentration is probably a key factor for mitochondrial toxicity. Increased intracellular concentrations of zidovudine and lamivudine were also found in women relative to men, and it should be noted that both women and patients with low nadir CD4+ T cell counts seem particularly susceptible to mitochondrial toxicity.68-70

To summarize, substantial differences in both phosphorylation rates and cellular uptake of NRTIs might explain the variability of mtDNA-related toxicity between cell types. This could explain the observed reduction of mtDNA in susceptible adipocytes from patients with lipatrophy, who apparently had no corresponding depletion of mtDNA in PBMC.52,71-73

**mtDNA mutations and reduction in gene expression**

NRTI may clearly cause mitochondrial dysfunction by mechanisms other than inhibition of DNA pol γ, for example by predisposing, pre-existing mutations or accumulation of mtDNA mutations as a result of NRTIs. Although the spontaneous mutation rate is generally higher in mtDNA compared with nuclear DNA, data on mtDNA mutations in HIV-infected patients have been inconsistent in relation to ART. First, in PBMC, a longitudinal study of HIV-infected patients found such mutations in PBMC soon after initiating combined ART,74 while another study found neither mtDNA mutations nor reduced mtDNA content in PBMC from patients who had or had not developed lipatrophy.75 Second, in muscle tissue, we found mtDNA deletions in NRTI-exposed patients,76 whereas reduced mtDNA in adipose tissue from NRTI-exposed patients with lipatrophy but no concomitant mtDNA mutations was found in another study.77

Another factor for reduced mitochondrial function is a decreased expression of mitochondrial genes, again detected in adipocytes78-80 and even in the absence of reduced mtDNA. In the study by Mallon et al.,81 decreased mRNA levels corresponding to the mtDNA-encoded subunits of the OXPHOS system were found, in addition to down-regulation of mitochondrial and nuclear genes involved in lipid metabolism.81 Another explanation for the differences between solid tissue and PBMC is that mitochondrial gene expression in PBMC was normal, despite concurrent depletion of mtDNA found in PBMC from patients exposed to stavudine and didanosine, suggesting a compensatory up-regulation of mitochondrial transcription or translation.37 Taken together, more in-depth studies are required to understand mitochondrial toxicity and the impact of deletions, mutations and regulation of mtDNA gene expression for clinical practice.

**Host factors**

Because not all individuals treated with specific NRTIs develop adverse effects related to mitochondrial toxicity, a number of host factors have been suggested, such as female sex, white race,
older age, hepatitis B and C co-infection, nadir CD4+ T cell counts, and decreased creatinine clearance.72–87

Gender
Lactic acidosis in stavudine-exposed patients has been reported more frequently in women,69,88,89 with a particular increased risk in obese women.90 Women might also be more susceptible to fat distribution changes.85,86 These phenomena could possibly be related to the previously discussed pharmacological differences between men and women.66 In fact, normal plasma concentrations but higher intracellular triphosphate concentrations of zidovudine and lamivudine were found in women compared with men.67 Recently, focus has also been placed on the stage of HIV infection, because nadir CD4+ T cell counts of <100 cells/mm³ appear to be associated with lipatrophy and polyneuropathy.70,87 It might be speculated that this could be attributed to the enhanced susceptibility due to dysregulated lipid metabolism in advanced HIV with high levels of tumour necrosis factor α (TNF-α).68

Inborn mitochondrial variants
Pre-existing mutations in both mtDNA and nuclear DNA as sub-clinical inborn variants will affect all mitochondria and, therefore, possibly predispose to overt mitochondrial toxicity in chronic HIV.75 Recently, a mutation in POLG, the nuclear gene encoding DNA pol γ, was found in a woman with stavudine-associated lactic acidosis, suggesting an increased risk because of the pre-existing mutation in this nuclear gene.90 Mitochondrial haplogroup T was associated with increased risk for peripheral neuropathy in individuals treated with stavudine and didanosine,55 whereas haplogroup J possibly protects from lipatrophy.91

Age
Older age, which is associated with accumulated mtDNA mutations in non-HIV,92 was also an independent predictor for peripheral neuropathy.55 Age-related mtDNA mutations might be even more challenged in the near future, because individuals treated with combination ART not only become older, but also have a long cumulative exposure period to thymidine analogues and didanosine, with some patients even having been exposed to zalcitabine, the first drug taken off the market due to profound mitochondrial toxicity.

Other factors
Co-morbidities and other concomitant medication may also contribute to an increased risk for mitochondrial toxicity in HIV-infected patients, such as individuals with HIV and hepatitis C co-infection treated with ribavirin in combination with didanosine, who have a substantial risk for lactic acidosis.93–95

HIV and its effect on mitochondria
HIV infection per se appears to affect mitochondria and may therefore contribute to the mitochondrial toxicity observed in NRTI-exposed individuals. In fact, reduced mtDNA content, decreased OXPHOS activity and reduced mitochondrial membrane potential (ΔΨm) have been found in ART-naive patients, mainly in PBMC.18,19,21,22,96 In fact, ΔΨm even correlated with the CD4+ T cell count.96 Further, mitochondrial changes have been observed also in tissue from treatment-naive patients. First, decreased mtDNA content was found in skeletal muscle in a few treatment-naive individuals.54 Second, reduced levels of both mitochondrial- and nuclear-encoded proteins necessary for mitochondrial function were found in adipocytes from both treatment-naive and NRTI-exposed patients.78

The underlying mechanisms for the mitochondrial changes observed in untreated HIV infection have been less clear; although, recently, we62 and others97 found that reduced mtDNA content was related to markers of persistent immune activation in ART-naive individuals, at least in PBMC. Although the hallmark of chronic HIV infection is substantial CD4+ T cell loss,98 it should be noted that lymphocyte proliferation and turnover rates become increased not only among CD4+ T lymphocytes (2-fold), but particularly among hyperactivated CD8+ T lymphocytes (6-fold)99 and B lymphocytes.100 In fact, the enhanced proliferation and turnover of CD4+ T cells probably overcompensates the actual HIV-related CD4+ T cell death rate. Moreover, the increased proliferation of both CD8+ T cells and B cells is probably the result of chronic immune stimulation, partly from HIV but also from other sources, such as antigen leaking across mucosal membranes.101,102 Such phenomena might play a role also in CD4+ T cells.98,103 Persistent immune activation in itself might therefore hamper mtDNA synthesis in untreated HIV-infected individuals, as supported by the observation by Casula et al.,18 who found mtDNA loss in PBMC after seroconversion in a retrospective longitudinal study. One explanation could be increased rates of mitochondrial apoptosis induced by proinflammatory cytokines as well as HIV proteins, as recently reviewed by Shedlock et al.104 Some HIV gene products may in fact aggravate mitochondrial dysfunction (Figure 1).34,105–109 Proinflammatory cytokines, such as TNF-α, interferon α and γ, and interleukin 2 and 6, may also in turn induce apoptosis.109,110 This notion is in agreement with a recent study in which mitochondrial dysfunction was associated with increased BMC apoptosis in HIV progressors but not in long-term non-progressors.111 Nevertheless, the complexity is illustrated by data suggesting that HIV proteins also may be antiapoptotic, since Tat might protect from apoptosis by down-regulation of caspase-10 activity.110 The expression of the activation marker CD38 on CD8+ T cells has long been the best predictor of disease progression in chronic HIV infection.112 Recently, programmed cell death-1 (PD-1) was suggested to play an important immunopathophysiological role through its involvement in reversible T cell exhaustion113 and we subsequently found that expression of PD-1 had even better prognostic significance.103 In our studies, mtDNA was substantially decreased in CD8+ T cells and B cells of ART-naive patients, both PBMC subsets known to be particularly hyperactivated in untreated chronic HIV infection. Even though mtDNA was less decreased in CD4+ T cells than in CD8+ T cells, mtDNA was significantly lower in untreated rapid progressors compared with slow progressors. In fact, mtDNA depletion in all these lymphocyte subsets correlated strongly with markers of persistent immune activation (CD38 and PD-1) on CD8+ T cells.62 Because HIV infection also induces B cell dysfunction,114,115 this might explain the highly significant depletion of mtDNA also in B cells from ART-naive
patients. We therefore suggested that persistent immune activation particularly hampers mtDNA in lymphocytes from untreated HIV-infected patients, whereas we previously did not find mtDNA depletion in muscle tissue from such patients.

Non-NRTIs and mitochondrial toxicity

Although non-NRTIs do not inhibit DNA pol $\gamma$, a recent study found that both efavirenz and nevirapine might reduce mitochondrial $\Delta\Psi_m$ in PBMC, and current treatment with nevirapine was also in fact associated with apoptosis of lymphocytes. In keeping with this notion, efavirenz was associated with apoptosis in $\textit{vitro}$, induced by the intrinsic mitochondrial apoptotic pathway. On the other hand, protease inhibitors probably protect $\Delta\Psi_m$, thereby preventing apoptosis induced by the mitochondria. This might be in agreement with clinical experience, because treatment with efavirenz rather than boosted lopinavir was more likely associated with lipoatrophy at 96 weeks of combination therapy with a backbone regimen of stavudine or zidovudine regimens, in contrast to a minimal risk for lipoatrophy when efavirenz was combined with either abacavir or tenofovir.

Conclusions and future perspectives

Although the pathogenesis of NRTI-related mitochondrial toxicity was first explained by the inhibition of DNA pol $\gamma$, a number of other factors might also contribute. For example, decreased mtRNAs but no concomitant depletion of mtDNA have been found, and also reduced mtDNA content but normal levels of mtDNA-encoded proteins have been observed in both PBMC and tissue. Until recently, quantification of mtDNA content was frequently used in studies of mitochondrial toxicity in HIV-infected individuals. However, the functional and thereby clinical significance of mtDNA changes, such as numerical loss, in terms of mitochondrial dysfunction are not always clear. MtDNA content might even be unaffected in PBMC while decreased in specific tissues and this in fact highlights the limitation of quantifying mtDNA content in PBMC in relation to clinically adverse effects. Finally, HIV-associated reduction of mtDNA in the ART-naive phase could possibly also contribute to mitochondrial toxicity even in NRTI-treated patients. However, this issue is not at all elucidated, suggesting the relevance for longitudinal assessments of mtDNA content in treatment-naive patients after initiation of ART. Such data could provide better understanding of the impact of reduced immune activation after starting effective ART, which might explain improved mtDNA content in peripheral blood in some patients, and the accumulating negative effects of certain NRTIs. Future studies should relate different mitochondrial assays to the quantification assay of mtDNA content. Also, more in-depth studies should elucidate the possible role of mtDNA loss in specific lymphocyte subsets in relation to drug-associated toxicity and possibly also disease progression in the ART-naive phase of HIV infection.

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