Proceedings of the 1st Meeting on Mitochondrial Toxicity and HIV Infection: Understanding the Pathogenesis for a Therapeutic Approach

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ORGANIZING & SCIENTIFIC COMMITTEE

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Proceedings of the 1st Meeting on Mitochondrial Toxicity and HIV Infection: Understanding the Pathogenesis for a Therapeutic Approach
May 19-21, 2005, Modena, Italy

**PROGRAMME**

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Introduction

Mitochondria, HIV infection and its treatment: where do we go from here?

Andrea Cossarizza* and Peter Reiss

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The idea of organizing a meeting on different aspects of the interactions between mitochondria, HIV infection and its treatment emerged as a result of the growing interest the scientific community has shown in this topic during recent years. Many of the scientists who have been conducting clinical as well as more basic research in this field responded enthusiastically to the invitation to present and discuss their ongoing research at the first international conference on “Mitochondrial toxicity and HIV infection: understanding the pathogenesis for a therapeutic approach”, held in Modena (Italy) from May 19-21, 2005. The initiative for this conference was taken by Professor Andrea Cossarizza, Chair of Immunology at the University of Modena and Reggio Emilia, and his colleagues from the Department of Infectious Diseases at the same University, Professor Roberto Esposito and Dr Cristina Mussini.

The meeting was made possible by an unrestricted educational grant from Gilead Sciences, Italy, and publication of part of the proceedings of the meeting in this supplement of Antiviral Therapy was made possible by an unrestricted grant from Sigma Tau, Italy. We wish to underline that the conference organizers had full responsibility for the content of the programme as well as the choice of speakers and session chairs, without any interference from the pharmaceutical sponsors. All papers appearing in this supplement underwent peer-review prior to being considered for publication.

The meeting started with a lectura magistralis given by Professor Vladimir Skulachev (State University of Moscow, Russia), one of the founding fathers of the field of “bioenergetics”, who provided an overview on programmed death in relation to ageing, at the level of individual cells (apoptosis), whole organisms (phenoptosis) as well as subcellular organelles including mitochondria (mitoptosis). This stimulated discussion on whether several of the adverse effects of treatment for HIV infection might be a reflection of “accelerated ageing” by mechanisms including mitochondrial toxicity.

The talks and related discussions that took place in the following days revealed that several issues concerning the detrimental effects which HIV, other concomitant infections such as those with hepatitis C, and HIV therapy may have on mitochondria warrant further investigation and clarification in the years to come.

What is becoming increasingly clear is that antiretrovirals, and nucleoside analogue reverse transcriptase inhibitors (nRTI) in particular, may affect mitochondria in more ways than one. Inhibition by nRTI of DNA γ-polymerase resulting in mitochondrial DNA (mtDNA) depletion remains one of the cornerstones by which nRTI may induce mitochondrial toxicity. It is evident however that nRTI and even other classes of antiretrovirals such as HIV protease inhibitors, may have additional effects on mitochondria, for instance by way of exerting direct effects on mtRNA transcription and mitochondrial enzymes. Differences between individual agents as well as differences in the extent to which these effects may play a role in different cell types remain to be further delineated.

An important problem still remaining is the choice of the type of cell in which to best measure mitochondrial markers as a possible reflection of treatment toxicity. Blood obviously remains the easiest tissue to obtain from patients, but contamination with platelets which contain numerous mitochondria may yield poorly interpretable results when using whole blood or peripheral blood mononuclear cells (PBMCs). Better purified cell populations such as isolated CD4+ or CD8+ T lymphocytes, or platelet-depleted lymphocytes...
or monocytes may provide more reliable results and need to be investigated further. Nevertheless, other tissues and cells coming directly from the organ systems affected by the treatment toxicity being studied (for instance adipose, hepatic or renal) may be more informative, and more appropriate when trying to assess the possible mitochondrial pathogenesis underlying a specific drug toxicity.

In order for any marker of mitochondrial toxicity to become clinically useful for the early detection of drug toxicity, its sensitivity, specificity and predictive value would have to be adequately demonstrated in the context of appropriate clinical studies. Several technologies, assays and methodologies are nowadays used by several groups to analyse different aspects of mitochondrial biology. Flow cytometry and confocal microscopy, utilizing specific fluorescent probes, can detect at the single cell level changes in several mitochondrial parameters (including changes in internal membrane potential or organelle mass), as well as modification in the morphology of the cell or tissue under investigation, and are widely used in different laboratories. Classic biochemical assays, which can measure different aspects of mitochondrial respiratory chain function, are also quite popular, even if they often require large amounts of biological material, classically skeletal muscle. Thanks to the improvement in molecular biological techniques, including assays based upon the real time polymerase chain reaction, the measurement of the amount of mitochondrial DNA per cell is becoming easier, and a variety of scientists – even if different genes are used as target – are obtaining similar results, also as far as the absolute number of mtDNA copies in a given cell is concerned. Nevertheless, adequate quality control and assurance programmes are key in order to allow comparison of results obtained by different groups of investigators. Of note, a task force focussing on the further standardization of mtDNA quantification, involving investigators in the US, Canada, Australia, and Europe, has just been created. Similar approaches should be advocated for the evaluation of additional mitochondrial markers such as the quantification of mitochondrial RNA.

Conferences such as the one held in Modena will hopefully contribute to the further unravelling of the role mitochondria play in the setting of HIV infection and its treatment, ultimately leading to safer treatments for our patients.
Possible ways nucleoside analogues can affect mitochondrial DNA content and gene expression during HIV therapy

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In recent years, research into nucleoside reverse transcriptase inhibitor (NRTI)-related mitochondrial (mt) toxicity in HIV therapy has led to conflicting results and many unanswered questions regarding the molecular mechanisms that lead to such toxicity. From the early hypothesis that inhibition of the human mt polymerase γ by NRTIs was responsible for the drugs’ mt toxicity, an increasingly complex picture is emerging that probably involves multiple mt pathways. Results have been presented suggesting that NRTIs affect not only mtDNA but also mtRNA, nucleotide phosphorylation and the mt respiratory chain. Based on the current level of knowledge, this overview addresses some of the potential mechanisms through which NRTIs could affect mitochondria and ultimately cause the toxicity symptoms observed in HIV patients receiving NRTI-containing antiretroviral therapy.

Introduction

Since mitochondrial (mt) toxicity was suggested as a common pathway for the adverse effects of nucleoside reverse transcriptase inhibitors (NRTIs) [1–3], a body of evidence has accumulated that, for the most part, supports the model. The intention of this review is to point out some of the limitations of HIV drug toxicity research, review the evidence relating NRTI toxicity to mtDNA damage (or not), and present the various pathways of mt toxicity that could potentially contribute to such damage.

It has been known for some time that mt toxicity tends to be drug and tissue specific [4–9,39]. More recently, evidence has been mounting that NRTI-related mt toxicity in HIV is probably a complex, multifactorial phenomenon. Drug- and tissue-specific differences in inhibitory activity toward polymerases could, of course, play a part, but a number of other variables are emerging. Different rates of NRTI uptake and/or intracellular phosphorylation between tissues could modulate specificity [11]. Patient characteristics such as gender, body weight or coinfection status can influence the incidence and severity of mt toxicity symptoms. A plausible association between proinflammatory and immune activation cytokine levels such as interferon and tissue necrosis factor, and the heightened risk of NRTI toxicity in specific populations has been suggested [11,12], but the mechanism behind this effect remains unclear. Co-morbidities, concomitant medications and genetic factors, as well as HIV therapy-independent mt damage can all influence and modulate mt toxicity and the development of symptoms.

Limitations of HIV mt toxicity research

Mitochondrial toxicity is a complex phenomenon, with multiple factors involved. Several factors may have contributed to the inconsistencies currently found in the literature on HIV-related mt toxicity (Table 1). Firstly, the relatively low incidence of severe mt toxicity-related adverse events has presented a challenge to studies in the HIV patient population. Secondly, many clinical symptoms suspected to have their aetiology in mt toxicity such as peripheral neuropathy, myopathy or even lipoatrophy can be challenging to quantify objectively in a standardized fashion. The majority of publications to date have studied relatively small observational cohorts, often in cross-sectional designs. In small studies, a lack of statistically significant association between mtDNA levels and a given clinical condition, although important to report, may be imputable to insufficient power, thereby limiting the conclusions that can be reached. A further issue is the potential time lag between the NRTI-induced mt damage taking place and the actual manifestation of the toxicity through clinical symptoms. As is the case of hyperlactataemia [16] and
probably lipoatrophy, this time lag can also present a challenge to the demonstration of any relationship, let alone causation. In certain tissues with rapid turnover such as blood cells, depleted mtDNA levels usually ‘rebound’ upon withdrawal of drug pressure [16]. This readily available sample is thereby susceptible to treatment interruptions, planned or not. Finally, differences in the type of sample studied as well as the techniques and methodologies used could also explain some of the discrepancies in the literature [13–15].

 Potential pathways of mt toxicity (Figure 1)

 mtDNA depletion

 Cellular mtDNA content is significantly lower in HIV-infected patients than in uninfected controls [16–21]. This important observation strongly suggests a virus-induced effect on the mitochondria and its genome, by an as yet unknown mechanism. That NRTI-containing therapy exacerbates this effect is highly plausible. It has long been known that NRTIs used in HIV therapy can inhibit the mt polymerase \( \gamma \) enzyme in vitro [22,23]. Cell culture [24–29] and animal studies [30–34] provide strong evidence in support of the NRTI inhibiting mtDNA synthesis model. However, whether this mtDNA depletion is the pathogenesis for all clinical symptoms of toxicity observed in HIV patients on highly active antiretroviral therapy (HAART) is less clear. The DNA polymerase \( \gamma \) hypothesis by itself fails to explain the entire array of metabolic deficiencies associated with NRTI-induced disorders [35]. Table 2 presents a summary of some of the published studies on NRTI and related mt toxicity, divided in two groups: consistent and inconsistent with mtDNA depletion as the pathogenesis of mt toxicity.

 mtDNA depletion was first reported in muscle [54], fat [55] and nerve tissue [4] of HIV patients experiencing drug-related symptoms of myopathy, lipoatrophy and neuropathy, respectively. We reported mtDNA depletion in peripheral blood buffycoat from HIV-infected patients receiving NRTIs and having symptoms of mt toxicity including hyperlactataemia.
The mtDNA depletion was reversible upon removal of the NRTIs suspected of causing mt toxicity, as are most toxicity symptoms if the antiretroviral therapy is changed or interrupted. We also found that the NRTI combination of stavudine (d4T) with didanosine (ddI), two drugs associated with a greater risk of hyperlactataemia [5,56,57], was also associated with greater blood mtDNA depletion than the other NRTI combinations studied [7]. These findings all appear consistent with the involvement of mtDNA depletion in NRTI-induced mt toxicity. However, not all studies of HIV therapy-related symptomatic mt toxicity show mtDNA depletion (Table 2). Discrepancy and controversy also remains with respect to the effect of NRTI-mediated mtDNA depletion on mt function. Several studies, using various tissues from HIV patients, have concluded that mtDNA levels and mt activities are positively correlated [38,44,58]. However, others would suggest otherwise and the relationship between mtDNA and mt function is somewhat unclear based on the current HIV literature. For example, one study found that a decrease in blood cell mt mass and mtDNA content was not accompanied by a decrease in cytochrome c oxidase gene expression or activity, leading the authors to suggest a compensatory mechanism up-regulating mt transcription or translation [59]. Another study from the same group found that decreased peripheral blood mononuclear cell (PBMC) mtDNA was accompanied by a decrease in mt respiratory chain (MRC) complex IV activity, but without evidence of mt dysfunction such as altered oxygen consumption or lipid peroxidation [51].

Indeed, results have also been inconsistent with respect to the association of mtDNA depletion with specific clinical adverse effects widely attributed to mt toxicity such as lipoatrophy and hyperlactataemia (Table 2). For example, in cases of hyperlactataemia and lipoatrophy, several studies have shown an association between the symptoms and mtDNA depletion in blood cells [16,10,18] while others have not [47,50,20]. In fat tissue, a few studies have suggested an association between fat mtDNA copies/cell and the presence of lipoatrophy [36,40,45]. Confocal microscopy analyses even offered direct evidence of a relationship between severity of adipose tissue toxicity and mtDNA depletion [40]. Recently, a significant decrease in fat cell mtDNA but not PBMC mtDNA levels was found in patients with peripheral neuropathy or lipodystrophy [43]. In contrast, another group found PBMC mtDNA decreased significantly in patients with lipoatrophy compared with those without lipoatrophy while mtDNA levels in adipose tissue did not differ significantly between the two groups [10]. Thus, contradictory results prevail in adipose tissue as well. These and other studies [60] clearly emphasize the importance of the tissue assayed.

Drug-related adverse events remain a common reason for intentional lack of adherence or for therapy change or interruption in adherent patients [61,62]. We carried out an observational study on a cohort of HIV-infected individuals starting their first regimen, to examine the relationship between blood buffycoat mtDNA and therapy change or discontinuation ‘for any reason’. Within the first 6 months on therapy, we measured a greater blood cell mtDNA decrease from baseline in those receiving the NRTI combination d4T/ddI. A greater proportion of these patients also eventually changed or discontinued therapy ‘for any reason’ during the study period. However, in the final analysis, despite the fact that all NRTI combinations studied showed a decline in mtDNA levels at the last follow-up, an inverse relationship was observed - a shorter time to therapy change/stop was associated with an increase in mtDNA from baseline, rather than a decline [63]. A number of confounding factors that could contribute to therapy changes or cessation could not be controlled for and may have contributed to this counterintuitive result. Alternatively, it could reflect some compensatory mechanism as suggested by others [59], which would be consistent with the in vitro observation that mt mass and mtDNA content initially increase in response to oxidative stress [64].

Overall, poor correlation between mtDNA levels and various mt toxicity symptoms strongly suggest additional mechanism(s), some of which are presented below.

**mtRNA depletion**

The mt genome encodes 13 polypeptides, all subunits of the mt respiratory chain (MRC) and essential for oxidative phosphorylation. It is tempting to assume that, in a manner similar to the effect of aging on muscle [65], depletion of mtDNA by NRTIs may lead to decreases in mt gene expression and ultimately a decrease in mt function. Recent work, however, has thrown doubt on this simplistic model. Non-HIV studies have shown that despite severe mtDNA depletion, mt genes can be transcribed at normal levels [66,67]. In HIV patients, results once again have been somewhat inconsistent. One study found that mtDNA depletion was not accompanied by a decrease in PBMC mt gene expression [59]. A second showed no change in PBMC mtDNA or mtRNA levels between two groups of patients initiating an antiretroviral regimen with or without d4T [52]. In contrast, Mallon et al. reported significantly decreased mt gene expression in adipocytes...
Table 2. Published studies with findings appearing consistent versus inconsistent with a relationship between mtDNA damage and NRTI-related mt toxicity

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<td>Symptomatic hyperlactataemia</td>
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<td>Decrease</td>
<td>D drugs</td>
<td>Cherry et al., 2002 [37]</td>
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<td>HAART +/- lipodystrophy, decline in MRC activity</td>
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<td>Specimen studied (tissue)</td>
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<td>Blood leukocytes</td>
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<td>Blood lymphocytes</td>
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<td>PBMCs</td>
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<td>Longitudinal PBMCs</td>
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<td>Subcutaneous fat</td>
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</table>

D drugs: didanosine, stavudine and zalcitabine (didexyynucleotides); COX, cytochrome c oxidase; HAART, highly active antiretroviral therapy; mt, mitochondrial; MRC, mt respiratory chain; NRTI, nucleoside reverse transcriptase inhibitor; PBMCs, peripheral blood mononuclear cells.
and blood monocytes after short periods on zidovudine (AZT)- or d4T-containing therapy [53,68]. Importantly, this was not accompanied by any significant change in mtDNA content or metabolic parameters, and was independent of HIV [53,68]. The latter results would imply that NRTIs can exert an effect, directly or indirectly, not only on mtDNA polymerization but could also regulate mt gene expression and/or inhibit mtRNA polymerization.

Interestingly, the DNA polymerase γ is capable of catalysing reverse transcription with a high efficiency, an activity that may be physiologically significant, especially considering that RNA-primed DNA synthesis activity is required for initiation of mtDNA replication [69]. Such activity could also be inhibited by NRTIs. Another potential target of NRTIs could be the mtDNA polymerase itself and the co-factors required for mt transcription, some of which are regulated through phosphorylation [70] and are, therefore, susceptible to MRC dysfunction. In addition, NRTIs could impair ribonucleotide synthesis and/or utilization in a manner similar to that hypothesized for nucleotides. All of the above could potentially translate into altered mt messenger RNA (mRNA) levels.

The mt genome also encodes 22 mt transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) that are necessary for the translation of the 13 mtDNA-encoded peptidyltransferase. Mutations in mt tRNA genes have been associated with various neuromuscular and neurodegenerative disorders, many sharing phenotypic similarities with HIV therapy mt toxicity symptoms [71,72]. Any NRTI-induced mtDNA damage or any transcription inhibition could also affect these other mtRNAs and participate in mt toxicity.

### Nucleotide pool imbalance

mtDNA depletion can be caused by NRTI incorporation into elongating mtDNA or inhibition of the polymerase γ, but even if these events occur, they are probably not the only mechanisms at play. Imbalances in the mt nucleotide pool have been suggested as the cause of mtDNA abnormalities in patients with inherited thymidine phosphorlase deficiencies [73,74]. In a similar manner, nucleoside analogues used to treat HIV infection can alter the endogenous cellular and mt nucleoside/nucleotide pools, potentially leading to the disturbance of a wide range of nucleic acid pathways that depend on these building blocks (Figure 1).

In its non-phosphorylated pro-drug form, AZT is a potent inhibitor of thymidine phosphorylase in heart and liver mitochondria [75–77]. Thus, the toxicity of AZT in some tissues may be mediated by disruption of the substrate supply of deoxynucleoside triphosphates (dNTPs) for mtDNA replication. De novo synthesis of pyrimidine nucleotides [dTMP, deoxycytidine triphosphate (dCTP) and deoxyuridine triphosphate (dUTP)] is coupled to the MRC [78]. Therefore, MRC dysfunction, whatever the cause, leads to decreased mt ATP regeneration that, in turn, impairs pyrimidine synthesis as well as its nucleotide phosphorylation [79], further enhancing the cycle of mt toxicity. Indeed, mtDNA-lacking cells do not synthesize pyrimidine nucleosides and require exogenous uridine for growth [80]. Interestingly, this is consistent with the observations that uridine can rescue mtDNA depletion induced by the pyrimidine analogues AZT, d4T and zalcitabine (ddC) in vitro [81,82].

### mtDNA damage (mutation, deletion)

Much attention has been paid to mtDNA quantity in recent research. Also important, but technically challenging to evaluate, is mtDNA quality. It is well-documented that mtDNA damage in the form of mutations and deletions accumulates over time, and plays a role in the conditions and diseases associated with aging [83–85]. NRTI therapy also provides conditions permissive for the development of peripheral blood mtDNA mutations in vivo [42]. Furthermore, NRTI or oxidative agents have been shown to directly impair the energy-producing system of mitochondria, causing dysfunction of cellular redox control, which eventually leads to loss of the mtDNA integrity [86].

In utero d4T exposure in mice leads to long-term mt damage and dysfunction in heart mitochondria, lasting to adulthood [87]. This effect would not be mediated by inhibition of the DNA polymerase γ but more probably result from persistent mtDNA mutations in the cardiac tissue [87], and it provides indirect evidence of NRTIs causing long-term decrease in mtDNA quality.

In the HIV population, large mtDNA deletions have been reported in a few cases of NRTI lactic acidosis [88,89]. Although it is unknown at this point whether these deletions predated HIV infection and HIV therapy, one can hypothesize that the presence of such deletions would, in all likelihood, diminish the mitochondria’s ability to compensate under NRTI drug pressure, thereby accelerating or exacerbating the development of mt toxicity symptoms.

### Other potential mt toxicity mechanisms

Apart from the various ways by which NRTIs can affect mt nucleic acids and their synthesis, there are a number of mtDNA-independent, poorly understood potential mechanisms that have been suggested for NRTI-mediated toxicity and a few are presented below.

Before any measurable effect on mtDNA, short-term AZT treatment of cultured rat myotube can induce a marked, yet reversible reduction in mt membrane...
This suggests that AZT can physically interfere with the membrane structure and could lead to modifications of its physical characteristics. This is consistent with the earlier observations that AZT exerts a short-term effect directly on the MRC [30], causing a decrease in ATP production [86], in addition to long-term alteration of the mtDNA. Similarly, in a rat heart model, ddC can decrease ATP, increase reactive oxygen species (ROS) formation and mediate cell damage before changes to mtDNA occur [91].

Recently, thymidine analogues and a thymidine catabolite were shown to enhance hepatic fat oxidation without affecting fat mtDNA, introducing NRTI catabolic products as possible players in HIV lipoatrophy [92].

Over time, ROS and free radicals damage the mtDNA, a phenomenon that forms the basis for one of the theories on aging. This damage in turn increases ROS production and leads to further oxidative damage to the mtDNA in human tissues, such as large-scale deletions and point mutations that accumulate with age [93,94,85]. There is also growing evidence that HIV itself can affect mitochondria and its nucleic acid [9,21] by an as yet unknown mechanism, possibly predisposing patients to mt toxicity [95,96]. Finally, it is now well accepted that NRTIs used to treat HIV infection can also damage mtDNA. This cumulative mtDNA damage eventually impairs MRC function, which itself can lead to further oxidative stress and mtDNA damage [85]. Long-term HIV therapy with NRTI-containing regimen in aging patients thus provides the context for a ‘triple’ insult to the mitochondria (Figure 2). One can hypothesize a synergistic, multifactorial model for mt toxicity, which would operate at different rates depending on the tissue, the drugs and the individual’s health and genetic background.

These are but a few of the various ways NRTIs and their intermediates are known or suspected to lead to mt toxicity in HIV patients receiving HAART. Further research with large controlled longitudinal studies, preferably with standardized assays [13,97], will be needed to elucidate the mechanisms of NRTI-induced mt toxicity and their relative contribution to clinical toxicity symptoms. This will be crucial in determining the marker(s) and samples most clinically relevant to mt toxicity testing, with the goal of preventing, predicting and monitoring mt toxicity in HIV patients on antiretroviral therapy.

Acknowledgements

Thanks to Christopher Alexander and Brian Scarth for their assistance with the manuscript.

Support

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37. Cherry CL, Gahan ME, M arthur JC, Lewin SR, H oy JF & Wesselingh SL. Exposure to deoxynucleotides is reflected in lowered mitochondrial DNA in subcutaneous
Mitochondrial nucleic acids in HIV therapy


Nucleoside reverse transcriptase inhibitors, mitochondrial DNA and AIDS therapy

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Introduction and overview

New, more effective antiretroviral therapeutic agents [1] and promise from HIV vaccine studies [2] have not prevented the AIDS epidemic’s global spread. Nucleoside reverse transcriptase inhibitors (NRTIs) in combinations called highly active antiretroviral therapy (HAART) are cornerstones of AIDS therapy in the developed world. Their extensive use has brought serious side effects to light that appear to relate to mitochondrial dysfunction. This review addresses some mitochondrial changes attributed to NRTI therapy as a brief overview of some of the relevant literature, with emphasis on the prevailing theories.

As alluded to above, the number of publications that relate to AIDS and mitochondrial dysfunction have grown rapidly since the original clinical and basic observations in the latter part of the 20th century [3–9]. Increased clinical interest in the entity coincided with increased frequency of side effects and increased patient survival. A number of reviews addressed different aspects of the problem from basic [10–19], clinical laboratory [20–22] and patient care [23–26] perspectives.

Predisposition to NRTI toxicity

Presently, there is limited understanding of genetic predispositions to NRTI toxicity or associated somatic mutations that may have pharmacogenetic implications. Such information may be crucial to complete understanding of toxicological mechanisms of NRTIs in humans and, conversely, to rationally guide effective therapeutic strategies in patients receiving HAART.

Mitochondrial DNA replication defects

Mitochondrial DNA (mtDNA) replication defects, including mtDNA depletion in target (or possibly surrogate) tissues, are frequently but not universally observed in both experimental and clinical reports.

Table 1 is an annotated list of some of the reports in the literature that have addressed issues of mitochondrial toxicity (MT) of individual NRTIs, target organs, basic and clinical findings, and diagnostic approaches to the clinical problem. The subcellular pharmacological mechanism or mechanisms of MT are incompletely understood, but are generally believed to relate (at least in part) to inhibition of DNA polymerase-γ (DNA pol-γ; the enzyme responsible for mtDNA replication in eukaryotes [27]) by the phosphorylated NRTIs. Enzyme kinetic changes in mtDNA with NRTI toxicity have been addressed by us [16,17] and other investigators [18,19].

Clinical and experimental findings related to MT from NRTI therapy with zidovudine (AZT) and related compounds were first presented in the latter part of the last century [3–5,8,9,28]. Hallmark features of mtDNA depletion and energy depletion related clinical [3,4,16] and in vivo [9,28,29] experimental findings to inhibition kinetics with DNA pol-γ and phosphorylated NRTIs in vitro [5,10,30–34]. Other observations suggested that mitochondrial energy deprivation is concomitant with, or the result of, mitochondrial oxidative stress in AIDS (for example, from HIV itself) or from NRTI therapy with its mitochondrial effects. In vivo studies with NRTI treatment of inbred mice [29,35] support this latter part of the hypothesis and data from our group and others employing transgenic mice revealed that oxidative stress results from transgenic expression of HIV Tat in the heart or liver [36–38]. The above-mentioned processes may result from oxidative mtDNA damage, aberrant mtDNA replication and/or altered mtRNA transcription. These events are cornerstones of the ‘mitochondrial dysfunction hypothesis’ [16,17] and remain foci in my laboratory’s studies of toxicity of NRTIs and events in cardiomyopathy (CM) in AIDS [8,39–41]. These principles apply to MT in other tissue targets demonstrated experimentally [32,42–44].
Table 1. Published reports of the mitochondrial toxicity of individual NRTIs

<table>
<thead>
<tr>
<th>NRTI</th>
<th>Anatomic or tissue target</th>
<th>Clinical evidence for MT</th>
<th>Experimental evidence for MT</th>
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<tbody>
<tr>
<td>AZT</td>
<td>Liver, heart, skeletal muscle and bone marrow</td>
<td>Mitochondrial myopathy; ragged red fibres; decreased mtDNA, paracrystals; phosphocreatine depletion ([3,4,24,52,64–71])</td>
<td>Reviews&lt;br&gt;Mechanisms of NRTI toxicity that focus on mtDNA replication ([13–16,18,33,47,62,69,103–111])&lt;br&gt;Absence of mitochondrial toxicity ([112])&lt;br&gt;Questioning the relationship to mtDNA ([218])</td>
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<tr>
<td></td>
<td></td>
<td>Polymyositis versus AZT myopathy; HIV vs NRTIs ([205,206])</td>
<td>DNA pol-(\gamma) hypothesis&lt;br&gt;Decreased mtDNA, mtRNA, mitochondrial polypeptides and mitochondrial ultrastructural damage in vivo and in vitro. Low (K_i) for AZT-TP with DNA pol-(\gamma). Failure of exonucleolytic excision of terminally incorporated AZT. Mixed (competitive and non-competitive) (K_i) with cardiac DNA pol-(\gamma) against various templates ([5,7–9,28,30–33,40,42,81,86,113–123])</td>
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<td></td>
<td>AIDS myopathy not AZT-related ([207,208])</td>
<td>Biochemical/cellular mechanisms proposed for DNA pol-(\gamma) inhibition ([14,16,18,19,106,219–222])&lt;br&gt;Mechanism of NRTI toxicity unrelated to NRTI triphosphate ([223])</td>
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<td>Cardiomyopathy with cardiac dilatation and failure; mitochondrial cristae dissolution; elevated serum lactate; Reye’s syndrome ([11,40,148–151])</td>
<td>Biochemical and cell biological findings&lt;br&gt;AZT is toxic to skeletal and cardiac muscle in vivo ([224])&lt;br&gt;AZT depletes mtDNA and releases lactate in vitro ([225])&lt;br&gt;AZT causes oxidative stress ([29,209])&lt;br&gt;AZT depletes glutathione ([129])&lt;br&gt;AZT inhibits adenylyl kinase ([125])&lt;br&gt;AZT inhibits adenine nucleotide translocator ([23,126])&lt;br&gt;AZT inhibits NADH-cytochrome c reductase ([226])&lt;br&gt;AZT inhibits mitochondrial permeability transition and apoptosis ([22,227])&lt;br&gt;AZT inhibits NADH oxidase ([127])&lt;br&gt;AZT effects on human deoxynucleotide carrier in vitro ([100])&lt;br&gt;AZT changes cellular nucleotide pools ([84,228])&lt;br&gt;AZT inhibits protein glycosylation ([82])&lt;br&gt;AZT inhibits ORPHOS early ([119])&lt;br&gt;AZT has no effect on mitochondria ([229])&lt;br&gt;AZT causes hepatic mitochondrial defects in vivo ([117])&lt;br&gt;Uridine abrogates NRTI toxicity in vitro ([230])&lt;br&gt;NRTIs decrease mtDNA replication in rats and humans ([180,231])&lt;br&gt;HepG2 cells are excellent models for NRTI dysfunction ([232])</td>
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<td>Hepatomegaly, steatosis, mitochondrial ultrastructural change ([66,144–147])</td>
<td>Other findings&lt;br&gt;NRTIs have no effects on mtDNA depletion ([109])&lt;br&gt;Cytoplasmic bystander gene effect ([128])</td>
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<td>Reduced mtDNA, mtRNA, mitochondrial polypeptides and mitochondrial ultrastructural change ([66,144–147])</td>
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<td>Polymyositis versus AZT myopathy; HIV vs NRTIs ([205,206])</td>
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<td>Cardiomyopathy with cardiac dilatation and failure; mitochondrial cristae dissolution; elevated serum lactate; Reye’s syndrome ([11,40,148–151])</td>
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<td>Reduced mtDNA, mtRNA, mitochondrial polypeptides and mitochondrial ultrastructural change ([66,144–147])</td>
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Table 1. Continued

<table>
<thead>
<tr>
<th>NRTIs</th>
<th>mtDNA and AIDS therapy</th>
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<tr>
<td>ddC</td>
<td>Peripheral nerve</td>
</tr>
<tr>
<td>3TC</td>
<td>3TC muscle toxicity [195]</td>
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<tr>
<td>Carbovir</td>
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<tr>
<td>ddI</td>
<td>Pancreas</td>
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<tr>
<td>ddI</td>
<td>Peripheral nerve</td>
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<tr>
<td>ddI</td>
<td>Liver</td>
</tr>
<tr>
<td>ddI+tenofovir</td>
<td></td>
</tr>
<tr>
<td>d4T</td>
<td>Peripheral nerve</td>
</tr>
<tr>
<td>d4T</td>
<td>Liver</td>
</tr>
<tr>
<td>Abacavir</td>
<td></td>
</tr>
<tr>
<td>Tenofovir</td>
<td>Mitochondrial side effects not clear; pancreatitis with co-administration of ddI may relate to MT [244,245]</td>
</tr>
<tr>
<td>(-)FTC</td>
<td></td>
</tr>
<tr>
<td>FIAU</td>
<td>Liver, skeletal and cardiac muscle, peripheral nerve</td>
</tr>
<tr>
<td>FddA</td>
<td>Newly developed antiretroviral for salvage [202]; plasma levels established [262]; safety concerns stop clinical trial [263,264]</td>
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<tr>
<td>FddA</td>
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<tr>
<td>All</td>
<td>Clinical testing</td>
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<td>All</td>
<td>with surrogate marker</td>
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NRTIs: nucleoside reverse transcriptase inhibitors; mtDNA: mitochondrial DNA; NADH: nicotinamide adenine dinucleotide; NRTI: nucleoside reverse transcriptase inhibitor; OXPHOS: oxidative phosphorylation; PBL: peripheral blood lymphocytes; -TP: triphosphate.
Assessing NRTI toxicity

It is possible to make analogies between studies that address mechanisms of NRTI-induced MT and those that examine defects in genetic mitochondrial illnesses in which defective mitochondrial gene product, oxidative stress and environmental factors contribute to disease [45]. This has been the approach utilized by many clinical studies in which mtDNA depletion in surrogate tissues (for example, blood cells) [22,46–53] and lactic acidemia [54–56] support NRTI MT. Some studies indicated depleted mtDNA in target tissue occurred, particularly in fat tissue and skeletal muscle of AIDS patients treated with NRTIs [3,4,24,52,57–71]. Others suggested that mtDNA depletion in surrogate tissues was not a useful marker [72,73] or emphasized the intrinsic importance of sample handling and data interpretation as potential pitfalls that could confound proper interpretation of results [21,74–77].

The DNA pol-γ hypothesis follows principles of mitochondrial medicine [78]. If the intramitochondrial pool of native nucleotides is disrupted by NRTI treatment, altered energetics may result from alterations in expression of electron transport proteins encoded by mtDNA. In another system, the importance of mitochondrial alterations in the development of low output congestive heart failure [79] related mitochondrial and cardiac dysfunction. Genetic mitochondrial illnesses manifest with clinical findings that occur at a threshold that is based in part on the heteroplasmic effects of the associated mtDNA mutation and the impact of oxidative phosphorylation (OXPHOS) on tissue function according to the OXPHOS paradigm [80]. Energy deprivation, possibly the initial phenotypic step of NRTI toxicity (based on mtDNA depletion), relates decreased energy abundance in tissues (for example, heart, as in our studies [8,39,40]) to decreased mitochondrial function. Such a threshold underscores phenotypic change, particularly in tissues like myocardium. It should be understood that this might not be the only mechanism utilized or available to explain toxicity of NRTI in mitochondria. Other mechanisms have been suggested that relate to mtDNA replication [81] or other aspects of mitochondrial and cellular homeostasis [82,83] and are addressed below.

Nucleotide pools, NRTIs and mtDNA replication

An overview of some aspects of NRTI cellular pharmacology is offered here, but it is not exhaustive. On a mass action basis, sufficient intramitochondrial NRTI concentration is required to affect mtDNA replication. Specifically, NRTI triphosphate must compete with the native moiety at the nucleotide-binding site of DNA pol-γ to yield inhibition of mtDNA replication. On a biochemical basis, the important active pharmacological (and toxic) element of AZT is the triphosphate (AZT-TP). AZT-TP inhibits both HIV reverse transcriptase [84,85] and mammalian DNA pol-γ in vitro [32]. It follows that stoichiometrically sufficient phosphorylated NRTI must be made available intramitochondrially for inhibition of mtDNA synthesis, subsequent depletion of mtDNA [86] and development of toxic manifestations. The various mechanisms of internalization of nucleosides, their transport and the homeostasis of mitochondrial nucleotide pools are key elements, as is the phosphorylation of NRTIs. To date, the intramitochondrial concentration of NRTIs has been difficult to determine.

AZT-like NRTIs are phosphorylated in three intracellular steps. Thymidine kinase (TK) phosphorylates AZT to AZT-MP. Thymidylate kinase phosphorylates AZT-MP to AZT-TP. Nucleoside diphosphate kinase yields the active AZT-TP [87] from AZT-TP. At each step, phosphatases exist to maintain homeostasis. Accordingly, a key step in the pathophysiology of NRTI pharmacology and toxicity is regulation of natural deoxyribonucleotide triphosphate (dNTP) and NRTI triphosphate pool sizes in mitochondria that affect mtDNA replication – dysregulation of phosphorylation and dephosphorylation could impact mtDNA replication. TK exists in isoforms: TK1 (cytosolic isoform) has relatively low activity in extracts of striated skeletal muscle [88,89], while TK2 activity is higher. TK2 is reported to have a broader substrate range and phosphorylates deoxyctydine (dCyd) and 5-substituted deoxothymidine (dThd) and dCyd analogues [90]. Dideoxynucleotides (ddNTPs) function as either competitive inhibitors of the natural substrates of polymerases (reviewed in [91]) or lead to chain termination [85,92]. Since mtDNA depletion is a hallmark of treatment with AZT in skeletal muscle of humans and rodents [3,9], and fialuridine (FIAU; 1-[2-deoxy-2-fluoro-β-D-arabinofuranosyl]-5-iodouracil) causes mtDNA depletion in woodchucks and humans [12,93] (see below), it is possible that genetic models exist to support the working hypothesis. TK2 mutations represent an aetiology for mtDNA depletion and have been associated syndromically with that genetic finding. Two substitution mutations in TK2 (H is90Asn and Ile181Asn), resulted in a phenotype of infantile myopathy and mtDNA depletion in muscle [94]. In contrast to its cytoplasmic counterpart, mitochondrial ribonucleotide reductase is not well documented, so import of deoxyribonucleosides and their phosphorylated products (or the analogous NRTIs) into mitochondria must occur in mammalian tissues. dNTPs synthesized by the cytosolic ribonucleotide reductase can be imported directly through the mitochondrial membrane [95]. Import of deoxynucleosides into
mitochondria allows for subsequent phosphorylation by mitochondrially localized kinases [96] to nucleotides. Steady-state abundance is balanced by mitochondrially localized dephosphorylations by phosphatases [97–99]. Import of NRTIs or NRTI phosphates could alter the stoichiometry of the intramitochondrial pool of native nucleotides [100].

Overview of current NRTI therapy

NRTIs used to treat HIV infection [101] include zidovudine (AZT; 3'-azido-2',3'-dideoxythymidine), zalcitabine (ddC; 2',3'-dideoxyctydine), didanosine (ddI; 2',3'-dideoxynosine), stavudine (d4T; 2',3'-didehydro-3'-dideoxythymidine), lamivudine (3TC; 3-thiacytidine; cis-1-[2'-hydroxymethyl-5'-1,3-oxathiolanyl]cytosine), entricitabine ([--]FTC), tenofovir and abacavir (these latter compounds are not NRTIs, but related moieties). Because a large number of NRTIs are administered in HAART, resultant combinations vary widely in their constituents [102]. This amplifies the complexity of the problem and makes interpretation difficult. Controls are not always available for some observations in clinical populations. From a commercial standpoint, NRTIs serve an important market with features that include significant long-term patient use of therapy, a growing patient population and absence of competition from preventative or curative vaccines in the near future. Because of this, the search for new NRTIs has led to development of many important compounds with therapeutic effectiveness but also some that exhibited profound clinical toxicity (addressed below). Additionally, the diagnosis, identification, prediction and amelioration of mitochondrial toxic effects have generated increasing attention.

Mechanisms of toxicity

As mentioned previously, the prevailing theory suggests AZT-induced MT involves defective mtDNA replication (reviewed in [13–16,18,33,47,62,69,103–111]). It should be noted that the hypothesis is not universally accepted [112] and alternatives may prove to be promising as data are published. On a biochemical basis, decreased mtDNA, mtRNA, mitochondrial polypeptides and defective mitochondrial ultrastructure, correlate with micromolar, mixed Ks for dideoxy-NRTI triphosphates in various experimental systems [5,7–9,28,30–33,40,42,81,86,113–124]. Some other explanations for MT from AZT and NRTIs include inhibition of adenylate kinase [125], adenine nucleotide translocator [23,126], NADH oxidase [127], protein glycosylation [82] and a ‘bystander effect’ [128]. It should be noted that other mechanisms unrelated to mtDNA replication have also been suggested (including, for example, glutathione (GSH) depletion [129]).

Zidovudine (AZT)

AZT was the first NRTI antiretroviral used in the treatment of AIDS and affords the greatest toxicological experience. Both in clinical [3,4,24,52,64–71] and experimental studies [13–16,18,33,47,62,69,103–111], AZT has been implicated in the development of mitochondrial diseases with features of myopathy, ragged red fibres, decreased mtDNA and defective mtDNA replication. AZT has worsened mitochondrial genetic illnesses, been implicated in the genesis of lactic acidosis [130–142] and has caused mtDNA mutations [143]. With respect to toxicity in various target tissues, observationally based clinical correlates were made in some of the early studies in which AZT liver toxicity was associated with obesity and female gender [116,133]. Refined genetic correlates were lacking. NRTI toxicity presents a variable and complex diagnostic phenotype in the treated population and mimics key features of mitochondrial diseases. NRTI toxicity may serve as an important model system for relevant pharmacogenetic studies. Such a pharmacologically based review is beyond the scope of this work, but has been addressed elsewhere [11,16,17,106]. Various organs and tissues have been described as susceptible (and resistant) to AZT toxicity. Table 1 enumerates some of these, including the liver. In ways that resemble FIAU (described below) hepatomegaly, steatosis and mitochondrial ultrastructural change [66,144–147] have been documented with AZT. It should also be noted that hepatic toxicity from didanosine (ddl) and zalcitabine (ddC) has been reported [64,66,67]. The toxic mechanism is presumed to relate to liver mitochondria. Fatal hepatomegaly with severe steatosis [66], severe lactic acidosis [67] and adult Reye's syndrome [64] in AZT-treated HIV-seropositive patients were all pathogenetically linked to AZT-induced hepatotoxicity. Clinical features resembled some of those seen in FIAU toxicity (below).

The cardiovascular system effects of AZT include CM with cardiac dilatation and failure, mitochondrial cristae dissolution and elevated serum lactate [11,40,148–151]. Conflicting reports have suggested resistance [13,67,152–155] or susceptibility to toxicity from AZT [156], but MT to cardiovascular tissues has been documented in primates and in utero [145,157–161] with AZT and other NRTIs. AZT therapy more recently has been implicated in lipodystrophy in AIDS patients [20,162].

Stavudine (d4T)

d4T emerged as a first-line HAART component. A number of documented MTs [145,157–161] were
attributed to d4T (see Table 1). Like ddC (below), painful peripheral neuropathy [163–165] has been described with d4T. One study suggested a lack of such a relationship. A clinical ‘proof of principle’ used treatment of d4T mitochondrial neuropathy with acetyl-l-carnitine. The trial was considered successful and suggested that MT was mechanistically related to d4T effects on mitochondria [166,167]. Preclinical and basic studies further support MT of d4T. Distorted cristae and decreased mtDNA in CEM cells occurred with d4T exposure [33,168,169] and d4T mitochondrial neuropathy was generated in vivo [170]. Kinetics of inhibition with d4T and DNA pol-γ [171] resulted in a nanomolar K, [124].

Clinical treatment with certain NRTIs (d4T/3TC) results in anion gap acidosis. Moreover, the lactic acidosis/hepatic steatosis syndrome may be more common than previously appreciated in adults and children treated with NRTIs. d4T treatment has also been associated with lipodystrophy [172]. Mechanisms were posited to involve altered mitochondrial biogenesis and/or oxidative changes, and adipocyte apoptosis [11,117].

d4T and other NRTIs have been suggested to relate to development of lactic acidemia. Some patients treated with NRTIs experienced lactic acidemia [67,93,133,135,145,154,174–178] and a phenotype of mtDNA depletion [46,123]. Depending on the biological system employed in the study [21,63,162,179,180], deleterious effects on mitochondrial structure and function in selected targets have been documented [8,11,44,181]. Although the specificity of blood cell mtDNA depletion as a surrogate marker for NRTI toxicity has been documented in some studies [58,156,162,182,183], the impact of the studies was confounded by the control groups used [50]. In principle, mtDNA depletion is mechanistically consistent with NRTI toxicity. However, the impact of mitochondrial dysfunction in surrogate tissues remains unclear [52], even in the face of a logical working hypothesis. Methods for diagnosis usually include examination of plasma lactate or lactate/pyruvate ratios [50,51], but require careful sample preparation and handling to assure meaningful results and interpretations. Overall, depletion of mtDNA appears to be accepted as an important marker of the toxic process, and may even serve as a diagnostic hallmark [3,9] to monitor successful HAART therapy [46]. It should be emphasized that the ideal surrogate tissue to monitor mtDNA depletion from NRTIs remains to be determined and is an active focus of clinical research and commercial enterprise.

Zalcitibine (ddC)

ddC is less popular today; nonetheless, painful peripheral neuropathy attributed to mitochondrial dysfunction has been associated with clinical ddC toxicity [184–189], and inhibition of mtDNA replication has been observed in vitro and in vivo [5–7,33,81,115,168,179,181,190–194].

Lamivudine (3TC) and emitracibine (–)FTC

3TC is widely used in HAART and (–)FTC is becoming an important therapeutic tool since its recent addition to the armamentarium. Of the commonly used NRTIs, 3TC appears to have a favourable safety profile, but like the others, requires co-administration in a HAART regimen, particularly because of induction of HIV resistance mutations. Toxicity to muscle is reported clinically with 3TC [195], but basic evidence for toxicity of 3TC monotherapy is lacking in our in vivo systems (Lewis et al., unpublished). Newly approved (–)FTC exhibits a relatively favourable safety profile [196] and kinetics with DNA pol-γ favour efficacy [197]. Studies with HepG2 cells also are supportive [198] of the safety of (–)FTC. It remains to be seen if long-term toxicity is to occur.

Didanosine (ddl)

ddl remains an important element in HAART. Two principal clinical toxicities have been recognized. As with ddC, a painful peripheral neuropathy has been documented with ddl therapy in humans [199,200]. Early in the development of ddl, severe pancreatitis was identified as an important side effect with mortality [199]. Experimental work documented pancreatic changes by flow cytometry [201]. Fatal hepatotoxicity was described and lactic acidosis has occurred with co-administration of tenofovir.

Other NRTIs

One more recent and serious adverse event occurred with a halogenated purine. Fluoro-dideoxyadenosine (FddA; 2’-fluoro-2’,3’-dideoxyadenosine) went into clinical trial but was discontinued about 1 year later because it exhibited severe adverse events including profound lactic acidosis [202,203]. A second serious adverse event occurred with a halogenated pyrimidine in a hepatitis B trial. This tragic event occurred at the Clinical Center at the National Institutes of Health. After in vitro studies documented significant efficacy, the pyrimidine nucleoside analogue FIAU went to clinical trial and experienced promising results early on. FIAU was later found in the trial to be extremely toxic to liver, skeletal and cardiac muscle, pancreas and peripheral nerves in treated patients. MT from FIAU was profound. Lactic acidosis and hepatic failure required heroic clinical interventions and necessitated
early termination of the protocol, but some deaths occurred. Abandonment of these compounds as pharmacological agents was subsequently confirmed by documenting MT in animal models [43,204] and in humans [93] and defining inhibition kinetics in vitro with DNA pol-γ that favoured toxicity to mitochondria [42,44]. These tragic trials underscore the necessity for examining MT of NRTIs extensively using in vitro and in vivo testing.

Summary

NRTIs are cornerstones of antiretroviral therapy and perhaps the most important drugs developed for AIDS treatment. Judicious use of NRTIs in the fight against HIV infection has afforded significant clinical advances in AIDS treatment. Nonetheless, it is axiomatic to expect side effects from NRTIs as well. A principal toxicity of NRTIs relates to chronic and cumulative MT in various tissues. The long-term impact of this toxicity on affected patients is not clear except in extreme cases where morbidity was severe and mortality occurred. Because of chronicity and potential for severity, NRTI MT remains an important clinical problem. Further studies will help unravel mechanisms of NRTI MT and the natural history of mitochondrial biogenesis in humans and other mammalian systems.

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1st Meeting on Mitochondrial Toxicity & HIV Infection: Understanding the Pathogenesis for a Therapeutic Approach

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HIV protease inhibitors prevent mitochondrial hyperpolarization and redox imbalance and decrease endogenous uncoupler protein-2 expression in gp120-activated human T lymphocytes

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1st Meeting on Mitochondrial Toxicity & HIV Infection: Understanding the Pathogenesis for a Therapeutic Approach

Introduction

Given the important role of apoptosis in the pathogenesis and progression of HIV infection, several studies have been focused specifically on the mechanisms of apoptosis in both HIV-infected and uninfected CD4+ cells [1,2]. As a general rule, two different apoptotic pathways leading to activation of cell-specific programs have been proposed [3]. These two pathways refer to different initiation patterns, that is, receptor-dependent or independent [4]. In particular, changes of mitochondrial membrane potential (MMP) have been hypothesized to play a key role in apoptotic cascade. In fact, alterations of MMP have been associated with the release of apoptogenic factors that directly or indirectly, that is, via apoptosome formation, lead to the execution of apoptosis. A major role of mitochondria has also previously been suggested in the process of CD4+ T cell death [5,6].

Several drugs employed in clinical practice powerfully counteract the reduction of CD4+ cells by apoptosis in HIV infection. In fact, an important aspect of highly active antiretroviral therapy (HAART) is represented by immune reconstitution [7]. This is a therapeutic approach that involves, among others, drugs of different natures, such as HIV reverse transcriptase inhibitors, for example, zidovudine (AZT), and HIV protease inhibitors (PIs). Some of these, for example, indinavir (IDV), saquinavir (SQV) and lopinavir (LPV), are known to induce viral load lowering as well as the reduction of cell loss. It has also been suggested that AZT might be considered as an apoptotic inducer [8] whilst various PIs can be considered as apoptosis-hindering drugs [7,9]. Some in vitro and ex vivo studies have in fact suggested that PIs are able to inhibit peripheral blood mononuclear cell loss and restore impaired T-cell proliferative response [10]. Strikingly, this occurred independently from any viral infection [10]. Although a target activity of PIs towards mitochondria was hypothesized [11,12], the mechanism
underlying this activity still remains poorly understood [9,13]. In the present work, we analysed the earlier mechanisms involved in the subcellular effects of PIs considered as 'mitochondriotropic' drugs. The results reported herein document a series of changes, that is, cell polarization, induced by gp120 HIV protein in freshly isolated human T lymphocytes and the consequent proneness to CD95/Fas-induced apoptosis. These events were accompanied by mitochondrial hyperpolarization. PIs infer, with these subcellular events, hijacking T cells towards apoptotic resistance via a target effect on mitochondrial homeostasis. Furthermore, they also suggest for the first time that the mechanisms involved in PI activity include the modulation of the expression of endogenous mitochondrial uncoupler proteins (UCPs).

**Materials and methods**

**Isolation and activation of peripheral blood lymphocytes**

Human peripheral blood lymphocytes (PBLs) from healthy donors (HDs) were isolated from freshly heparinized blood through a Ficoll-Hypaque density gradient centrifugation and washed three times in phosphate-buffer saline (PBS), pH 7.4. (Lympholyte-H; Cedarlane Laboratories, Hornby, O, N, Canada). PBLs were subcultured in 25 cm² or 75 cm² Falcon plastic flasks at a density of approximately 1×10⁶ cells/ml in RPMI 1640 (Gibco-BRL Life Technologies, Milan, Italy) containing 10% fetal calf serum, (Flow Laboratories, Irvine, Scotland), 1% non-essential amino acids, 5 mM L-glutamine, penicillin (100 IU/ml) and streptomycin (100 mg/ml) at 37°C in a humidified 5% CO₂ atmosphere.

In vitro treatments

Purified T cells were activated for 72 h, in the presence or absence of 100 nM IDV (Merck Sharp & Dohme Ltd, Hotteson, UK), SQV (Roche Registration, Welwyn Garden City, UK) or LPV (Abbott Laboratories, Queenborough, UK), with i) interleukin 2 (IL2, 60 IU/ml; Gibco-BRL); ii) IL2 and phytohaemagglutinin (PHA 2 µg/ml; Sigma Chemical Co, St Louis, MO, USA); iii) HIV-1 gp120 (3 µg/ml; Intracta Corp, Cambridge, M, A, USA); and iv) IL2 and HIV-1 gp120. Isolated human lymphocytes (resting or differently activated) were treated with 500 ng/ml of an anti-human Fas IgM monoclonal antibodies (mAbs) (α-Fas, clone CH11; Upstate Biotechnology, Lake Placid, N Y, USA). For dose-dependent studies, T lymphocytes were treated with 100 nM, 1 µM and 10 µM IDV for 72 h. Because of overlapping results obtained by using the three different PIs, only results obtained with IDV, thus considered as representative, are shown in this paper.

**Evaluation of cell surface receptors**

The surface expression of molecules associated with T cell activation (CD69, CD38 and HLA-DR) and cell death (CD95/Fas) was verified by flow cytometry on resting and activated lymphocytes. For this purpose, mAbs directly conjugated to fluorochromes PE, FITC or PerCP to human CD95, CD38, HLA-DR and CD69 (Becton Dickinson, Mountain View, CA, USA) were used. Appropriate fluorochrome-conjugated immunoglobulins were used as negative controls.

**Apoptosis evaluation**

Quantitative evaluation of apoptosis was performed by using the following flow and static cytometry methods: i) double staining using the annexin V-FITC apoptosis detection kit (Eppendorf, Milan, Italy). This technique allows cells that have lost membrane integrity (and are therefore considered necrotic) to show red staining with propidium iodide (40 µg/ml) throughout the nucleus and to be easily distinguished from the living cells and ii) staining with the chromatin dye Hoechst (Molecular Probes, Eugene, OR, USA) as previously described [14].

**Mitochondrial membrane potential (MMP) in living cells**

The MMP of control and treated lymphocytes was studied by using the JC-1 probe. Following this method, cells were stained with 10 µM of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1; Molecular Probes) as previously described [15].

**Redox balance**

Resting and differently activated lymphocytes (5×10⁵), either treated or untreated with IDV, were incubated in 495 µl of Hank's balanced salt solution (HBSS) (Gibco BR2, Burlington, O, N, Canada) pH 7.4 with 10 µM dihydroorhodamine 123 (DHR 123; Molecular Probes) or 1 µM dihydroethidium (DHE; Molecular Probes) in polypropylene test tubes for 15 min at 37°C. The median values of fluorescence intensity histograms were used to provide semi-quantitative analysis of reactive oxygen intermediate (ROI) production.

**Immunocytochemistry**

Immunofluorescence analyses were performed by double staining as follows. Control and treated lymphocyte samples were incubated with mAb to CD95/Fas (Chemicon International, Temecula, CA, USA) directly conjugated with FITC for 1 h at 4°C. Cells were then washed, fixed with 4% paraformaldehyde (w/v in PBS) for 1 h at room temperature and then made permeable.
by 0.5% (v/v) Triton X-100 for 5 min. After washing, samples were incubated for 1 h at 4°C with a mAb to ezrin (Becton Dickinson, Mountain View, CA, USA). Negative controls were incubated with isotypic immunoglobulins. After washing, samples and isotypic controls were incubated for 45 min at room temperature with anti-mouse IgG TRITC-conjugate (Sigma Chemical Co). All samples were mounted with specific medium and analysed by intensified video microscopy (IVM) as previously reported [16].

Evaluation of uncoupler protein 2 (UCP-2) expression

Western blot. Aliquots of total protein extracts (20 µg) from T cells after different treatments were size fractioned by 12% SDS-PAGE and, after transfer to nitrocellulose membrane, filters were incubated with primary polyclonal antibody (Pab) against UCP-2 (Calbiochem Co, Darmstadt, Germany). Detection was achieved using HRP-conjugated secondary Pab and by the ECL detection system (Amersham-Pharmacia, Arlington Heights, IL, USA). Densitometric analyses were carried out by using a densitometer (Biomed Instruments, Inc, Fullerton, CA, USA) and results were expressed as arbitrary units (AU).

Flow cytometry. Control and IDV-treated lymphocytes were washed, fixed with 4% paraformaldehyde (w/v) in PBS for 1 h at room temperature and then permeabilized by 0.5% (v/v) Triton X-100 for 5 min. For quantification of uncoupler protein 2 (UCP-2), samples were incubated for 1 h at room temperature with specific Pab (Calbiochem). Negative controls were incubated with normal rabbit serum. After several washings, samples and isotypic controls were incubated in the dark for 45 min at room temperature with Alexa488-conjugated anti-rabbit IgG (Molecular Probes), washed and analysed on a cytometer.

Transmission electron microscopy (TEM)

For TEM examination, cells were fixed in 2.5% cacodylate-buffered (0.2 M, pH 7.2) glutaraldehyde for 20 min at room temperature and post-fixed in 1% Oso4 in cacodylate buffer for 1 h at room temperature. Fixed specimens were dehydrated through a graded series of ethanol solutions and embedded in Agar 100 (Agar Aids, Cambridge, UK). Serial ultrathin sections were collected on 200-mesh grids and then counterstained with uranyl acetate and lead citrate. Sections were observed with a Philips 208 electron microscope at 80 kV.

Preparation of isolated mitochondria

Gp120-, IDV+gp120- and carbonyl cyanide p-trifluoromethoxyphenyl hydrazone (FCCP)+gp120-treated PBL were collected by centrifugation. After three washings in PBS, cells were resuspended in Homo buffer (10 mM Hepes, pH 7.4; 1 mM ethylene glycol-bis(β-aminopropyl ether) N,N′,N′′-tetraacetic acid (EGTA), 0.1 M sucrose, 5% bovine serum albumin (BSA), 1 mM phenylmethyl-sulphonyl fluoride and complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and maintained for 10 min on ice. After this time, cells were homogenized with about 100 strokes of a Teflon homogenizer with B-type pestle as previously reported [17] for 10 min at 4°C to remove intact cells and nuclei, and the supernatants were further centrifuged at 10 000 × g at 4°C for 10 min to precipitate the heavy membrane fractions (enriched in mitochondria). These fractions were then purified by standard differential centrifugation. The mitochondrial pellet obtained was resuspended in swelling buffer (SB) containing 0.1 M sucrose, 0.5 M sodium succinate, 50 mM EGTA at pH 7.4, 1 M phosphoric acid (H3PO4), 0.5 M 3-(N-morpholino)propanesulphonic acid (MOPS) and 2 mM rotenone, kept on ice and used within 2 h of the preparation. Protein content in the mitochondrial preparation was determined by a spectrophotometric method using BSA as standard. The purity of the mitochondria preparation was assessed by Western blot, checking subunit I of cytochrome c oxidase (mAb; Chemicon, Temecula, CA, USA).

Swelling experiments

Mitochondria (0.5 mg protein/ml) from each sample (gp120, IDV+gp120 and FCCP+gp120) were resuspended in SB at a final volume of 3 ml and separated into two samples. In the first sample, to induce mitochondrial swelling mimicking physiological conditions, we used 1 µg/ml of recombinant Bid protein cleaved by caspase 3 (t-Bid; PeproTech, Inc, Rocky Hill, NJ, USA). Mitochondria from gp120, IDV+gp120-treated and FCCP+gp120-treated lymphocytes were incubated with 1 µg/ml t-Bid 2 h at 37°C. In the second, control sample, mitochondria from the same samples were incubated in SB without t-Bid.

Flow cytometry analysis. We analysed the ΔΨ of isolated mitochondria by using a cytofluorimetric method after staining with 1 µM TMRM. Using this method, the incorporation of the dye TMRM was monitored in the FL3 channel: low levels of TMRM incorporation (revealed by a decrease of red fluorescence) indicated a low ΔΨ. On this basis, in our experiments we tested the effect produced by t-Bid on the ΔΨ of gp120-, IDV+gp120- or FCCP+gp120-treated samples. As a general rule, we recorded 5 min control samples (without t-Bid) and, after this time, we started to record t-Bid-treated samples for a total recording time of 25 min. All samples were analysed with a FACScan cytometer (Becton Dickinson) equipped with a 488 argon laser. To exclude debris, samples were gated
based on light-scattering properties in the side scattering and forward scattering modes during analyses. The red fluorescence emission (due to TMRM dye) of untreated mitochondria was set up in correspondence of the 10^{2} channel and considered as basal emission. Dot plots of red fluorescence emission as a function of the time, obtained in each condition we used, were statistically analysed by using CellQuest™ software (Version 3.3) on a Macintosh (Becton Dickinson) in order to determine the percentage of mitochondria with depolarized membrane.

Analysis of cytochrome c release
Cytochrome c (CytC) was evaluated in supernatants from isolated mitochondria by a sensitive and specific immunoassay, using a commercial ELISA kit (R&D Systems, Minneapolis, M.N., USA) according to the manufacturer's instructions. The light emitted was quantified by using a microtitre plate reader at 405 nm. CytC concentration was expressed in ng/ml.

Data analysis and statistics
All samples were analysed with a FACScan cytometer (Becton Dickinson) equipped with a 488 argon laser. At least 20,000 events were acquired. Data were recorded and statistically analysed using CellQuest™. The expression level of considered proteins was expressed as the median value of fluorescence emission curve and the statistical significance was calculated using the parametric Kolmogorov-Smirnov (K/S) test. Statistical analysis of apoptosis data was performed by using Student's t-test or one-way variance analysis by using the StatView® program (Version 5.0) for Macintosh (SAS Institute, Inc. Cary, N.C., USA). All data reported in this paper were verified in at least four different HDs and expressed as mean ± standard deviation (SD). Only P<0.01 was considered as significant.

Results
IDV hinders gp120-induced T-cell polarization
Viral envelope protein gp120 was able to induce a wide variety of changes in human T lymphocytes that are typical of activation, for example, i) the increased expression of T-cell activation markers and ii) the redistribution of molecules of importance in determining lymphocyte polarization and fate, that is, survival or apoptosis. In particular, regarding the first point, we evaluated the surface expression of CD69 (Figure 1A), HLA-DR (Figure 1B) and CD38 (Figure 1C). A significant increase of these molecules on the surface of T cells was detected after gp120 administration (Figure 1).

Interestingly, these results were comparable with those obtained by using typical activating factors such as IL2 and PHA (Figures 1A–C). Importantly, pre-exposure of human lymphocytes to PIs was ineffective in this respect and the up-regulation of these activation markers remained still detectable (Figure 3A–C). Regarding the molecules of importance in determining T cell fate, the following results were obtained. Firstly, CD95/Fas expression was significantly increased by administration of gp120 either given alone or in association with IL2 (Figure 1D). Furthermore, in consideration of the well-known ability of various PIs to impair T cell apoptosis [11,18], specific measurements of CD95/Fas expression were carried out in the presence of IDV. Notably, as described above for activation markers (Figures 1A–C), the presence of IDV did not influence this parameter and the gp120-induced over-expression of CD95/Fas was welldetectable either in the presence or absence of IDV (Figure 1D). Secondly, a peculiar rearrangement of this molecule was observed. In particular, cellular localization of CD95/Fas was found to be modified by gp120. In fact, whilst control resting T cells appear non-polarized (Figure 2A, first row), gp120-treated cells underwent profound shape changes mainly represented by cell polarization. They appeared characterized by an accumulation of CD95/Fas molecules at one pole of the cell – positive patches at the cell surface, confined in the subcellular erniations called uropods were detectable by IVM analyses (Figure 2A, second row, left panel).

This polarization of the CD95 molecule was paralleled by a rearrangement of a cytoskeletal molecule previously demonstrated to play a key role in CD95/Fas-induced cell death, representing a pre-requisite for Fas signalling to occur: the ezrin molecule [16,19]. In fact, after gp120 exposure, in most of the lymphocytes the ezrin molecule appeared localized at one pole of the cell. Importantly, this cytoskeleton protein also appeared to co-localize with CD95/Fas at the uropod level (Figure 2A, second row, middle and right panels; compare with resting cells shown in the first row). Notably, IDV treatment was capable of hindering these processes and both CD95/Fas and ezrin remained randomly distributed at the cell surface or in the cell cytoplasm, respectively, and no co-localization of these molecules was detectable (Figure 2A, third row).

Altogether these results seem to indicate that: i) gp120 activation can predispose T lymphocytes to Fas-mediated apoptosis via uropod formation, CD95 polarization and ezrin cytoskeleton reorganization and ii) the presence of IDV was capable of impairing these processes. Ultrastructural analysis by TEM also point out some morphogenetic changes caused by gp120, such as cell shape remodelling, as well as mitochondria ultrastructural changes. In particular, gp120 administration led to: i) a redistribution of organelles, mainly of mitochondria, at one pole of the cell and ii) an alteration of their ultrastructural features (Figure 2B, middle
The first was characterized by the appearance of clusters of coalescent mitochondria migrated to one pole of the cell (Figure 2B, middle panel). The second was represented by ultrastructural modifications of mitochondria in terms of increased electron density and changes in the morphological features of cristae (Figure 2B, middle panel, inset; compare with control cell mitochondria shown in the inset in Figure 2B, left panel). Importantly, IDV administration (Figure 2B, right panel) prevented the mitochondrial morphology and distribution changes being similar to those detected in resting lymphocytes (compare left and right panels in Figure 2B).

Mitochondrial membrane potential

It has previously been reported that during T lymphocyte activation, an early increase of MMP clearly occurs [20,21]. This alteration was associated with T cell sensitization to Fas-induced apoptosis [18,22,23]. On the basis of these published data, the aim was to assess the early effects of HIV-1 gp120 protein on mitochondrial ∆Ψ. Thus, MMP was evaluated in T cells exposed to gp120 by using two different probes specifically employed in flow cytometry studies, that is, TMRM and JC-1 [15]. Figure 3A shows the results obtained in lymphocytes from a representative HD, whereas in Figure 3B the mean of values obtained by analysing four different panels; compare with resting cells shown in Figure 2B, left panel). The first was characterized by the appearance of clusters of coalescent mitochondria migrated to one pole of the cell (Figure 2B, middle panel). The second was represented by ultrastructural modifications of mitochondria in terms of increased electron density and changes in the morphological features of cristae (Figure 2B, middle panel, inset; compare with control cell mitochondria shown in the inset in Figure 2B, left panel). Importantly, IDV administration (Figure 2B, right panel) prevented the mitochondrial morphology and distribution changes being similar to those detected in resting lymphocytes (compare left and right panels in Figure 2B).
Figure 2. IDV hinders gp120-induced T cell polarization

(A) Immunofluorescence analysis of CD95 and ezrin molecules after double staining of T lymphocytes with specific antibodies. Micrographs show that, after gp120 administration, the CD95 molecule was polarized at the uropod (second row, green fluorescence) where it co-localized with ezrin (second row, red fluorescence) as demonstrated by merge pictures in the right column (second row, yellow fluorescence). In IDV/gp120-treated lymphocytes, the uropod formation was inhibited and no CD95/ezrin co-localization was detectable (third row) as in resting cells (first row).

(B) Ultrastructural analysis by TEM of resting and gp120-activated T lymphocytes in the presence or absence of IDV. Control (untreated cells) display mitochondria randomly distributed throughout the cell cytoplasm (left panel). After gp120 treatment, mitochondria appeared to have migrated to one pole of the cell, characterized by electron dense matrix and rearrangement of cristae (middle panel). These alterations were prevented by IDV (right panel). Results obtained from a representative HD out of four are shown. IDV, indinavir.
HIV protease inhibitors supply mitochondria homeostasis

Figure 3. IDV hinders mitochondria hyperpolarization and redox changes associated with T lymphocyte activation

(A) Quantitative cytofluorimetric analyses of MMP in resting (left panel) and in gp120-activated lymphocytes in the presence or absence of IDV as detected using the JC-1 probe. J-aggregates (red fluorescence, ordinate) increased after gp120 administration, when mitochondrial membrane became hyperpolarized (middle panel). This mitochondria hyperpolarization was significantly (P<0.01) prevented by IDV (right panel). Numbers in the boxed areas represent the percentage of cells with hyperpolarized mitochondria, while in the area under the dashed line, the percentage of cells with depolarized mitochondria is reported. Results obtained from a representative HD out of four are shown. (B) Flow cytometry MMP analysis performed in different activating conditions. Results obtained by pooling together data from four different HDs are reported. (C–F) Semiquantitative cytofluorimetric analyses of ROI production performed by using DHE (C and D) and DHR123 (E and F) in resting and gp-120-activated lymphocytes in the presence (or absence) of IDV. Values reported represent the median values of the fluorescence intensity histograms. In (E) and (F), the means±SD of the results obtained from four different HDs are reported. Note that, independently from the activating condition used, IDV was capable of significantly (P<0.01) preventing either anion superoxide (C,D) or hydrogen peroxide (E and F) production. HD, healthy donors; IDV, indinavir; IL2, interleukin 2; MMP, mitochondrial membrane potential; PHA, phytohaemoagglutinin; ROI, reactive oxygen intermediate.
HDs are reported. We found that, with respect to control samples (Figure 3A, left panel, boxed area and Figure 3B), gp120 induced a marked increase of MMP (hyperpolarization) in a significant percentage of cells (Figure 3A, middle panel, boxed area and Figure 3B). Of note is that the mitochondrial hyperpolarization phenomenon, as detected by flow cytometry, seems to correspond to changes in the mitochondrial structural features, as revealed by TEM analysis and shown in Figure 2B (middle panel). Importantly, pre-exposure to IDV completely impaired this mitochondrial hyperpolarization state and the percentage of cells with increased MMP was similar to that found in control samples (Figures 3A, compare right and left panels, boxed areas, and histograms in Figure 3B).

IDV hinders redox changes associated with gp120 administration

Production of reactive oxygen species was associated with mitochondrial changes occurring during T cell activation [23,24]. We thus evaluated two of the major parameters of importance in defining the redox state of a cell: superoxide anion and hydrogen peroxide production by a semiquantitative flow cytometry analysis. It has been found that gp120 was able to act as a mild pro-oxidant compound leading to an increased production of both superoxide anion (Figure 3C, a representative HD; Figure 3D, mean results obtained from lymphocytes from four different HDs) and hydrogen peroxide (Figure 3E, a representative HD; Figure 3F, mean results obtained from lymphocytes from four different HDs). Interestingly, IDV treatment was able to significantly protect lymphocytes from gp120-induced oxidative imbalance, as demonstrated by fluorescence median values very similar to those found in control samples.

IDV hinders T cell sensitization to CD95/Fas-mediated apoptosis

The increase of CD95/Fas cell surface expression, the ezrin/CD95/Fas polarization and co-localization, as well as mitochondria hyperpolarization and pro-oxidant conditions are known to be associated with an increased susceptibility to Fas-induced apoptosis [19,22,25]. In the following experiments we evaluated whether gp120 administration was able to sensitize T cells to various apoptotic stimuli. The analysis of apoptosis, after double staining of cells with annexin V-FITC and propidium iodide, clearly indicated that i) gp120 was not able to induce apoptosis per se (Figure 4A, first row, middle panel) but ii) it was able to sensitize T cells to CD95/Fas-mediated apoptotic triggering (Figure 4A, second row, middle panel) and that iii) IDV was capable of significantly preventing lymphocyte apoptosis (Figure 4A, second row, right panel). Furthermore, the anti-apoptotic activity exerted by IDV was also assessed in T lymphocytes activated by standard protocols and exposed to triggering α-Fas (Figure 4B). Data reported in Figure 4B, clearly show that IDV also significantly (P<0.01) prevented Fas-induced apoptosis in lymphocytes activated by IL2, IL2/PHA or IL2/gp120.

On the basis of these results, we then considered whether IDV treatment was capable of hindering apoptosis associated with agents specifically influencing mitochondrial homeostasis. To this purpose, the cytokine interferon-α (IFN-α) and the drug staurosporin (STS), known to be able to modify mitochondrial homeostasis [26,27] were used. However, these agents were used at low concentrations and were, therefore, unable to induce any pro-apoptotic effect per se. The results obtained are shown in Figures 5A and 5B and can be summarized as follows. Firstly, in the presence of low concentrations of IFN-α (Figure 4A, boxed area) and STS (Figure 4B, boxed area) the percentage of cells with hyperpolarized mitochondria was significantly increased with respect to resting control lymphocytes without any sign of cell death (see Figure 3A, left panel). Secondly, according to the above results (see Figure 3), lymphocytes were hypersensitive to α-Fas-triggering that also induced, as a late event, depolarization of mitochondrial membrane, typical of apoptotic execution. This was demonstrated by the high percentage of cells characterized by low red fluorescence emission when stained by JC-1 (see area under dotted line). Thirdly, and most importantly, in contemporaneous treatments with IDV and IFN-α (IDV/IFN-α) or IDV and STS (IDV/STS), we observed a significant impairment of IFN-α and STS-mediated increase of ΔΨm, a reduction of T lymphocyte α-Fas-induced apoptosis and, accordingly, an inhibition of ΔΨm loss, that is, mitochondria depolarization typical of apoptotic execution phase (Figures 5C and 5D, respectively; in Figure 5E mean values from four different HDs are shown).

IDV inhibits t-Bid-induced swelling in isolated mitochondria

The above results seem to support a direct effect of IDV on mitochondria polarization state. In order to further analyse this point, we first investigated whether IDV was able to prevent mitochondrial swelling in isolated mitochondria. To this end, we treated mitochondria isolated from human T lymphocytes with recombinant Bid protein cleaved by caspase 3 (t-Bid). It is in fact well known that Bid, a pro-apoptotic protein belonging to the Bcl-2 family, represents a key effector of the CD95-induced apoptotic pathway acting on mitochondria [28]. Figure 6A shows a representative profile of the swelling induced by 1 µg/ml t-Bid on mitochondria.
isolated from gp120-activated lymphocytes monitored by means of variations in TMRM fluorescence. It is very evident that t-Bid induced a rapid decrease of MMP (swelling phenomenon) in a significant percentage of cells (19.6%, grey area). In particular, the MMP, expressed as a median value of the TMRM fluorescence curve significantly decreased from 249.4 to 89.4 (Figure 6B; control: plain black histogram; t-Bid: empty black histogram). Importantly, in accordance with the above results obtained in intact cells, mitochondria derived from IDV-treated gp120-activated lymphocytes were more resistant to t-Bid-induced swelling, being the percentage of depolarized mitochondria significantly lower than that found in

Figure 4. IDV hinders CD95/Fas-mediated apoptosis in activated T cells

(A) Percentage of apoptotic cells (as revealed by annexin V/propidium iodide double staining) in gp120-activated PBL from a representative HD. Note that gp120 i) did not induce apoptosis per se, ii) sensitized T cells to CD95-induced apoptosis and iii) that IDV was able to significantly reduce apoptosis (P<0.01) independently from the activating stimulus taken into consideration. (B) Mean ±SD of the results obtained in four different HDs. PI, propidium iodide.
Figure 5. IDV is capable of hindering apoptosis associated with agents specifically influencing mitochondrial homeostasis.

Cytofluorimetric analysis of MMP (by JC-1) and of CD95-induced apoptosis (by annexin V/propidium iodide double staining) in PBL of a representative HD treated with 1000 IU IFN-α (A,C) or 1 nM STS (B,D) in the absence (A,B) or presence (C,D) of IDV. In the JC-1 dot plots, numbers in the boxed areas represent the percentages of cells with hyperpolarized mitochondria, while in the area under the dashed line, the percentage of cells with depolarized mitochondria is reported. In annexin V/propidium iodide dot plots, numbers reported in the lower and upper right quadrants represent the percentages of annexin V single positive cells and annexin V/propidium iodide double positive cells, respectively. In (E) the analysis of CD95-induced apoptosis is reported as mean ± SD of the results obtained from four different HDs. Note that IDV was capable of significantly (P<0.01) preventing apoptosis either in INF-α- or in STS-treated lymphocytes. HD, healthy donors; IDV, indinavir; IFN, interferon; IL2, interleukin 2; MMP, mitochondrial membrane potential; PBL, peripheral blood lymphocytes; STS, staurosporine.
**Figure 5 continued**

**C**

![Graph C](image)

**D**

![Graph D](image)

**E**

![Graph E](image)
Figure 6. IDV inhibits t-Bid-induced mitochondrial swelling

Cytofluorimetric dot plots represent the profile of the mitochondrial swelling monitored by means of variations in TMRM fluorescence as a function of time in mitochondria isolated from untreated lymphocytes. (A) Swelling was evaluated after treatment with t-Bid [histograms in (B) show the corresponding MMP values], (C) after treatment with IDV [histograms in (D) show the corresponding MMP values], or (E) with the uncoupler FCCP [histograms in (F) show the corresponding MMP values]. Results obtained in freshly isolated T lymphocytes from a representative HD are shown. (A,C,E) Numbers in grey areas represent the percentage of mitochondria that underwent MMP decrease. Note that i) t-Bid induced swelling in a high percentage (19.6%) of mitochondria of untreated lymphocytes [(A), grey area], while ii) mitochondria derived from IDV- and FCCP-treated lymphocytes were significantly more resistant to t-Bid-induced swelling as shown by the percentages reported in grey areas of (B) and (C), and iii) parallel evaluations indicated the MMP loss induced by t-Bid (B) was counteracted by both IDV (D) and FCCP (F). (G) MMP mean values ±SD obtained by evaluating lymphocytes from three different healthy donors. Note the significant protection (P<0.001) exerted by both IDV and FCCP against t-Bid induced depolarization. Calcium chloride, able to induce MMP loss, represented a positive control. Gp120 was not able per se to induce mitochondrial membrane depolarization. (H) Results obtained by evaluating CytC release from isolated mitochondria treated with gp120 are reported. Calcium chloride, used as positive control, as well as recombinant t-Bid were able to induce the release of a significant amount of CytC. Conversely, treatment with IDV significantly (P<0.001) decreased the amount of CytC released from isolated mitochondria. Gp120 was not able to induce CytC release per se. CytC, cytochrome c; IDV, indinavir; FCCP, carbonyl cyanide p-trifluoromethoxyphenyl hydrazone; MMP, mitochondrial membrane potential, TMRM, tetramethylrhodamine ester.
gp120-treated samples (Figure 6C; 5.4%, grey area). In fact, the median value of the TMRM fluorescence curve obtained from mitochondria isolated from IDV-treated lymphocytes was only slightly modified (median values reduced from 196.3 to 165.4, Figure 6D; control: plain black histogram; t-Bid: empty histogram). As a control for this series of experiments, we used the protonophore uncoupler carbonyl cyanide fluorophenyl-hydrazone (FCCP), known to be capable of hindering MMP loss at low concentrations [18,29]. Interestingly, FCCP, similarly to IDV, significantly prevented t-Bid-induced mitochondrial swelling (Figure 6E, grey area) as well as the decrease of MMP as measured by TMRM fluorescence emission (Figure 6F; control: plain black histogram; t-Bid: empty histogram). Mean values ±SD obtained from three independent experiments are shown in Figure 6G. A significant ‘protection’ exerted by both IDV and FCCP towards MMP changes induced by t-Bid was detected.

We have also evaluated the ability of t-Bid to induce the release of CytC from mitochondria purified from human lymphocytes. The supernatants obtained from gp120-treated mitochondria (swelling experiments), before any TMRM staining, were analysed by means of ELISA methodology. Figure 6H shows i) as expected, calcium chloride 300 µM, used as positive control, induced the release of a significant amount of CytC; ii) recombinant t-Bid also induced CytC release, although in a minor extent; and iii) either IDV or a ‘baby dose’ of the FCCP compound (not shown), significantly (P<0.01) decreased the amount of CytC released from isolated mitochondria.

These data suggest that IDV could act similarly to FCCP given at low concentration, that is, via a mitochondrial uncoupler-like activity. A family of endogenous uncoupling proteins are normally detectable in the inner mitochondrial membrane of different tissues. These are called uncoupler proteins (UCPs) and are able to regulate cell energy and mitochondrial membrane potential ‘protecting’ cells from ROI production and apoptosis [30,31]. On the basis of these considerations, an analysis of the expression of UCP-2 (an ubiquitously expressed UCP isoform) was thus performed either by flow cytometry or by Western blotting (WB) analyses.

The results obtained clearly indicated an IDV-dependent down-regulation of UCP-2 protein in gp120-activated T cells (Figure 7A, empty grey histogram) with respect to gp120-activated control cells (Figure 7A, empty black histogram; UCP-2 expression in resting T cells is shown as a plain histogram). Statistical analyses of these data by K/S tests also indicated that this IDV-induced UCP-2 decrease was highly significant (Figure 7A, right panels). Mean values ±SD obtained on T cells from four different HDs are reported in Figure 7B. These results show a clear-cut dose-effect response of IDV in gp120-activated lymphocytes. In fact, a increased down-regulation of UCP-2 expression was detectable with increasing IDV concentrations. Notably, as expected [18], no effect of IDV was found in resting T cells. These results were confirmed by WB analyses, as demonstrated by the use of increasing doses of IDV, that is, 0.1, 1 and 10 µM (see densitometric measurements in Figure 7C).

Discussion

Freshly isolated lymphocytes represent a peculiar model for studying apoptotic cell death. In fact, resting T cells have been considered as apoptotic-resistant cells [32]. By contrast, once activated, for example, by IL-2 and PHA, they become susceptible to different pro-apoptotic stimuli, including the CD95/Fas ‘physiological’ receptor triggering. Interestingly, among various changes occurring in activated T cells, two of them are gathered the attention of immunologists involved in the study of immune system homeostasis: cell polarization (the migration of organelles and surface molecules at one pole of the cell) and an early mitochondrial change detectable by different flow cytometry methods – an increase of MMP (a mitochondrial hyperpolarization) [21,33]. In this context, several studies have been carried out as regards the activity of HIV PIs. These compounds have been demonstrated to impair apoptosis, improving T cell survival and to act independently from the presence of HIV particles inside T cells. The ability of various PIs to lead to the so-called immune reconstitution has in turn stimulated a plethora of experimental studies aimed at the comprehension of their mechanisms of action. In these studies, PIs have been demonstrated to be able to hinder apoptosis via a target effect on mitochondria [7,18]. In particular, the mitochondrial hyperpolarization state as detectable in T lymphocytes following activation with IL-2/PHA administration, was suggested to represent a prerequisite for apoptotic susceptibility of T cells and was found to be inhibited by PI treatments [18]. Accordingly, in the present work, we shepherd through the mechanisms underlying this peculiar effect exerted by PIs by using a model system that, in our opinion, could be of great relevance in AIDS pathogenesis studies, that is, gp120-treated, freshly isolated human T lymphocytes. Engagingly, the administration of HIV envelope protein gp120, which is responsible for HIV-host cell interaction and detectable in the blood of HIV-infected patients, was capable of inducing a series of changes. Namely, gp120 induces a significant increase of some activation markers and overexpression of CD95/Fas; organelle, cytoskeletal components and surface molecule polarization; mitochondrial
Figure 7. IDV decreases UCP-2 expression in gp120-treated T lymphocytes

(A) Quantitative flow cytometry analysis of UCP-2 protein expression in gp120-activated lymphocytes treated with IDV in comparison with resting untreated lymphocytes. Note UCP-2 down-regulation detectable after IDV treatment (grey curve). Statistical analyses (K/S test) indicated that IDV significantly ($P<0.01$) decreased UCP-2 expression in gp120-activated cells only, whilst resting lymphocytes remained unaffected (right hand panels). Numbers reported indicate the median values of fluorescence intensity histograms obtained in T lymphocytes isolated from a representative HD.

(B) Semiquantitative flow cytometry analysis of UCP-2 expression in both resting and gp120-activated lymphocytes treated with different doses of IDV. Results are shown as means ±SD of the data obtained from six different HDs. Note the dose-dependent decrease of the UCP-2 protein.

(C) Western blotting analysis of UCP-2 protein shows an IDV-induced dose-dependent decrease of UCP-2 expression in gp120-activated lymphocytes. Densitometric analyses confirmed the dose-dependent activity of IDV. These results are expressed in arbitrary units (AU). IDV, indinavir; UCP-2, uncoupler protein 2.
ultrastructural changes and hyperpolarization; and an increased apoptotic susceptibility. Conversely, the presence of PIs, particularly IDV, was irrelevant with regards to cell surface molecule overexpression, including that of CD95/Fas, while it significantly reduced cell polarization state and also blocked both MMP changes and apoptosis. Hence, PIs do not act by down-regulating cell surface molecules of importance in apoptotic triggering, that is, CD95/Fas, but, conversely, they specifically act by impairing cell morphogenetic changes influencing apoptotic prion-ness and blocking MMP increase. The importance of cell re-modeling, that is, uropod formation and cell polarization, as well as of mitochondrial ‘stabilization’, that is, the blockade of hyperpolarization state, was previously described in other in vitro and ex vivo systems [18,34,35]. Hence, our results clearly indicate that HIV-gp120 may act as a sort of ‘booster’ of T cell alterations associated with the ‘activation’ of apoptotic machinery and that PIs can lead to apoptosis hindering via a specific inhibitory effect on cell polarization and maintenance of mitochondrial homeostasis. In consideration of the role of apoptosis occurring in bystander cells of HIV-infected patients and of its role in the onset of immunodeficiency, our data seem to partially explain the possible mechanisms underlying the beneficial effects of HAART. They support, in fact, the concept that the immune reconstitution detected in vivo after PI exposure could also be due to a protective activity of these drugs against gp120-induced apoptosis sensitization of bystander cells. Moreover, these results seem also to provide further evidence for a reappraisal of PIs in the clinical management of human immune deficiencies other than AIDS.

Data herein reported also indicate some important new findings regarding the target effects of these drugs. One is represented by the ability of PIs to act by impairing reactive oxygen species formation detectable after gp120 administration. Hence, in consideration of the key role played by the redox imbalance in apoptotic cell death program [22], these results allow us to hypothesize that the IDV-induced cell survival can be the outcome of a sort of antioxidant activity exerted by PIs. It is in fact well known that mitochondria are the major source of free radical species originated during the execution phases of apoptotic cell death. On these bases, we cannot rule out the possibility that PIs, by acting as ‘quenching’ agents, could contribute to maintaining the intracellular redox balance via their target activity on mitochondria, that is, via unexpected antioxidant properties. These hypotheses seem to be confirmed by the protective role of PIs towards apoptosis in model systems alternative to gp120-induced apoptosis sensitization. In fact, T cell treatment with non-cytotoxic doses of IFN-α or STS, both considered as ‘mitochondriotropic’ agents [23,36], lead to an increased apoptotic prion-ness, which was significantly counteracted by IDV. Hence, an immunoregulatory cytokine such as IFN-α as well as a drug, such as STS, directly acting on mitochondrial homeostasis, were both capable of sensitizing T cells to Fas-mediated apoptosis. Thus, the target activity of PIs to mitochondria could be responsible for their protective effects in the maintenance of mitochondrial homeostasis and, as a consequence, in the inhibition of T cell apoptosis. In consideration of the importance of IFNs in infectious diseases, these results also point to PIs as useful pharmacological modulators in a number of human innate and acquired immune diseases.

Some interesting considerations derive from the analyses of the results obtained by low concentrations of the protonophore uncoupler FCCP. High concentrations of this compound were demonstrated to induce apoptosis in tumour cell lines [37]. In contrast, very low doses of FCCP could protect human T cells from CD95-induced apoptosis [38]. Our results emphasized that PIs and FCCP show impressive similarities in their mitochondrial effects. In fact, the results obtained in isolated mitochondria have shown the ability of both drugs to ‘stabilize’ MMP after t-Bid administration. The truncated form of the Bid molecule was in fact considered as a pro-apoptotic signalling molecule reaching mitochondria after CD95/Fas stimulation. A partial confirmation of the above hypothesis was further offered by the PI-induced down-modulation of UCP-2 mitochondrial protein. This can reinforce the idea that mitochondria represent specific targets of PIs and also favour the intriguing hypothesis that PIs, for example, IDV, could exert a uncoupler-like activity, minimizing mitochondrial hyperpolarization occurring in the early phases of apoptosis. In fact, the mechanisms implicated in the maintenance of mitochondrial homeostasis and redox balance involve the coupling activity of the respiratory chain and are physiologically regulated by UCPs. The decreased UCP expression in the presence of PIs could also represent indirect evidence for a peculiar role of PIs that, vicariating UCP activity in activated lymphocytes, might sustain mitochondrial hyperpolarization, reduce redox imbalance and, later, inhibit ∆Ψ loss. In addition, UCPs seem to play an important role also in some pathophysiological states [39] including obesity, insulin resistance [40] and atherosclerosis [41]. Finally, it is well known that UCPs also play a pivotal role in regulating the energy metabolism also in the adipose tissue, where UCPs have been isolated for the first time [42,43]. Thus, on the basis of the well-known adverse effects associated with HAART, that is, lipodystrophy, atherosclerosis and diabetes, the present results on the modulatory activity...
exerted by PIs on UCP molecule expression would also stimulate further studies on the pathogenetic mechanism underlying HAART-associated disease contributing, in the long run, to a refining of the therapeutic strategies.

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Mechanisms of HIV and nucleoside reverse transcriptase inhibitor injury to mitochondria

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Introduction

Mitochondria perform a range of biological functions and carry a number of factors involved in cell apoptosis. In particular, mitochondria are the key organelles in energy production in all human cells except erythrocytes. Energy, in the form of ATP, is produced through the oxidative phosphorylation pathway. HIV and other infectious agents as well as some nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) are known to affect mitochondrial (mt) function. A number of important clinical events occurring in individuals with HIV infection and on antiretroviral therapy (ART) have been linked to mt injury and dysfunction.

In vitro studies have demonstrated that NRTIs may differ in their effects on mitochondria and may affect mitochondria in different cell lines in different ways. This is likely to influence the clinical syndromes associated with toxicity to these agents. Dideoxy-NRTIs (d-NRTIs) have the greatest affinity for mtDNA polymerase-γ [1], the enzyme responsible for mtDNA replication, whereas other nucleoside analogues may influence mt function through other mechanisms. These differences may be important when choosing techniques to evaluate the impact of antiretroviral agents on mitochondria.

Clinical toxicities with a possible mt aetiology

A wide range of adverse events occurring in individuals with HIV infection, particularly those receiving NRTI-based therapy, have been suggested as relating to diminished mt function. The development programs of zidovudine (AZT), didanosine, zalcitabine and stavudine have all required dose de-escalations due to toxicities thought to be related to drug impact on mitochondria. Due to limitations in measurement technology, clinical studies of NRTIs, while collecting adverse event data, have not specifically evaluated mt toxicity in a systematic way.

Lactic acidosis is the event that establishes proof that clinically important mt toxicity occurs in individuals receiving NRTIs, although causation is not necessarily established by this observation. For lactic acidosis to occur, the mt oxidative phosphorylation system, which normally removes H⁺ generated by the hydrolysis of ATP and the conversion of glucose-6-phosphate to pyruvate, must be dysfunctional (Figure 1). Lactic acidosis in the critical care setting has a range of causes and these are not always reliably excluded in reports of individuals with HIV on ART who develop lactic acidosis. Not surprising, lactic acidosis has also been reported in individuals with HIV not currently receiving ART [2].

Other NRTI-associated adverse events where the weight of evidence favours mt injury include peripheral neuropathy, myopathy and hepatic steatosis. However, multiple aetiologies for these adverse events exist and should be routinely sought in individuals presenting with these effects. Other adverse effects observed during ART that may represent manifestations of, or be contributed to by mt toxicity include hypogonadism, pancreatitis, diabetes mellitus, proximal renal tubular dysfunction, anaemia and neutropaenia, and peripheral fat atrophy [3].

Manifestations of NRTI-related mt toxicity in adults do not commonly involve the brain, the organ most commonly affected in inherited mt disorders. The possibility of CNS mt toxicity arising in infants after in utero exposure to NRTIs has been raised in reports from France [4,5] but has not been found in a large retrospective analysis in the USA [6]. Certainly, an extremely high incidence of hyperlactataemia in infants after in utero NRTI (mainly AZT plus lamivudine) exposure has been reported [7,8], suggesting mt dysfunction may be common in infants exposed to nucleoside analogues in utero, possibly due to differential effects of NRTIs during ontogeny. Additionally,
G Moyle

Figure 1. (A) L-Lactic acid synthesis: lactic acid accumulates if pyruvate or NADH accumulates. (B) DCA effects: DCA activates PDH, favouring lactate oxidation

A

Glycogen → Glucose → Pyruvate + H+ + NADH + H+ + LDH → Lactate + H+ + NAD+

Protein → Amino acids → Urea

B

Glucose → Lactate → Pyruvate → Acetyl-CoA → ATP, ADP

Fatty acids → PDH → O2, ADP

Activated by DCA


In patients with untreated HIV, samples from a range of tissues have been observed to have diminished mtDNA relative to age-matched uninfected individuals [20–24]. Muscle and nerve biopsies from untreated individuals presenting with HIV-associated myopathy [22] or distal symmetrical peripheral neuropathy may have low mtDNA, abnormalities in mt ultrastructure or respiratory chain abnormalities [22]. These changes are similar to those reported with NRTI therapy in vitro, animal and human nerves and muscle cells. Declines in mtDNA in adipose tissue of untreated individuals have also been described [20,22–25]. Changes in the mtDNA:nDNA ratio in peripheral blood mononuclear cells (PBMCs) (derived from buffy coat) were proportionally more pronounced when comparing HIV-negative controls to HIV-infected individuals (a 44% reduction) relative to the addition of antiretroviral agents known to inhibit DNA polymerase-γ. Changes in mtDNA content or mitochondria ultrastructure in NRTI-treated individuals, with or without clinical disease, are generally greater than in untreated individuals. Thus, reductions in mtDNA can occur with HIV infection alone and these changes may precede the use of NRTI therapy or symptoms. Indeed, mtDNA in PBMCs may rise following the initiation of effective therapy [24], even when that therapy includes agents known to inhibit DNA polymerase-γ. Changes in mtDNA content or mitochondria ultrastructure in NRTI-treated individuals, with or without clinical disease, are generally greater than in untreated individuals.

The impact of HIV infection on mitochondria

In patients with untreated HIV, samples from a range of tissues have been observed to have diminished mtDNA relative to age-matched uninfected individuals [20–24]. Muscle and nerve biopsies from untreated individuals presenting with HIV-associated myopathy [22] or distal symmetrical peripheral neuropathy may have low mtDNA, abnormalities in mt ultrastructure or respiratory chain abnormalities [22]. These changes are similar to those reported with NRTI therapy in vitro, animal and human nerves and muscle cells. Declines in mtDNA in adipose tissue of untreated individuals have also been described [20,22–25]. Changes in the mtDNA:nDNA ratio in peripheral blood mononuclear cells (PBMCs) (derived from buffy coat) were proportionally more pronounced when comparing HIV-negative controls to HIV-infected individuals (a 44% reduction) relative to the addition of antiretrovirals (a further 24% decline) [20]. Thus, reductions in mtDNA can occur with HIV infection alone and these changes may precede the use of NRTI therapy or symptoms. Indeed, mtDNA in PBMCs may rise following the initiation of effective therapy [24], even when that therapy includes agents known to inhibit DNA polymerase-γ. Changes in mtDNA content or mitochondria ultrastructure in NRTI-treated individuals, with or without clinical disease, are generally greater than in untreated individuals.

These data raise the possibility that HIV may directly (or via cytokines released in response to HIV infection or immune reconstitution) injure mitochondria, potentially making them more vulnerable to the effects of NRTIs.

Several HIV gene products, most notably tat and viral protein R (vpr), have been demonstrated to decrease or damage mitochondria and cause clinical disease in vitro or in animal models. For example, expression of TAT may lead to cardiomyopathy with mt destruction in mice [26]. The vpr directly affects the mt permeability transition pore and may trigger cell apoptosis through a mt pathway independent of caspase [27]. The HIV-1 protease may also process pro-caspase to cause mt release of cytochrome c triggering apoptosis [28].

As muscle, nerve or fat cells may not be infectable by HIV, cytokines may represent the key mediator of
Mitochondria and NRTIs

Effects of NRTIs on mitochondria

The most prevalent theory regarding the effects of NRTIs on mitochondria is that these agents act as competitive inhibitors of DNA polymerase-γ, the enzyme responsible for copying mtDNA. The d-NRTIs, zalcitabine (ddC), didanosine (ddl) and stavudine (d4T), have the greatest affinity for this enzyme [1]. Inhibition of polymerase-γ is likely to lead to decline in quantity and, most likely, quality of mtDNA and eventually trigger a cellular energy crisis.

With regards to peripheral neuropathy and the dose-limiting toxicity of the d-NRTIs, the weight of evidence indicates that this is indeed a mt toxicity with mtDNA decline. Using an in vitro model of nerve cells, ddC and ddl have been shown to reduce mtDNA, leading to destruction of mitochondria and an increase in intracellular lactate levels [44]. Nerve biopsies from patients with NRTI-associated neuropathy have show abnormal mitochondria with enlarged size, excessive vacuolization, electron-dense concentric inclusions and degenerative myelin structures. Abnormal mitochondria represented the majority (55% vs 9% in controls) of nerve cell mitochondria in biopsies from individuals with ddC-related neuropathy and mtDNA was reduced by as much as 80% compared with the controls [45]. This extent of mtDNA depletion is consistent with loss of functional mtDNA observed in familial mt disorders and hence is consistent with causation. Mitochondrial changes are also found in nerves of untreated individuals presenting with HIV-related peripheral neuropathy [46], hence neuropathy in individuals on therapy may in some cases represent an unmasking or exacerbation of a pre-existent mt neuropathy.

However, with myopathy, a probable mt toxicity associated with AZT therapy, the effects on mtDNA mass are less evident. As with neuropathy, specific changes in muscle mitochondria have been observed in clinical biopsy samples and in animal or in vitro models. Effects have been demonstrated in both skeletal and cardiac muscle [47]. Histological features suggested to be distinguishing include ragged-red fibres and abnormal mitochondria with paracrystalline inclusions [48,49], although similar mt abnormalities have also been reported in therapy-naïve individuals with HIV-related myopathy [22,50]. Tissue-specificity studies with AZT have demonstrated that this drug has greater impact on muscle cells, compared with kidney or liver cells, and may involve inhibition of succinate transport or cytochrome oxidase activity, reduction in carnitine, oxidative damage to mitochondria and destruction of myotubes rather than reduction in mtDNA [47,51–55]. Thus, myopathy (and presumably other toxicity events) with AZT may relate to this agent's impact on mt enzyme systems rather than on DNA polymerase-γ. Other studies have found that AZT may affect a range of mt functions including strongly inhibiting the ADP/ATP antiport in a competitive manner [17], and adenylate kinase [18].

These differences in mechanism of toxicity with AZT relative to d-NRTIs probably relates to the observation that some of its cytological and mt toxicities are mediated through its intermediate metabolite AZT-monophosphate rather than the triphosphate metabolites responsible for the toxicity (and activity) of other NRTIs [56]. This has important implications when assessing ways in which drug impact on mitochondria is measured, as standard measures of mtDNA content are likely to underestimate the effects of AZT on mt function.

More recently, a further mechanism by which AZT may cause mt toxicity has been evidenced. In non-mitotic cells, AZT inhibits thymidine phosphorylation by thymidine kinase (TK) type 2, the TK expressed in mitochondria. TK2 is the only constitutively expressed TK in non-mitotic cells. The reduced supply of the natural thymide triphosphate (TTP) leads to limited mtDNA replication and subsequent mtDNA depletion.
[57]. As d4T is a much poorer substrate of TK2 than AZT, but a more potent inhibitor of polymerase-γ, it is unlikely that this mechanism is critical to d4T toxicity [58]. In vitro studies indicate that lactic acid production begins in AZT-treated cells before the decline in mtDNA, whereas declines in mtDNA coincide with lactic acid production increases in d-NRTI-treated cells [59]. In vivo, the rate of mtDNA reductions over time appear somewhat slower and may be less severe in TK-dependent cells (such as adipocytes) with AZT than with d4T [25,60,61], an issue that may be linked to the different modes of toxicity.

Summary

Available evidence suggests that a number of important clinical events in individuals with HIV infection are related to mt dysfunction. Several factors may contribute to the development of these events and the tissue(s) in which the event occurs. Some individuals are likely to have important genetic predispositions for mt disease, which may be unmasked by the presence of HIV infection or the introduction of NRTI antiretrovirals. HIV infection per se is associated with reduction in mtDNA content and changes in mt morphology and function, which in some cases leads to clinical events such as myopathy or peripheral neuropathy. NRTI antiretrovirals may impact mtDNA content and function through a number of different mechanisms and have been demonstrated to be causative of a number of clinical toxicities. In in vitro and in clinical studies, newer nucleoside and nucleotides agents such as lamivudine, emtricitabine, abacavir and tenofovir appear to be much weaker inhibitors of mtDNA polymerase-γ or other mt functions, and appear to be associated with a lower risk of events thought to be related to mt toxicity.

Simple, non-invasive tests for mt function are not available at present in the clinical routine, and assays of mtDNA content in blood cells may miss key aspects of mt function, require careful sample handling and may not reflect events occurring in other tissues. There remains a need for the development of rapid, cheap and clinically applicable assays that would enable the prediction of increased likelihood of mt events.

References


Nucleoside analogues toxicities related to mitochondrial dysfunction: focus on HIV-infected children

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Introduction

Survival in HIV-infected children has greatly improved with the introduction of highly active antiretroviral therapy (HAART) [1]. Concomitantly, morbidity from the long-term effects of antiretroviral therapy (ART) has grown in importance. Children are more vulnerable than adults to the metabolic side effects of therapy because of the potential impact on growth and the likelihood of greater cumulative exposure. A number of important clinical events have been described in HIV-infected adults receiving antiretroviral nucleoside analogues that have been linked to mitochondrial (mt) injury and dysfunction. This review discusses these complications individually in an attempt to summarize the current understanding of their pathogenesis, with a focus on HIV-infected children.

Hyperlactataemia syndromes

Use of nucleoside analogue reverse transcriptase inhibitors (NRTIs) by HIV-infected adult patients may be complicated by hyperlactataemia with a frequency ranging from 8–18.3% in cohort studies [2–6]. The precise biochemical pathways involved in NRTI-related hyperlactataemia (NRH) are not yet completely understood or defined. Nevertheless, a leading theory based on several in vitro data is that NRH derives from direct inhibition of mtDNA polymerase-γ by NRTIs and, consequently, mt dysfunction [7–10]. Three different hyperlactataemia syndromes have been described in HIV-infected adults: lactic acidosis syndrome (LAS), symptomatic hyperlactataemia and subclinical hyperlactataemia.

LAS is a rare, life-threatening disease characterized by severe lactic acidosis (lactate levels usually >5.0 mmol/l), hepatic steatosis, occasional liver failure and sometimes by other NRTI-related toxicities believed to have a mt genesis, such as pancreatitis, sensory neuropathy and skeletal myopathy [10–14]. In HIV adult cohorts, LAS shows an incidence ranging from 1.3–3.9 cases per 1000 person-years, a mortality rate greater than 50% and is highly correlated with NRTI use [2,14].

In some cases, hyperlactataemia (≤5.0 mmol/l) is associated with clinical symptoms (nausea, emesis and vague abdominal pain), mild liver abnormalities without hepatic failure and is not complicated by metabolic acidosis [2–6]. Symptomatic hyperlactataemia is rare in HIV-infected adults (8–14.5 cases per 1000 person-years) and is highly correlated with NRTI use, showing a mild clinical course usually with a good prognosis, provided that NRTIs are promptly discontinued [2,12,15].

Most (>85%) of the 8–18.3% of HIV-infected adults who test positive for hyperlactataemia are symptom-free [2–6]. Subclinical hyperlactataemia is characterized by lactate levels ranging from 2.1–5.0 mmol/l, is usually transient and has a poor positive predictive value for identifying cases of future symptomatic lactic acidosis/hepatic steatosis [3–5]. Moreover, in the largest published prospective cohort study, the detection of subclinical hyperlactataemia in 2% of ART-naive patients indicated that this syndrome might be relatively non-specific for current NRTI use [5]. Finally, a recent prospective study showed that chronic subclinical mild hyperlactataemia with a lactate concentration of 1.5–3.5 mmol/l occurs in a significant proportion of HIV-infected adults started on NRTI-containing regimens. Initiation of HAART containing either zidovudine (AZT) or stavudine (d4T) was associated with a mild increase in group mean lactate levels during the first 6–8 months of therapy and with a new serum lactate ‘steady state’ after 12 months of therapy [3]. The cause(s) of subclinical hyperlactataemia remain(s) unclear. Given its transitory pattern, however, sampling variability might play a major role. In addition, the superimposition of sampling variability on an already increased serum lactate setpoint may also account for the higher...
Lactic acidosis is an important cause of metabolic acidosis in children and may be divided into two categories: type A, which is associated with tissue hypoperfusion and hypoxaemia secondary to cardiopulmonary arrest, septic, cardiac or hypovolaemic shock and type B, which occurs without any clinical evidence of tissue hypoxia but is due to impairment of mt function caused by drugs, toxins and inherited metabolic disease. The first group is relatively common in the paediatric intensive care unit; the second is uncommon [16].

During 1997–2003, three cases of type B lactic acidosis were identified in HIV-infected children treated with antiretroviral drugs. The first paediatric case of lactic acidosis and hepatic steatosis was described by Miller et al. [17]. The author reported on four patients, one of whom was an HIV-infected adolescent who developed severe hepatic steatosis and lactic acidosis while receiving an antiretroviral regimen containing d4T. The paediatric case was a 16-year-old HIV-infected girl with a 3-day history of nausea, vomiting and abdominal pain. She had severe metabolic acidosis (arterial blood pH 7.33), an elevated serum lactate level (9.9 mmol/l), hepatic steatosis [liver density, at computed tomography (CT), consistent with diffuse fatty infiltration], elevated liver enzymes [alanine aminotransferase (ALT), 120 U/l; aspartate aminotransferase (AST), 166 U/l], pancreatitis (pancreatic imaging, at CT, showing inflammation and necrosis) with increased levels of amylase (561 U/l) and lipase (6150 U/l), and myopathy (increased fat droplet in myocytes, cytochrome oxidase-negative fibres, degenerating fibres at light and electron microscopy assessment of quadriceps biopsy specimen). Therapy with d4T, didanosine (ddi) and nelfinavir (NFV) (which the patient had been taking for 3 months) was discontinued. Serum lactate level initially decreased from 9.9 to 19.5 mmol/l; however, soon after this, lactate level increased and bicarbonate decreased again and this tendency persisted until the death of the child 36 h after hospital admission. The comparison between paediatric and adult cases of LAS seems to identify several overlapping features concerning incidence, risk factors and clinical presentation and, unique to paediatric cases, the more frequent concomitant presence of other signs of NRTI-related toxicities.

Recently, Rey et al. described the first paediatric case of fatal lactic acidosis [19]. The child was a 5-year-old HIV-infected girl receiving ritonavir, d4T and ddI with a 10-day history of nausea, vomiting and a 12-hour history of abdominal pain with severe tachypnea and hypoponca. Initial laboratory studies revealed a pH of 7.27, bicarbonate of 2.8 mmol/l and a lactate level of 13.6 mmol/l. Signs of liver dysfunction included elevated bilirubin levels, very decreased fibrinogen levels (37 mg/dl) with prolonged prothrombin time (21.5 sec) and partial thromboplastin time (40 sec), mild elevation of AST and ALT (57 and 67 U/l, respectively), and elevated lipase (3222 U/l) and amylase levels (805 U/l). She was admitted to the paediatric intensive care unit, where ART was discontinued and high-dose intravenous bicarbonate, haemodiafiltration and empirical therapy for mt failure with essential cofactors (L-carnitine, riboflavin and thiamine) was started. Serum lactate level initially decreased from 21.2 to 12.4 mmol/l and serum bicarbonate rose from 9.5 to 19.5 mmol/l; however, soon after this, lactate increased and bicarbonate decreased again and this tendency persisted until the death of the child 36 h after hospital admission.

Thus, the above-mentioned paediatric cases of LAS share several features with those described in adult patients; the duration of exposure to NRTIs was in the expected range of 3–20 months, the most common complaints were gastrointestinal (except in the infant who could not be expected to be able to refer these symptoms), signs of liver dysfunction were present in...

...disorderly mitochondria with...
Of the total 251 lactate readings, 56 (25.6%) were above 2 mmol/l. Only one patient was symptomatic, with abdominal pain, nausea and vomiting, but these symptoms disappeared after a temporary discontinuation of ART. Elevated lactate levels were associated with therapy with NRTIs (P=0.01) or PI (P=0.04) when considered as classes.

Further studies are obviously needed to fully evaluate the incidence of subclinical hyperlactataemia and to assess the exact contributory role of factors other than NRTIs in this syndrome in HIV-infected children. However, both the published studies deserve some comment. Noguera et al. [24] showed a 17% prevalence of subclinical hyperlactataemia; however, if the nine cases with transient hyperlactataemia are excluded, the frequency would further decrease to 11%. In addition, younger age was a significant risk factor for hyperlactataemia. This observation needs further confirmation, namely, it may arise either from a greater susceptibility of younger children to NRTI toxicity or from more frequent difficulties in blood sampling in infants and toddlers compared with older children.

Desay et al. [25] claim a 32% prevalence of subclinical hyperlactataemia; however, no detailed information on the blood sampling method is given or whether hyperlactataemia was also detected in the 23 untreated children included in the study. The study also raises the novel possibility that therapy with PIs may contribute to elevated lactate levels. However, since almost all patients (102/104) included in the analysis were receiving at least one NRTI, it is unclear if the observation came from a true evidence or from a bias in the statistical analysis.

Cardiomyopathy

Several patterns of cardiovascular involvement have been reported in HIV-infected children [26–32]. A continuum from asymptomatic left ventricular (LV) dysfunction to dilated cardiomyopathy to congestive heart failure (CHF) to hypotensive pump failure with cardiac associated mortality has been suggested [33]. Abnormalities of LV hypertrophy have also been suggested in which LV mass is excessive for body surface area but insufficient for LV dimension, resulting in a sustained elevation of LV peak wall stress, a mediator of mechanically induced hypertrophy [28,34]. Other reported cardiac problems include haemodynamic abnormalities, conduction abnormalities, dysrhythmias and sudden death, as well as pericardial and vascular involvement [26–32]. Dilated cardiomyopathy appears to be more common in HIV-infected children than in seroverted children and increases in frequency as HIV-infected children progress to AIDS [35]. CHF appears to occur chronically in some 10% of HIV-infected children and transiently in another 10%
The relationship between AZT use and the development of cardiomyopathy has been carefully examined by Lipshultz et al. [47]. Serial echocardiograms were performed in 24 children with symptomatic HIV infection before they started AZT and a mean of 1.32 years since therapy began, 27 age-matched children with symptomatic HIV infection not treated with antiretrovirals and 191 healthy controls (HCs). As compared with HCs, children treated with AZT had progressive LV dilatation and peak wall stress; dilatation and stress were significantly elevated both before and during AZT treatment. The ratio of ventricular thickness to internal dimension was below normal before ART began. After AZT treatment, overall LV mass as well as peak wall stress increased while LV fractional shortening decreased. No significant differences were detected at follow-up in any of these measurements between HIV-infected children treated with AZT and those not treated. These data show that a progressive LV dilatation with compensatory hypertrophy inadequate to maintain normal peak systolic wall stress occurred in children with symptomatic HIV infection. AZT treatment did not appear to worsen or ameliorate these cardiac changes.

In a P2C2 HIV Study Group trial, 196 children with severe and mild symptomatic HIV infection and with a median age of 2.1 years were followed with a longitudinal 2-year echocardiographic assessment. The results confirmed that subclinical cardiovascular abnormalities were common in HIV-infected children and most remained throughout follow-up, with some progression. Unfortunately, the study did not perform a separate analysis for the 124 AZT-treated and the 72 untreated children, and so the possible role of this drug cannot be inferred [48].

In another P2C2 HIV Study Group trial, infants born to HIV-infected mothers were followed from birth to the first 14 months of age [49]. Data on 382 infants without HIV infection (all exposed to AZT perinatally and 36 postnatally, for a median of 42 days) and 58 HIV-infected children (all exposed to AZT perinatally and 12 postnatally during the first 12 months of age) were analysed. No association with acute or chronic abnormalities in LV structure or function was found with perinatal and postnatal exposure to this antiretroviral drug. Domanski et al. retrospectively reviewed echocardiograms and clinical records (from January 1987–December 1992) on 137 HIV-infected children (13 of whom had never received ART) with symptomatic disease in the large majority. At the date of the first echocardiogram, the antiretrovirals administered included AZT (52 patients), ddI (13 patients), both drugs (one patient) and no treatment (71 patients). M any of the 71 untreated patients were receiving their echocardiograms as a part of pre-treatment evaluation.
Children treated with AZT had a lower average in LV fractional shortening than those untreated with AZT. The odds that a cardiomyopathy would develop were 8.4 times greater in children who had previously used AZT than in those who had never taken this drug. ddI was not associated with the development of a cardiomyopathy [50].

Overall, the great majority of the studies performed in the pre-HAART era suggest that cardiomyopathy is quite common in HIV-infected children with symptomatic disease and that it is not associated with AZT use. The conflicting data obtained by Domanski et al. need to be interpreted with caution. The authors have mainly compared serial echocardiographic measurements from children with symptoms of HIV disease receiving AZT with those from children receiving ddI. The echocardiograms on 58/71 children, who were supposed to represent a group who had received neither therapy, were actually "baseline" measurements before the start of ART with no follow-up studies without therapy. Only 13 never-treated patients were included to represent the effect of HIV alone and these were specified as having 'no symptoms'.

Finally a recent case report showed benefits of a HAART regimen including AZT in a child with severe cardiomyopathy [51]. A dilated cardiomyopathy was diagnosed between 9–12 months and it severely deteriorated in the ensuing 2 years. When the child was 3.5 years old, AZT, lamivudine (3TC) and ritonavir therapy was started. Six months after therapy, CD4 count increased, viral load became undetectable and LV shortening fraction increased from 12 to 25%. One year later, the heart size was still slightly large on chest X-ray, but echocardiography revealed normal cardiac function with an LV shortening fraction of 33%.

**Distal symmetric polyneuropathy**

Peripheral nerve disorders are frequent complications of HIV disease and distal symmetric polyneuropathy (DSP) is the most common form of neuropathy with reported estimates of 15–50% in HIV-infected adult populations [52]. While DSP is relatively uncommon early in the course of HIV disease, its incidence increases as the degree of immunosuppression progresses [53]. The major presenting complaints of DSP are paraesthesia, numbness and burning sensations in the feet, usually in a symmetrical pattern. Unexpected peripheral neuropathies have been described in Phase I/II dose-finding studies with zalcitabine (ddC), ddl and d4T. The percentage of patients who developed DSP during ddC therapy ranged from 25–66% [54–56]. The occurrence of ddC-associated neuropathy as well as the severity and progression of symptoms were clearly dose-dependent. The incidence of DSP in patients treated with ddI varied from 12–34% of the subjects [57]. The onset and progression of neurological toxicity were both related to the daily and cumulative doses of this drug [58]. The occurrence of DSP has been described in 6, 15 and 31% of patients receiving d4T at a daily dose of 0.5, 1.0 and 2.0 mg/kg, respectively (Bristol-Meyers Squibb, data on file). The temporal relationship of onset and resolution of DSP with drug intake and discontinuation clearly indicates a toxic effect of NRTIs. There are several theories for the mechanism of this neurotoxicity but most evidence focuses on mt toxicity. Using an in vitro model of nerve cells, ddC and ddl have been shown to reduce mtDNA and cause mt damage [59].

Nerve biopsies from patients with ddC-related DSP, as compared with nerve biopsies from patients with AIDS-related neuropathy never treated with ddC, have shown a majority of abnormal mitochondria (55 vs 9%) and mtDNA depletion as high as 80% [60]. However, mt changes were also observed in nerves of untreated individuals presenting with DSP [61]. In addition, depleted levels of acetyl-L-carnitine have been noted in patients with DSP receiving ddC, ddl or d4T therapy [62]. This depletion may impair peripheral nerve regeneration and disrupt mt metabolism providing a further mechanism for NRTI neurotoxicity [63].

Children with HIV infection develop a variety of neurological complications, but peripheral nervous system involvement is relatively infrequent compared with the frequent occurrence of peripheral neuropathy described in HIV-infected adults [64].

Neuropathy has never been observed or has been rarely reported in paediatric populations treated with ddC [65]. In 1991, a case of a 5-year-old boy with AIDS who developed an inflammatory demyelinating polyneuropathy was reported by Raphael et al. [66].

In 1997, Floeter et al. described the electrophysiological data of 50 HIV-infected children under 18 years of age referred for evaluation of suspected neuropathy to the EMG laboratory at the National Institutes of Health during 1989–1995 [67]. Most children had moderate to severe manifestations of HIV infection, with 31/50 classified as C3 – the clinical and immunologically most severe category of the disease. All but three of the children had been treated with one or more NRTIs (AZT, ddI, ddC, 3TC) before the development of symptoms of neuropathy and before referral for nerve conduction studies (NCSs). Overall, 12 children had abnormal NCSs and a few common patterns of abnormalities were detected: DSP in seven cases, median mononeuropathy in three cases and unusual patterns in two cases (central and peripheral nervous system dysfunction in one and subacute lumbosacral polyradiculopathy following a varicella zoster infection).
in the other one). All children with DSP had fairly advanced HIV infection (five in CDC class C3 and two in CDC class B2) and had received ART prior to experiencing symptoms suggestive of neuropathy. Interestingly, a stepwise logistical regression of risk factors (age, % CD4 count and number of NRTIs received) for the development of neuropathy found that age alone was significantly associated with DSP. Children with DSP had a mean age of 14.4 years compared with a mean age of 8.2 years for children without DSP. Thus DSP in children, as in adults, seems to be the most common pattern of peripheral neuropathy and it occurs in subjects with advanced HIV infection. The role of NRTIs, particularly ddI and ddC, in causing distal neuropathy could not be clarified by this study. In this series, children with more severe HIV infection had received more antiretroviral drugs overall, and all children with DSP received more than one antiretroviral drug prior to developing symptoms. Although these data cannot truly untangle the role of NRTIs in the development of neuropathy in these children, most children who received NRTIs did not develop peripheral neuropathy.

In 2000, a cross-sectional study on 39 children (age range 5–14 years) was conducted by means of a structured questionnaire (regarding symptoms of pain, paraesthesia or weakness) and a physical examination [68]. Thirteen (34%) cases had symptoms and signs of DSP. Distal paraesthesia and/or pain plus diminished ankle jerks and/or diminished vibration sense were the most common clinical findings. Symptoms were chronic and fluctuating, but pain was, in general, not severe. In eight of the 13 patients, the use of potential neurotoxic drugs was absent before the beginning of DSP, while in the remaining five patients, clinical features developed after having been on ddI therapy.

The paucity of reports on DSP in HIV-infected children does not allow a definitive assessment of its incidence and correlation with the use of neurotoxic drugs in this population. However, the prevalence reported in the more recent study [68] is similar to that reported in HIV-infected adults. The lack of peripheral neuropathy in studies conducted at the beginning of the epidemic could be explained by the early mortality of children at that time. Dying before acquiring adequate language skills would preclude symptoms such as paraesthesia or pain from being confirmed. In addition, symptoms in children seem less severe and they could not be referred spontaneously to the clinicians. An interesting question, which deserves further research, is the role of age in the development of DSP. In animals, age alone is a risk factor diminishing regenerative capacity of the growing peripheral nervous system [69]. In humans, peripheral nerves normally exhibit very rapid growth shortly after birth followed by slower growth over the first few years, leading to adult nerve conduction velocities by about the fourth year [70]. If regenerative capacity in animals parallels that in humans, the peripheral nervous system may no longer be able to compensate for chronic low-level nerve injuries from HIV infection by the time the child reaches adolescence.

**Peripheral lipoatrophy**

Body fat abnormalities, summarized under the term lipodystrophy, are common in HIV-infected adults receiving potent ART, occurring in 30–50% or more of individuals in several large, prospective studies [71–73]. These abnormalities include, singularly or in combination, central fat accumulation evidenced by increased abdominal girth, development of a dorsocervical fat pad and breast enlargement, as well as loss of peripheral subcutaneous fat (lipoatrophy). The latter designation includes subcutaneous fat loss in the limbs, buttocks and face. There is no universally accepted definition of lipodystrophy, but most descriptions define this syndrome on the basis of clinical features of fat redistribution, as assessed with physical examination, CT or magnetic resonance imaging (MRI) scans, dual-energy X-ray absorptiometry (DXA) scans and anthropometry.

The clinical diagnosis of lipodystrophy in children is hampered by the lack of questionnaires appropriate to different paediatric ages and by the fact that alterations in body fat frequently escape clinical detection. The latter observation stems from studies which clearly showed that children without clinical signs of lipodystrophy demonstrate a decrease in limbs fat and an increase in trunk fat with DXA assessment [74,75]. Lumbar single-slide CT or MRI scans have shown great accuracy in quantifying visceral and subcutaneous adipose tissue in patients affected by body composition abnormalities [76]. However, the use of these methods without sedation is only suitable in children older than 4–5 years and there are no reference values in the paediatric population. DXA quantifies the lean and fat compartments precisely and it is presently considered the gold standard for in vivo body composition studies in children; moreover, it allows a regional analysis of the fat distribution between limbs and trunk [77]. Once again, this technique is only suitable in children older than 4–5 years and the few available reference values are mainly derived from a population including overweight children [78]. Skinfold thickness can measure the subcutaneous fat layer, allowing a comparison with reference values at well-defined points at all paediatric ages [79]. However, this method suffers from wide inter-intraperson variability, requires considerable training for the results to be reproducible and may also be inappropriate in subjects with severe lipoatrophy.
Reference values are available for circumferences in children; waist circumference may be a useful parameter to identify trunk adiposity although limb circumferences are a useful marker of peripheral atrophy, being a measure of the sum of lean and fat mass. In addition, the lack of comparative studies between simple anthropometry (peripheral or central skinfolds and circumferences) and gold standard techniques (DXA and MRI) in HIV-infected children currently limits its diagnostic power, especially in subtle, early forms of lipodystrophy.

Overall, the measurement of change in adiposity in children is challenging because of the effects of maturational growth and on lean muscle mass, fat mass and hydration status. This is particularly true in age periods when subcutaneous adipose tissue is minimal (pre-school and school age) or when important inter-individual gender-related variabilities in fat compartment are common (infancy and puberty) [79]. Longitudinal body composition analysis, possibly starting before beginning ART, may be required even more in children than in adults to properly assess lipodystrophy and, consequently, to adopt therapeutic strategies in order to avoid this relevant clinical and metabolic complication.

Studies evaluating body composition by DXA, MRI and skinfold thickness have indicated that changes in body fat content and distribution do occur in HIV-infected children. The prevalence of these changes varies from 18–33% and increases with longer duration of exposure to ART [74,75,80–82]. Recently, a clinical assessment of body fat abnormalities, according to an agreed protocol, was performed on 477 HIV-infected children aged ≥3 years in 30 European paediatric HIV clinics [83]. Prevalence was 26.0% for any fat redistribution, 8.81% for central lipohypertrophy, 7.55% for peripheral lipodystrophy and 9.64% for the combined subtype. Independent predictors of fat redistribution included advanced stage of HIV disease, female gender, ever used versus never used PIs and d4T. Increasing time since initiation of ART was associated with increased severity of fat redistribution.

Considerable controversy exists concerning the pathophysiological mechanisms underlying the development of lipodystrophy. Although many studies support the view that this syndrome is mainly a drug-related side effect mediated from both the NRTI and PI classes, some studies have demonstrated no evidence of antiretroviral class-specific effects [84]. PIs were initially believed to be the most likely cause of this syndrome, but more recently the use of NRTIs, d4T in particular, has been linked specifically to the development of the lipopathic component of lipodystrophy [85,86]. Non-drug factors such as HIV itself, older age, Caucasian race, sex and genetics may also modulate this syndrome.

It has been proposed that mt toxicity induced by NRTI-mediated inhibition of DNA γ polymerase plays a role in the development of the lipodystrophy component of the lipodystrophy syndrome. This theory was suggested by the phenotypic similarity in fat maldistribution and metabolic abnormalities to what is seen in some patients with inherited mt enzyme deficiency. However, the precise mechanism by which NRTIs contribute to the syndrome is not yet completely understood [87]. Several data support mt toxicity as the mechanism of lipodystrophy whilst other data refute this hypothesis [88]. Mitochondrial structural abnormalities and reduced mtDNA in subcutaneous fat biopsies taken from adult patients with lipodystrophy have been observed [89,90]. Reduction of mtDNA content and respiratory chain activity in adipocytes of patients on NRTI therapy has been demonstrated and these effects were noted 6–12 months after the beginning of NRTI therapy [91]. Moreover, the ability to reverse peripheral fat loss to some extent following the substitution of d4T with abacavir or AZT, and the fact that such improvement is associated with decreased adipose cell apoptosis may further support the role of NRTI-induced mt toxicity in the development of lipodystrophy [92–94]. On the other hand, samples from subjects with lipodystrophy have normal levels of mtDNA in some cases, while some of the samples from HCs showed diminished mtDNA [89,90]. In addition, another study demonstrated a higher mtDNA content in CD4 T cells from patient with lipodystrophy as compared with patients without lipodystrophy and HCs [95].

The existence of possible association(s) between mt toxicity and the lipodystrophy syndrome has been assessed in only one paediatric study so far [96]. Mitochondrial functionality and apoptosis were studied in peripheral blood lymphocytes (PBLs) in 18 HAART-treated (d4T, 3TC and one protease inhibitor), HIV-infected children, 12 with (LD+) and six without (LD−) lipodystrophy, and in 10 HCs. Using flow cytometry, mt membrane potential, mt mass, intra-mt cardiolipin distribution, and early and late apoptosis in fresh PBLs or in PBLs cultured with different stimuli were assessed. MtDNA content in fresh PBLs was also evaluated by competitive quantitative PCR. PBLs from LD+ and LD− children and HCs were similar in mt functionality and their tendency to apoptosis. MtDNA content showed diminished mtDNA [89,90]. In addition, another study demonstrated a higher mtDNA content in CD4 T cells from patient with lipodystrophy as compared with patients without lipodystrophy and HCs [95].

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was too short to induce relevant damage. PBLs have a low metabolic rate with a consequent low replication rate of mtDNA, which in turn provokes a low incorporation rate of damaging drugs into mtDNA and a low requirement for γ polymerase. Lastly, adipocytes may be more representative, as compared with PBLs, to assess the role of NRTI-related mt damage in lipoatrophy.

**Treatment of possible mitochondria-related events**

The manifestations of NRTI-related mitochondrial toxicity are generally, at least partially, reversible upon withdrawal of the causative agent(s). However, choosing agents that appear to have a low risk of these toxicities and avoiding the use of combinations with overlapping mt toxicities may be the best approach for limiting the incidence of these problems. The severity of the toxicity and the degree of tissue damage may, at least in part, influence the extent of recovery from mt toxicities. Moreover, mt toxicity may recur following the reintroduction of the same or similar agents.

As concerns hyperlactataemia, given the controversial role of serum lactate levels and mtDNA/nuclear DNA assessments as screening tools, maintaining a high level of vigilance remains key to the prevention and diagnosis of this condition. Children (when appropriate) on NRTIs (mainly d4T, ddI and AZT) and their families should be instructed on signs and symptoms to look out for and strongly recommended to seek prompt medical evaluation. Physicians should regularly screen children taking these agents for symptoms (particularly during the first 12 months of therapy) such as unexplained weight loss, gastrointestinal complaints, myalgia and paraesthesia, and they should pay particular attention to unexplained tachypnea and neurological deterioration in infants and toddlers. A high index of suspicion is specifically warranted during episodes of infection, as antecedent respiratory infection may precede symptomatic lactic acidosis. In all cases with symptomatic hyperlactataemia and lactic acidosi, ART should be discontinued if no other causes are evident [97]; children who are more seriously ill may require supportive care in an intensive care unit, haemodialysis and mechanical ventilation.

Administration of essential cofactors such as thiamine, riboflavin, l-carnitine, coenzyme Q10 and antioxidants have been used as a therapy for congenital mt diseases [98]. The beneficial effect of these agents in subjects with NRTI-related symptomatic hyperlactataemia is largely anecdotal and dosing schedules have not yet been established. However, the low toxicity potential of these agents make them a possible adjunct to standard measures. Even after the interruption of ART, normalization of lactate levels may take 3–6 months. Rechallenge after symptomatic hyperlactataemia should be performed with caution but carries a low risk of recurrence as long as the culprit NRTI is replaced by either a non-NRTI antiretroviral agent or one less frequently associated with hyperlactataemia, such as abacavir, 3TC or tenofovir [97]. The management of NRTI-related peripheral neuropathy may involve discontinuation or dose reduction of the causative agent, however the latter approach not only lacks a proven beneficial effect but also cannot be recommended in HAART regimens. Even after drug withdrawal, symptoms may persist and require treatment with ibuprofen, acetaminophen, tricyclic antidepressants, topical lidocain, narcotics for refractory pain or acupuncture. The choice of which intervention needs to be carefully tailored to the age of the paediatric patient.

Treatment of lipodystrophy is evolving, with no clear standard of care. No proven treatment exists for HIV lipodystrophy and no studies have found that limb fat increases spontaneously over time in patients with lipodystrophy. In vivo studies have suggested that thymidine analogues, especially d4T, are important in the lipodystrophy component of the lipodystrophy syndrome. The effect on fat loss of replacing d4T with abacavir or AZT has been recently assessed in HIV-infected adults with lipodystrophy [92,93]. These studies showed that an improvement of lipodystrophy, albeit modest, occurs in the short-term (24–48 weeks) and lipodystrophy is partially reversed in the long-term follow-up (104 weeks). NRTI switches could be an attractive option in HIV-infected children with lipodystrophy, however they cannot be formally suggested at the moment due to the lack of studies in this population.

**Conclusions**

HIV-infected children treated with potent ART can exhibit a large decrease in HIV-RNA levels and a subsequent increase in CD4 cells. These changes have resulted in significant improvements in clinical outcome and quality of life, dramatically decreasing disease progression and mortality. For many, HIV disease has been transformed from an acute life-threatening illness into a manageable chronic disease, allowing children, who were once not expected to survive, to live well into adulthood. Some clinical events related to NRTI-related mt toxicity have been reported in HIV-infected children but the available studies are scanty as compared with those performed in HIV-infected adults and further research is required to clarify the entity and the consequences of NRTI-related mt toxicity in paediatric patients. Therefore, close monitoring of these syndromes is highly advisable due to the possible relevant damage to liver, muscle and nervous system, and the negative impact on growth related to a persistent subclinical mt toxicity.
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Introduction

Mitochondrial (mt) disorders are a complex group of diseases due to malfunctions of the mitochondrial respiratory chain (MRC). As mitochondria are ubiquitous within body cells, clinical manifestations of mt diseases are very polymorphous; classically, they have been considered primary or secondary. Primary mt defects are considered to be those caused by mutations in genes encoding subunits of the MRC [1]. Because mitochondria have a dual genetic control, these defects include mutations of mtDNA as well as nuclear DNA (nDNA) genes. Mitochondrial dysfunctions not related to mutations in genes encoding subunits of the MRC are considered secondary mt disorders [2,3]. They include defects of nuclear-encoded mt proteins responsible for the maintenance of MRC subunits and mtDNA biogenesis, or those involved in many other mt biochemical pathways. They also include non-genetic defects that produce derangements of mt homeostasis that have an impact on MRC function. This last situation is generally due to exposure to an environmental toxin or drug, which may interfere with MRC either directly or through a series of mechanisms including secondary genetic defects, which may appear as a consequence of mt derangement.

Since the introduction of highly active antiretroviral therapy (HAART) the prognosis of HIV infection has changed dramatically, shifting the concept of HIV disease from that of a highly mortal disease to that of a chronic illness. However, the chronic use of HAART has also been associated with an increase in adverse drug effects, such as hyperlactataemia, polyneuritis and lipodystrophy. It has been proposed that some of these effects could be mediated by mt toxicity and, actually, a decrease in mtDNA copy number and a MRC dysfunction has been repeatedly described [4–12].

A frequent problem in clinical practice is that the diagnosis of mt dysfunction is not easy to establish. A classic and relatively simple way to approach this issue is the measurement of ketone body ratios. An enzymatic defect of MRC produces a modification of redox potential status due to the accumulation of reduced equivalents (NADH and FADH₂). This accumulation partially inhibits the Krebs cycle that, in turn, favours the production of β-hydroxybutyrate with respect to acetoacetate inside mitochondria, and lactate with respect to pyruvate in cytoplasm. These ratios were measured some years ago in children with perinatal HIV infection treated with zidovudine without any significant result, possibly because its sensitivity is very low [13]. To our knowledge, its measurement has not been reported in HIV infection since. A series of more sophisticated techniques have been used since then, including morphological, molecular genetic and enzymatic studies. The most important findings using these approaches are the presence of ragged-red fibres on muscle histochemical studies [4], a depletion of mtDNA and a dysfunction of MRC in diverse tissues [5–12]. A problem with these techniques is that they are usually not routine. Moreover, to establish a diagnosis of mt dysfunction, a combination of these studies is frequently required, and only a few medical centres in each country are equipped for thorough mt evaluation. The suspicion of a possible mt dysfunction in the context of HAART treatment is, consequently, difficult to prove and frequently only suspected. This may have serious implications because the suspicion of mt toxicity often induces a change in HAART regime with the consequent discontinuation of useful antiretrovirals.

In this review, we analyse a series of techniques that may suggest the existence of a mt dysfunction not based on pathology, biochemical tissue or molecular genetic analyses. Several of these techniques have been used for the evaluation of classic mt diseases and, with some modifications, could be applied to the diagnosis of HAART-associated mt dysfunction.
Most have not been used for this purpose, their utility being speculative.

**Breath tests**

Breath tests are simple, cost-effective and safe. For these reasons, they have been proposed for many ‘dynamic’ evaluations, especially related to liver disease either of genetic or acquired origin. The rationale for their use to assess mt function is that oxidative metabolism of some substrates, such as methionine and ketoisocaproic acid, need the integrity of the hepatocyte electron transport chain and/or ATP synthesis for decarboxylation [14,15]. Methionine is the best-studied carbon-labelled substrate; the isotope usually used is $^{13}$C, which is non-radioactive, although $^{14}$C can also be used. The procedure consists of measuring the exhalation of $^{13}$CO$_2$ after administration of 2 mg/kg body weight of [methyl-$^{13}$C]-labelled methionine. Breath $^{13}$CO$_2$ is measured with a mass spectrometer at baseline and every 15 min thereafter for 120 or 180 min [15]. What the study reflects is hepatic mt function, although to date there is still no general consensus as to its usefulness in the clinical setting.

In the absence of liver disease, the $^{13}$C-methionine breath test has been used to assess mt function in patients under HAART. Decreased intramitochondrial decarboxylation capacity has been reported in HIV-infected patients with antiretroviral drug-related hyperlactataemia compared with healthy controls [16,17]. Drug discontinuation or regimen modification led to a recovery of this capacity (Figure 1).

The most important limitation in the evaluation of HIV-infected patients is the high prevalence of coinfection with hepatitis C and B viruses, as well as possible alcohol abuse or hepatotoxicity from drugs, making it difficult to completely exclude an undiagnosed hepatic disease. Another minor problem in the use of breath tests is the need for mass spectrophotometry. However, this problem can be easily overcome with the development of a kit similar to those used to diagnose *Helicobacter pylori* infection. This kit uses $^{13}$C-urea as a substrate and breath-exhaled samples are collected into bags and mailed to a reference laboratory [18]. The general impression is that this is a young but promising field and that further studies are needed to assess the suitable substrate or substrates required for the evaluation of the complex mt metabolism.

**Cardiopulmonary exercise tests**

Cardiopulmonary exercise testing is an objective method for evaluating exercise limitations either of cardiac, pulmonary or metabolic origin, in which subjects exercise, preferably on a bicycle ergometer.

During exercise they breathe through a mouthpiece that is a miniaturized pressure differential pneumotachygraph. The inspired and expired gas is continuously sampled and O$_2$ uptake and CO$_2$ elimination are computed [19]. Typical recordings also include cardiac output, dynamic flow volume loops and ventilation-perfusion measurements performed at rest and during an incremental workload exercise test. Serum lactate can also be measured. A series of derived parameters can be obtained and correlated with workload, measured in watts. The respiratory exchange ratio (RER) is the quotient of the amount of CO$_2$ produced to the amount of O$_2$ consumed. Peak oxygen consumption (VO$_2$max) in ml oxygen/kg body weight/min denotes cardiovascular or ‘aerobic fitness’. The final workload (peak watts/kg) achieved by patients is considered their ‘peak work capacity’. The point during exercise at which anaerobic metabolism is used to supplement aerobic metabolism as a source of energy is termed the ‘anaerobic threshold’. It normally occurs at >40% of VO$_2$max [19,20].

In mt myopathies, exaggerated circulatory and ventilatory responses to exercise are governed by skeletal muscle oxidative capacity, in which more severely impaired oxidative phosphorylation elicits more active systemic responses. Exercise capacity varies widely, which is attributable to different levels of oxidative impairment, but in general, the average peak work capacity and VO$_2$max are lower than in control.
subjects [21,22]. Low anaerobic threshold in a subject with unimpaired cardiovascular fitness provides additional evidence of mt dysfunction [22].

In a group of HIV-positive patients on HAART, classified according to their level of venous lactate levels, there were no significant differences with respect to peak work capacity, expired volume/min or VO$_2$max when compared with a group of controls. However, patients with abnormal venous lactate levels had a higher RER at the peak of exercise and tended to have a lower anaerobic threshold [23]. In another group of HIV-positive patients on HAART, lactate production was higher in patients with lipodystrophy than in patients without lipodystrophy at the same level of VO$_2$max. In the same way, peak work capacity on the cycloergometer was reduced in the lipodystrophic group. The anaerobic threshold occurred earlier in the lipodystrophic group [24]. Finally, a group of HIV-infected patients with lipodystrophy and elevated P-lactate levels had a significantly lower peak work capacity and a trend towards reduced VO$_2$ max compared with controls [25].

One problem in interpreting these results is that a series of HIV-positive patients analysed in the late 1980s before the introduction of HAART, also exercised to a significantly lower peak work capacity compared with a group of non-infected patients. The ventilatory anaerobic threshold values and VO$_2$max were also significantly lower [26]. There are actually several possible mechanisms to explain exercise limitations in HIV-positive patients aside from mt dysfunction: cardiac, ventilatory, peripheral nerve or muscle abnormalities, anaemia, smoking, deconditioning, decreased motivation resulting from chronic disease or a combination of these factors [27]. The extreme difficulty in including all these circumstances in HIV-positive patients, together with the need for well-trained personnel to interpret the results and the relatively sophisticated material used, makes cardiopulmonary exercise testing far from being an easy method to implement in usual clinical practice.

**Forearm exercise tests**

Functional blood tests

The best known is the ischaemic forearm exercise test introduced with the specific aim of screening for abnormalities of muscle glycogen metabolism such as myophosphorylase deficiency or McArdle's disease [28,29]. Although not designed for the study of mt defects, it has been suggested that the rate of lactate clearance in the post-exercise period may be significantly slower in patients with mt myopathies than in healthy subjects [30,31]. To our knowledge, there are no systematic studies that sustain this hypothesis. Furthermore, in a study not designed to study the rate of lactate clearance, the authors did not find any difference between HIV-infected patients with lipodystrophy and elevated P-lactate levels and a group of controls [25]. This possibly indicates that the test is not useful in the analysis of mitochondria in patients under HAART.

An aerobic forearm exercise test specifically developed for the study of mt performance [32] seems more interesting for the study of HAART-related mt dysfunction. The protocol consists of intermittent handgrip exercise (squeeze 1 sec, rest 1 sec) for 3 min at 33% of intended maximal voluntary contraction (MVC) force, which is determined 30 min before initiation of data collection. Oxygen saturation is analysed in blood samples collected from the median cubital vein of the exercised arm. Patients with a mt myopathy do not develop a significant desaturation during exercise compared with healthy subjects or patients with other muscular diseases [32]. We recently had the opportunity to study a patient with symptomatic hyperlactataemia due to HAART [33]. During the acute phase of hyperlactataemia, O$_2$ saturation did not decrease as expected in response to exercise. Antiretroviral therapy was initially stopped and afterwards modified. The patient clinically recovered and after 6 months, the aerobic forearm test was completely normal (Figure 2). Although promising and relatively easy to implement in clinical practice, more studies are required to determine the sensitivity and specificity of this method.

**Figure 2.** Venous oxygen saturation before a forearm aerobic exercise test (minute 0), during aerobic exercise (minutes 1, 2 and 3) and after resting (minute 4)

A patient on HAART who developed symptomatic hyperlactataemia did not present a normal venous desaturation during exercise as compared with healthy subjects and patients on HAART without hyperlactataemia. This is an indication of poor O$_2$ utilization during exercise. After 6 months and once the patient was clinically recovered, the aerobic forearm test was normal. SHL, symptomatic hyperlactataemia; HAART, highly active antiretroviral therapy.
Near-infrared spectroscopy (NIRS)
NIRS is a non-invasive optical method for continuous monitoring of oxygenation and haemodynamics in tissue. It is based on the capacity of light in the near-infrared range to penetrate tissues to a depth of several centimetres and on absorption characteristics of oxyhaemoglobin plus oxymyoglobin, compared with deoxyhaemoglobin plus deoxymyoglobin, with differential near-infrared light spectrometry. A series of optical fibres are placed on top of the muscles of the exercising limb and the difference in the absorption at 760 and 850 nm estimates the relative change in oxyhaemoglobin versus deoxyhaemoglobin. The sum of these spectra provides an estimate of the blood volume. Thus, the net extraction of oxygen from oxyhaemoglobin relative to oxygen delivery by blood circulation can be determined over the area of the tissue sampled by the device, and the effect of exercise on muscle oxygenation can be assessed [34]. Since data can be collected continuously, this device provides unique information regarding the kinetics of oxygen utilisation relative to delivery in the transition from rest to exercise, during sustained exercise and during recovery. This technique has been used to assess patients with classic mt diseases. It seems that these patients present a decrease in $O_2$ consumption compared with controls in both low-intensity exercise and at rest [34,35]. To our knowledge, NIRS has not been used to assess the possible mt toxicity in HAART-treated patients. The advantages of the non-invasiveness of the procedure are possibly counterbalanced by the difficulties in interpreting the results. It seems, nonetheless, to be a technique with potential utility in this setting.

Imaging techniques
Imaging studies have changed our approach to medical diagnosis in the last 20 years. Many modalities may contribute toward the diagnosis of mt diseases. Table 1 summarizes the most important methods [36]. Among them, magnetic resonance spectroscopy (MRS) seems the most promising for the diagnosis of mild mt dysfunction due to its ability to detect metabolic changes. MRS can be used to monitor tissue bioenergetic changes in both the brain and the skeletal muscle.

Proton-MRS is useful in studying bioenergetic changes in the brain. The main resonances observed in the spectrum arise from N-acetylaspartate (a neuronal marker), lactate, creatine and choline. Proton-MRS is less useful in the investigation of skeletal muscle because of the large signal from the protons in the subcutaneous fat, which can obscure other signals of interest [36]. For this reason, proton-MRS seems to be of low utility in the investigation of the effects of HAART regimes.

The spectra of phosphorus-MRS (P-MRS) contain several resonances: three arise from the $\gamma$, $\alpha$ and $\beta$ phosphate groups of ATP, one from phosphocreatine (PCr) and one from inorganic phosphate (Pi) (Figure 3); two additional smaller resonances can sometimes be observed from phosphomonoesters and phosphodiesters. The area under each resonance is proportional to the amount of the corresponding metabolite. The spectral distance between Pi and PCr provides information about intracellular pH.

In normal exercising muscle, there is a PCr decrease linked to Pi accumulation, while the ATP signal remains

| Table 1. Clinical imaging techniques useful in the diagnosis of mitochondrial diseases |
|-------------------------------------|---------------------------------|--------------------------|
| Imaging modality                   | Contribution to diagnosis       | Comments                |
| Magnetic resonance imaging (MRI)   | Structural changes              | No insight into tissue function |
| Computed tomography (CT)           | Structural changes              | No insight into tissue function |
| Diffusion weighted imaging         | Differentiation between ischemic and MELAS stroke | Limited relevant studies to date |
| Single-photon-emission computed tomography (SPECT) | Changes in central blood flow | Low spatial resolution |
| Positron emission tomography (PET) | Detection of glucose/oxygen and oxygen/blood flow ratios | Limited availability |
| Phosphorous and proton magnetic resonance spectroscopy (MRS) | Metabolic changes | Measurement of metabolite concentrations at rest or under exercise |

MELAS, mitochondrial encephalopathy with lactic acidosis and stroke-like episodes. Modified from Parry & Matthews [35].
unchanged due to the continuous resynthesis of ATP through different metabolic pathways. After exercise, mt oxidative phosphorylation remains the main source of ATP, which continues for a while at an accelerated rate to replenish the high-energy phosphates utilised during physical activity. Thus, during this period of recovery, PCr gradually increases, Pi and ADP decrease, and pH returns to its resting level. The initial rate of PCr recovery provides a measure of maximal oxidative rate in the tissue and the recovery of ADP is now considered as one of the most sensitive and reliable indexes of mt dysfunction in vivo [37].

P-MRS of muscle is particularly interesting in the evaluation of patients with mt diseases, which present, at rest, an increase in Pi and, less often, a decrease in PCr resulting in a low PCr/Pi (Figure 3). Abnormalities in resting skeletal muscle and in brain can be detected in patients even with relatively mild disease and normal serum lactate, or no clinical evidence of CNS involvement [38]. Muscle dynamic P-MRS studies during exercise can increase the specificity of the examination (Figure 4). Patients with mt myopathies display a rapid rate of PCr depletion. After stopping exercise, there is a prolonged rate of PCr recovery, which is a direct consequence of slower oxidative rephosphorylation of ADP to ATP. These changes do not seem to be specific for primary mt diseases. They have also been described to occur secondary to traumatic muscle injury or in inflammatory myopathies [40,41]. It is conceivable that P-MRS may be a useful tool for the evaluation of HIV-infected patients on HAART, although no studies have been reported to date.

Conclusions

There is currently a general belief that mt dysfunction plays a pivotal role in some adverse effects observed in patients receiving HAART. The demonstration of this hypothetical dysfunction is complex and relies on a combination of pathological, biochemical and molecular genetic studies that are far from being considered routine in most general clinical settings. In the present review we have analysed some further diagnostic procedures that may help in deciding whether mitochondria are really affected in a given patient. Although some of these procedures are expensive and also limited to a few high technology centres (for example, MRS), others can be easily performed in more conventional outpatient clinics (for example, functional forearm exercise tests). The major inconvenience of all these methods is that they have either been introduced relatively recently or their use in diagnosing mt dysfunction is only a proposition. For these reasons their sensitivity, specificity and positive and negative predictive values remain to be elucidated in the diagnosis of HAART-related mt toxicity.

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Mitochondrial studies in HAART-related lipodystrophy: from experimental hypothesis to clinical findings

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Chronic use of antiretrovirals (ARVs) to treat HIV infection, along with more prolonged patient survival, has been associated with an increase in adverse drug effects in HIV-infected patients on treatment. It has been proposed that some of these adverse effects (including myopathy, cardiomyopathy, anaemia, hyperlactatemia/lactic acidosis, pancreatitis, polyneuritis and lipodystrophy) could be mediated by mitochondrial (mt) toxicity. From the experimental data, it has been proposed that nucleoside analogue reverse transcriptase inhibitors (NRTIs) also inhibit γ3-polymerase, the enzyme devoted to replicate (and, to a lesser extent, repair) mtDNA. It is now widely accepted that the use of most NRTIs in HIV-infected patients is associated with mtDNA depletion.

Although cross-sectional studies suggest that certain ARVs, especially stavudine, are more toxic to mitochondria, the differences among different highly active ARV therapy (HAART) schedules detected in the analysis of longitudinal studies are not so clear. These types of study in previously untreated individuals suggest that the greatest mtDNA loss appears at the beginning of the treatment. Conversely, in ARV-experienced patients, the potential beneficial effects of HAART changes in terms of mtDNA content remain controversial and must be further investigated. Functional studies accompanying genetic investigations are needed for the correct pathogenic interpretation of the mtDNA abnormalities.

Introduction

The clinical use of antiretrovirals (ARVs) to treat HIV infection began in 1986 with the introduction of zidovudine (AZT), an analogue of thymidine nucleoside with proven capacity to inhibit HIV reverse transcriptase (RT). Since then, more than 20 ARVs that act at different steps of the HIV life cycle have been approved. With this wide therapeutic arsenal, the current standard treatment for HIV infection consists of a combination of several ARVs in so-called highly active ARV therapy (HAART). As a consequence, HAART use has achieved a marked decrease in patient mortality and has shifted the concept of HIV infection from a highly mortal disease to a chronic illness.

However, chronic use of ARVs together with prolonged patient survival has also been associated with an increase in adverse drug effects. It has been proposed that some of these adverse effects (including myopathy, cardiomyopathy, anaemia, hyperlactataemia/lactic acidosis, pancreatitis, polyneuritis and lipodystrophy) could be mediated by mitochondrial (mt) toxicity. Nonetheless, despite the large number of basic and clinical studies published on this hypothesis during the last 10 years, a unifying theory that explains the clinical manifestations of ARV mt damage is still lacking. One of the main reasons for this is that most of these studies have been performed in vitro and were either non-reproducible or have not been examined in the setting of human studies. Consequently, it is currently unknown why certain patients develop adverse effects in a particular tissue or why patients with similar accumulated doses of ARVs express different patterns of adverse effects. It is possible that ARV-related factors (different rates of tissue incorporation, transportation into mt compartment or intra-mt phosphorylation for each ARV), patient-related factors (mtDNA polymorphisms) or tissue-related factors (oxidative phosphorylation (OXPHOS) dependence) play a significant role in this heterogeneity. Therefore, while for some of these adverse effects (such as toxic myopathy or lactic acidosis) a strong relationship with mt dysfunction has been proven [1–6], in others (especially lipodystrophy) controversies remain. In this review, we cover the field from experimental hypothesis to clinical studies and analyse the level of evidence with respect to the relationship between ARV adverse effects and mt toxicity, with particular attention to mt participation in lipodystrophy.
Molecular basis of ARV toxicity on mitochondria

In addition to inhibiting HIV-RT, in vitro studies suggest that nucleoside analogue RT inhibitors (NRTIs) are also able to inhibit β and γ human DNA polymerases, although a current demonstration of such direct inhibition by NRTIs in humans is lacking. From a pathogenic point of view, whereas the inhibition of β-polymerase (devoted to the repair nuclear of DNA) seems not to be of clinical relevance, the inhibition of γ-polymerase (γ-pol, devoted to the replication and, to a lesser extent, the repair of mtDNA) has been proposed to participate in most of the secondary effects associated with the clinical use of NRTIs [7]. Although γ-pol inhibition is considered a specific class effect of these drugs, the magnitude is not the same for all NRTIs, at least under experimental conditions. Based on experimental laboratory data, different NRTIs have been classified from a higher potency to impair γ-pol activity to a lower potency as follows: zalcitabine (ddC) > didanosine (ddl) > stavudine (d4T) >>> AZT > lamivudine (3TC) > abacavir (ABV) = tenofovir (TDF) [8-10].

NRTIs inhibit γ-pol through four different mechanisms encompassing their effects as: i) mtDNA chain terminators (once incorporated into a growing strand, DNA replication is abruptly halted), ii) competitive inhibitors (competing with natural nucleotides to be incorporated into growing DNA chains by γ-pol), iii) inducers of errors in the fidelity of mtDNA replication (inhibiting the exonucleolytic proofreading function of γ-pol) and iv) contributors to the decrease of mtDNA reparatory exonuclease activity (resisting exonuclease activity of γ-pol because of the lack of the group 3′-OH in NRTIs) [11]. As a final consequence, the efficiency of γ-pol is decreased and the whole cellular mtDNA content is reduced, while the percentage of point mutations [12] and deletions [13] in such genomes increases. Since mtDNA only codifies for functional proteins corresponding to mt respiratory chain complexes I, III, IV [cytochrome C oxidase (COX)] and V, the final and only possible consequence of mtDNA damage (if the magnitude is great enough to surpass compensatory mechanisms) is to cause respiratory chain dysfunction and low ATP synthesis. Other studies carried out on different cell lines have also demonstrated decreased synthesis of some of these mt-encoded proteins [14,15]. Furthermore, respiratory chain dysfunction can cause a loss of mt membrane potential and an increase in mt-driven apoptosis, a cascade of events that has been demonstrated for AZT and d4T [16].

Experimental studies have also invoked other additional mechanisms aside from γ-pol inhibition to completely explain NRTI-associated mt toxicity. For example, AZT is able to i) inhibit the ATP/ADP translocator from rat liver and heart in vitro, thereby limiting the OXPHOS mt capacity [17,18], ii) reduce protein glycosylation [19] and iii) to decrease protein synthesis [20].

The effects of ARV drug classes other than NRTIs against mitochondria have been less frequently studied and evaluated. Although it seems that non-NRTIs (NNRTIs) have no toxic effects on mtDNA, they could interfere with apoptotic pathways and eventually lead to some secondary harmful effects against mitochondria. Recent data indicate that in laboratory assays, efavirenz (EFV) acts as an inducer of the caspase-dependent apoptotic mt pathway [21]. Also, few and controversial data have been reported regarding mt effects of protease inhibitors (PIs). While some authors have found that PIs can induce loss of mt membrane potential [22], others have suggested that PIs could exert a beneficial role due to their anti-apoptotic properties [23]. In a very recent review, Badley identified at least five distinct mechanisms for the anti-apoptotic effects of PIs: i) decreasing expression of apoptosis regulatory molecules, ii) caspase inhibition, iii) altering proliferation, iv) inhibiting calpain and v) avoiding loss of mt transmembrane potential [24]. However, under certain circumstances (particularly high doses of PIs) a paradoxical pro-apoptotic effect can be observed in transformed cell lines in vitro and implanted mouse models [24]. Clearly, further studies are required with this drug class to define the exact interactions with mitochondria, especially with apoptotic pathways. Finally, there are no current reports evaluating the effects of fusion inhibitors on mitochondria.

The bulk of data generated under experimental laboratory conditions, briefly mentioned above, have been of crucial relevance in order to propose and, in some cases define, the exact mechanisms by which ARV cause mt side effects. However, results from in vitro experimental studies on cultured cells or animals cannot always be directly extrapolated to what occurs in vivo in HIV-infected individuals. Laboratory models usually consist of non-HIV infected cells or animals and, nowadays, there are data consistently indicating that HIV itself causes some mt disarrangements, including mtDNA depletion [4,25-28] and respiratory chain dysfunction [25-29]. Thus, experimental studies do not take into consideration the role of HIV in facilitating or magnifying ARV-related mt toxic effects and do not include the beneficial effects of ARV in limiting HIV-related mt damage by controlling HIV infection. Additionally, ARV pharmacokinetics are not accounted for in models based on cell cultures, and the effects of the long-term use of ARVs are probably not accurately evaluated either. Moreover, experimental studies are
mainly limited to ascertaining the effects of an isolated ARV and, in clinical practice, ARVs are managed as combinations of three or four drugs that are concomitantly administered to patients. Finally, the exact pathogenic significance of experimental findings to explain certain adverse effects (such as lipodystrophy) which appear during HAART, is not established by cellular models. Therefore, this review encompasses mt data from studies performed in HIV-infected patients receiving ARV who have developed lipodystrophy.

Cross-sectional studies in HIV-infected individuals developing lipodystrophy

The first direct data of mt involvement in lipodystrophy was described in a case report of an HIV-infected woman suffering from lipodystrophy. The patient exhibited multiple mtDNA deletions in skeletal muscle and subcutaneous fat along with a marked respiratory chain dysfunction in skeletal muscle and peripheral blood mononuclear cells (PBMCs) [30]. Soon after this, mtDNA depletion was found in subcutaneous fat of eight patients developing lipodystrophy [31]. Since then, case series and cross-sectional studies have both progressively appeared. Currently, mt abnormalities (and especially mtDNA depletion) have been identified in several tissues of HIV-infected patients with lipodystrophy on HAART, including skeletal muscle [13,32,33], liver [32], adipose tissue [13,31,34,35], sperm [36] and PBMCs [12,37]. However, some other authors have not found mt abnormalities [38–41]. Table 1 summarizes the published data from transversal studies of HIV patients developing lipodystrophy. As one can see, the evaluated tissues are diverse and there is great heterogeneity of the methodologies for studying mitochondria. For example, Southern blot methodology to measure mtDNA content has been replaced by real-time PCR technology, which renders greater sensitivity, accuracy and reliability than the former. Moreover, it is highly unusual for a single work to provide morphological, genetic and functional data on mitochondria. These facts can explain, at least in part, the lack of current consensus regarding the exact role of mt dysfunction in lipodystrophy. Additionally, since the majority of data comes from HIV lipodystrophic patients who have been receiving ARV for many years, there are important limitations in interpreting the results. For example, the prior drug exposure is different in practically every patient and other uncontrolled patient-related factors [such as age, smoking habit, intake of drugs (other than ARV) which can interfere with mt function] can act as confounding factors, which may severely limit the power of the cross-sectional studies.

In addition, mtDNA depletion has also been described in patients not showing clinically relevant ARV adverse effects [42–44]. Some cross-sectional studies have reported that, in asymptomatic individuals, mtDNA depletion is greater in patients receiving d4T as the backbone of HAART in comparison with other HAART regimens [43–45]. This is consistent with the greater in vitro affinity of this drug for γ-pol than other currently used NRTIs. However, the finding of mtDNA depletion in asymptomatic individuals limits the pathogenic conclusions from the aforementioned transversal studies of patients with lipodystrophy, since mtDNA depletion could be interpreted as a class drug effect of NRTI that, if investigated, may be demonstrable in most patients receiving such drugs, irrespective of the presence or absence of adverse effects. In fact, it has been reported very recently that mtDNA depletion is even present in asymptomatic HIV-uninfected infants born to HIV-infected women receiving ARV during pregnancy [46]. There are, therefore, reasonable doubts regarding the real value of abnormal findings in HIV-infected individuals with lipodystrophy. In this setting, data from longitudinal studies evaluating the same mt parameters using the same tissues from the same individuals should help to better understand the findings reported by cross-sectional studies.

Longitudinal studies in previously untreated HIV-infected individuals

Sequential studies performed in naive patients starting ARV are of special interest because they eliminate the hypothetical effects of ARV taken by patients prior to the current ARV or HAART regimen that is being evaluated. The main endpoint evaluated in such studies is usually the mtDNA content, since this is the direct effect of NRTIs. In a pioneering study, Reiss’ group [47] reported the effects on the mtDNA content of PBMCs of starting ARV therapy with different ARV strategies, prior to the HAART era. They observed that, after 1 year of treatment, patients receiving AZT in monotherapy exhibited a 27% decrease of mtDNA with respect to baseline and that, when AZT was associated with ddC or ddI, the decrease was 44% and 49%, respectively. It is remarkable that the greatest part of this molecular side effect was already present as early as 4 weeks after starting the NRTIs. More recently, these investigators have also studied HIV-naïve patients starting HAART and have found that, after 4 years on treatment, both d4T- and AZT-based HAART are associated with a profound mtDNA depletion in PBMCs, with this decrease being greater in the former HAART schedules (75% of decrease) than in the latter (63% of decrease) [48]. Our laboratory has
studied 11 patients naive for ARV, four of them starting with d4T+ddI-based HAART and seven of them starting with AZT+3TC-based HAART. As with previously discussed studies, we have also found greater harmful effects of d4T on PBMCs’ mtDNA abundance, with a 28% decline in mtDNA content 6 months after starting HAART containing d4T+ddI, a situation that is not observed in those who started HAART containing AZT+3TC.

However, not all longitudinal studies have shown greater mtDNA depletion in HAART schemes containing d4T. Petit et al. [45] also evaluated the effect of starting four different HAART regimens on mtDNA content of PBMCs after a variable time on treatment (from 50–80 weeks). They found more modest negative effects on mtDNA abundance (ranging from 15–38% of reduction) than those found by Reiss et al. and they did not find clear differences between schemes containing d4T and those containing AZT. Interestingly, the sequential analysis of mtDNA content evolution demonstrates again that the greatest decay is achieved during the first weeks of treatment. Nolan’s group has been the only team to study the effects of the introduction of HAART on mtDNA content in the subcutaneous...
fat of naive patients [49] - the target tissue of the lipodystrophy syndrome. They found that when HAART included AZT+3TC, patients developed a 78% decrease in mtDNA after 24 weeks of treatment, whereas when HAART included d4T+3TC, the magnitude of the decrease was 58%. They have further reported that, in such patients, the decrease in mtDNA fat content is accompanied by COX deficiencies demonstrated through immunohistochemical reactions [50].

Taken as a whole (Figure 1), the aforementioned results indicate that commencing HAART is uniformly associated with mtDNA depletion. As previously discussed, the vast majority of cross-sectional studies have found greater mt toxicity for d4T-based HAART than other HAART schedules. However, no clear conclusions regarding a greater capacity to induce mtDNA depletion by a particular ARV or HAART scheme can be depicted from the data reported in longitudinal analysis of previously untreated patients. Additionally, such a decline seems to mainly occur early after starting ARV. This observation is consistent with the cross-sectional study performed in liver by Walker et al. [51]. They found that mtDNA content declines during the initial 6 months of treatment with dideoxynucleotides, with no further decline beyond this time. One possible explanation is that while HAART adverse effects on mtDNA kinetics due to \( \gamma \)-pol inhibition are clearly manifest at initial stages of treatment, they are further counteracted by the reduction of HIV viraemia achieved by HAART. Since it has been demonstrated that HIV infection per se is associated with a decrease in mtDNA content [4,25,28], the control of HIV infection may limit the mt side effects of HIV itself. As a result, the initial exponential decline in mtDNA content may be followed by a steady-step phase (Figure 2).

**Longitudinal studies of the effects of HAART switching in HIV-infected individuals**

From a clinical point of view, it seems that once lipodystrophy appears, changes in HAART schedules do not lead to a rapid improvement of the syndrome. One possible explanation could be that adipocytes involved

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**Figure 1.** Changes in mtDNA content in HIV-naive patients starting antiretrovirals

![Figure 1](image-url)

**Figure 2.** Proposed model of action of HIV and ARVs on mtDNA content

![Figure 2](image-url)

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ARV, antiretroviral; mt, mitochondrial; HAART, highly active antiretroviral therapy.
in lipodystrophic changes are severely and, for the most part, inevitably damaged. Since patients with lipodystrophy usually exhibit a mixed pattern of peripheral lipoatrophy and central adiposity, it has been postulated that apoptotic activation could be responsible for lipoatrophy while decreased mt metabolism could result in lipid accumulation in visceral and central adipocytes [49].

Based on experimental data regarding the affinity of NRTIs for $\gamma$-pol, it has been proposed that switching to a HAART scheme that does not contain dideoxynucleotides could eventually improve lipodystrophy. Very recently, data from the MitoX study [52] have shown that, in patients receiving HAART who have developed lipodystrophy, the substitution of d4T or AZT by ABV (one of the NRTIs with the weakest affinity for $\gamma$-pol), is associated with a significant increase in body fat content 2 years after ABV introduction. Unfortunately, no molecular studies of mtDNA content at 2 years accompanied this report. The demonstration of the reversibility of mtDNA depletion associated with HAART changes occurring concurrently with the regression of lipodystrophy would strengthen the existence of an mt role in the development of this syndrome. This close relationship between clinical manifestations and laboratory findings has been better approached for HAART-related hyperlactataemia. For example, Montaner et al. [53] studied eight patients receiving d4T-based HAART who developed hyperlactataemia, and demonstrated that, after discontinuation or substitution of d4T, the disappearance of the clinical signs and symptoms correlated with an increase of over 200% in the mtDNA content in PBMCs. Similarly, we have reported that reversibility of mt abnormalities with HAART interruption is not limited to mtDNA depletion in patients developing hyperlactataemia, but also implicates the restoration of previously altered respiratory chain function [54].

Unfortunately, the data regarding the benefits in terms of mtDNA content of switching a HAART schedule are scarce and have often been obtained in a very limited number of patients with contradictory results [49,55–58] (Figure 3). On one hand, Carr’s group [57] reported the largest cohort in which sequential mtDNA content has been determined and did not find evidence of any significant mtDNA change after switching from HAART containing a thymidine analogue to HAART containing ABV, at least after 24 weeks of follow-up. Additionally, the behaviour of these patients was the same as that of a control group in which no HAART changes were introduced. Consistent with Carr et al.’s results, we have found that the introduction of a nucleoside-sparing HAART (nevirapine plus lopinavir/ritonavir) to ARV-experienced patients is associated only with a trend toward increasing mtDNA without changes in respiratory chain function evaluated through COX activity [58]. Indeed, this group of patients showed very similar changes as those in patients who continued with the same NRTI-containing HAART scheme (Figure 4) [57]. It is noteworthy that these non-relevant effects of switching the HAART regimen obtained by our group and Carr’s were achieved using PBMCs of asymptomatic individuals. Although some doubts have been raised regarding the sensitivity of this biological sample to detect mt abnormalities, we have demonstrated that changes in mtDNA are present in asymptomatic patients receiving ARV, thereby demonstrating that PBMCs are reliable in the investigation of mt disorders [44].

In a clash with these results, Nolan’s group [49] found a mean increase of 650% of mtDNA in subcutaneous fat from three patients with lipodystrophy who were switched from d4T-based HAART to a HAART combination consisting in AZT+3TC+ABV. Similarly, McComsey’s group [55] has reported that mtDNA increased around 100% in the subcutaneous fat of patients with lipodystrophy in whom a d4T-based HAART was replaced by an AZT- or ABV-based HAART scheme. In a more recent and extensive paper, they studied mtDNA content in patients treated with
d4T-based HAART and lipodystrophy, and found that the replacement of d4T by AZT or ABV was accompanied by a rebound in mtDNA of 141% in skeletal muscle, 146% in adipose tissue and 369% in PBMCs at week 48 [56]. Additionally, they also observed a restoration of mt respiratory chain complex I activity in skeletal muscle [56], as well as a decrease in apoptosis scores in adipose tissue after the switching. It is noteworthy that this study simultaneously investigates, for the first time, sequential genetic and functional mt parameters along with clinical data from patients with lipodystrophy in whom HAART was switched, and it constitutes the first evidence that the substitution of ABV or AZT for d4T improves the subcutaneous fat content as well as reverting abnormal mt indices and fat apoptosis. The demonstration of deficiencies in the diverse tissues evaluated, as well as a close correlation between mtDNA content in PBMCs and in fat, reinforces the usefulness of PBMCs as subrogates of other more appropriate, but more difficult to obtain, tissues. As the only caveat, the McComsey et al. study [55] lacks a control cohort group to assess the exact effects of pharmacological intervention.

Overall, it is important to note that investigations of mtDNA content are not sufficient to correctly ascertain the effects of ARV on mitochondria. The study of functional repercussions of the eventual genetic deficiencies appearing during HAART is probably a more physiopathological approach to knowing the real involvement of mitochondria in the development of lipodystrophy [59]. For example, we have recently reported the presence of up-regulatory mechanisms that compensate for mtDNA depletion developed by patients on treatment with ddI+d4T. Such mechanisms allow maintenance of correct COXII expression (a protein codified by mtDNA, which was depleted in the patients studied), as well as a normal COX activity [60]. Therefore, mtDNA studies only cover one of the multiple aspects of mt function and, consequently, can only offer partial explanations. On the other hand, lipodystrophy probably has a multifactorial mt aetiology where events other than mtDNA depletion play a pivotal role. Among them, ARV modifications of mt-dependent apoptotic pathways, as well as of overall biology of adipose tissue, are being intensively investigated [61]. Regardless of the mechanism, the important question that remains to be answered is how altered mt function leads to the massive modifications observed in adipose tissue of patients developing HAART-related lipodystrophy.

Conclusions

Nowadays, it has been demonstrated that the use of most of NRTIs in HIV-infected patients is associated with mtDNA depletion. Although cross-sectional studies suggest that certain ARVs, especially d4T, are more toxic to mitochondria, the differences among different HAART schedules detected in the analysis of longitudinal studies are not so clear. This kind of study in previously untreated individuals suggests that the greatest mtDNA loss appears at the beginning of the treatment. Conversely, in ARV-experienced patients, the potential beneficial effects of HAART switches in terms of affecting mtDNA content remain controversial and must be further investigated. Functional studies accompanying genetic investigations are needed for the correct pathogenic interpretation of mtDNA abnormalities.

Grants

Fundación para la Investigación y la Prevención del Sida en España (FIPSE 3102-00 and 3161/00A), Fundació La Marató de TV3 (020210), Redes de Investigación en Mitochondrias (V2003-REDC06E-0) y Sida (Rg-173) and Suport a Grups de Recerca 2001/SGR/00379.

Figure 4. Longitudinal study comparing the effects of being maintained on a NRTI-containing HAART (NRTI group) or switched to a NRTI-sparing HAART (NVP group) on (A) mtDNA content and (B) complex IV activity

n, number of patients studied; NVP, nevirapine; HAART, highly active antiretroviral therapy; mt, mitochondrial; NRTI, nucleoside reverse transcriptase inhibitor.
References


Mitochondrial DNA levels of peripheral blood mononuclear cells and subcutaneous adipose tissue from thigh, fat and abdomen of HIV-1 seropositive and negative individuals

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Mitochondrial dysfunction has been demonstrated in subcutaneous adipose tissue from lipoatrophic HIV-1-infected patients treated with nucleoside reverse transcriptase inhibitors (NRTIs). To further assess mitochondrial toxicity, mitochondrial DNA (mtDNA) copies/cell were measured in subcutaneous fat from various sites. Peripheral adipose tissues were obtained from the abdominal wall near the umbilicus, anterior lateral thigh, and dorsal cervical region of the neck of individuals from four cohorts: 1) seven lipoatrophic HIV-1-infected patients receiving a regimen with nucleoside reverse transcriptase inhibitors (NRTIs) as part of highly active antiretroviral therapy (HAART) for >6 months; 2) seven non-lipoatrophic HIV-1-infected patients receiving NRTIs-containing HAART; 3) five HIV-1-infected patients on antiretroviral therapy <2 weeks (naive); and 4) five HIV-1-negative participants. Along with the adipose tissue samples, peripheral blood mononuclear cells (PBMC) were also obtained from each patient for mtDNA depletion examination. Total DNA was isolated and mtDNA copies/cell quantitated by competitive and real-time PCR. MtDNA copies/cell in abdomen, thigh, and neck fat were depleted in lipoatrophic HIV-1 seropositive compared to the seropositive naive and seronegative cohorts. MtDNA copies/cell in thigh and neck fat were also decreased in non-lipoatrophic subjects exposed to NRTIs compared with NRTI-naive and HIV seronegative controls. PBMC values did not differ among the cohorts and there was no correlation with lipoatrophy state or HIV-1 serostatus. Additionally, differences in mtDNA copies/cell were observed in the fat depots from seronegative subjects. Thigh fat mtDNA levels were 45–55% lower than abdomen and neck. These studies help demonstrate that mtDNA levels can vary in different subcutaneous adipose depots suggesting possible metabolic differences.

Introduction

HIV-1 lipodystrophy is a syndrome characterized by alterations in the normal distribution of fat tissue resulting in subcutaneous lipoatrophy of the face, arms and legs as well as increases in visceral fat of the abdomen and back of the neck. Metabolic findings of hyperlipidaemia and insulin resistance often accompany these disturbing body morphology changes [1,2]. Epidemiological data suggests a strong association between the use of NRTIs and the development of lipoatrophy [3–7]. Mitochondrial involvement in the pathogenesis of HIV-1 lipodystrophy has been hypothesized via their role in regulating energy metabolism in adipose tissue [8] and to their known sensitivity to NRTIs [9]. Mitochondria contain their own DNA consisting of a circular, double stranded DNA molecule of 16 kb. Although NRTIs preferentially inhibit HIV-1 reverse transcriptase, NRTIs can also inhibit cellular DNA polymerases, most notably mitochondrial DNA (mtDNA) polymerase γ. DNA polymerase γ is a key regulatory enzyme of mtDNA replication. It has been hypothesized that inhibition of this enzyme by NRTIs results in mtDNA depletion and/or impairment in the production of mitochondrial enzymes within the adipocyte [10,11]. It is hypothesized that tissue toxicities due to NRTIs will occur if mtDNA falls below a critical level, so that the production of mtDNA-encoded protein subunits of the mitochondrial respiratory chain and RNAs are insufficient to meet cellular energy requirements.

Recently several studies have shown decreases in mtDNA levels in subcutaneous adipose tissue in lipoatrophic patients taking NRTIs [12–15]. This has led
investigators to query whether mitochondrial parameters could be monitored in a non-invasive manner, for example in peripheral blood mononuclear cells (PBMCs), as in some genetically inherited mitochondrial diseases [16]. In this study, we measure mtDNA copies/cell by quantitative PCR in subcutaneous adipose tissues from the abdominal wall near the umbilicus, anterior lateral thigh and dorsal cervical region of the neck, and PBMCs from HIV-1 seropositive lipoatrophic patients, HIV-1 seropositive non-lipoatrophic, HIV-1 seronegative naïve, and seronegative patients.

**Materials and methods**

**Patients and specimens**

As per guidelines established by the University of Hawaii Institutional Review Board, specimens for this study were obtained without personal identifiers from a previously described cross-sectional 4-cohort study [12] and 5 additional HIV seronegative age and gender matched controls for the PBMC measurements. The cohorts consisted of: 1) HIV-1 (+) individuals on NRTI (zidovudine, stavudine and/or didanosine)-containing HAART for more than six months with self-reported changes in lipodystrophy which included peripheral lipodystrophy following initiation of such therapy (n=3 were taking stavudine); 2) HIV-1 (+) individuals on similar NRTI-containing HAART without self-reported changes of lipodystrophy (n=4 were taking stavudine) (Table 1); 3) HIV-1 (+) individuals with less than two weeks total exposure to antiretroviral therapy (naïve); and 4) individuals seronegative for antibodies to HIV-1 by ELISA.

Subcutaneous adipose tissue specimens were previously obtained by core needle technique from the abdominal wall near the umbilicus, anterior lateral thigh, and dorsal cervical region of the neck. The tissues were flash frozen and were stored at –70°C. Available sample numbers from the four cohorts of the study were obtained without personal identifiers from a previously described cross-sectional 4-cohort study [12] and 5 additional HIV seronegative age and gender matched controls for the PBMC measurements. The cohorts consisted of: 1) HIV-1 (+) individuals on NRTI (zidovudine, stavudine and/or didanosine)-containing HAART for more than six months with self-reported changes in lipodystrophy which included peripheral lipodystrophy following initiation of such therapy (n=3 were taking stavudine); 2) HIV-1 (+) individuals on similar NRTI-containing HAART without self-reported changes of lipodystrophy (n=4 were taking stavudine) (Table 1); 3) HIV-1 (+) individuals with less than two weeks total exposure to antiretroviral therapy (naïve); and 4) individuals seronegative for antibodies to HIV-1 by ELISA.

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PCR preparation and cycling was identical for both sets of competitive PCRs. Each reaction tube was prepared in a final volume of 25 µl and contained 1× PCR Buffer, 0.2 mmol/l of each dNTP, 0.2 µmol/l of each primer, 1 unit of AmpliTaq (Applied Biosystems, Foster City, CA, USA), 1 µl of a known pMITO dilution (ranging from 10⁸ copies per µl to 10⁶ copies per µl) and approximately 100 ng of patient DNA. Cycling conditions were as follows: 94°C for 5 min, 50 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, with a final hold at 72°C for 7 min. PCR products were visualized on an ethidium bromide stained 1% agarose gel. In order to determine the number of mitochondrial DNA copies per cell, it was necessary to perform two QC-PCR, one to detect mitochondrial primers, and one with genomic primers. Serial dilutions of the competitor (10⁵–10⁷) were set up with the same amounts of DNA from the patient sample. The mitochondrial primers, mt192R (GGCTCTAGAAAGAGATCAAGGGTCTTTAGTG) and mt27D (AAA ATGAACGAAATCTGGTCCGT) amplified a 180 bp fragment of the gene encoding for the subunit VI of FOF1 ATPase. 276R (GGGGATCCGGTGCCACCG-GTAGTGGAG) and 60D (GGGAATTCCTGAGAAAGATGAAACC GTTTGCTG), the genomic primers, amplified a 259 bp fragment of the Fas Ligand.

**Analysis of mtDNA copies/cell by real-time PCR**

All fat tissues and PBMCs were assayed using real-time PCR as previously described [18]. Standardization of real-time PCR was performed using iQ SYBR green supermix with the BioRad iCycler real-time instrument (Bio-Rad Laboratories, CA, USA). The mitochondrial primers, mtDIR (GGATCAGGTTGCTCTTTATAGTG) and mtREV (GGGATCCGGTGCCACCG-GTAGTGGAG) and 60D (GGGAATTCCTGAGAAAGATGAAACC GTTTGCTG), the genomic primers, amplified a 259 bp fragment of the Fas Ligand. mtDNA copies/cell by real-time PCR

A competitive PCR assay to semi-quantitate mtDNA copy per cell was set up as previously described [17] and used to assay the abdominal tissue. A plasmid containing the human mitochondrial gene encoding the subunit VI of the F₂F₆ ATPase region and a gDNA insert from the human FasL gene, spanning the first exon, was used as a standard. The competitor plasmid with the embedded mtDNA and gDNA is smaller than the target DNA from the specimens, which allows for differentiation when size-fractionated on an agarose gel. In order to determine the number of mitochondrial DNA copies per cell, it was necessary to perform two QC-PCR, one to detect mitochondrial primers, and one with genomic primers. Serial dilutions of the competitor (10⁵–10⁷) were set up with the same amounts of DNA from the patient sample. The mitochondrial primers, mt192R (GGCTCTAGAAAGAGATCAAGGGTCTTTAGTG) and mt27D (AAA ATGAACGAAATCTGGTCCGT) amplified a 180 bp fragment of the gene encoding for the subunit VI of FOF1 ATPase. 276R (GGGGATCCGGTGCCACCG-GTAGTGGAG) and 60D (GGGAATTCCTGAGAAAGATGAAACC GTTTGCTG), the genomic primers, amplified a 259 bp fragment of the Fas Ligand.

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Table 1. Cohort characteristics of lipoatrophic and non-lipoatrophic cohorts

<table>
<thead>
<tr>
<th>Case, n</th>
<th>ART at time of biopsy</th>
<th>Prior additive exposure time on ART</th>
<th>Age</th>
<th>Gender</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-lipoatrophic (n=7)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1006</td>
<td>11 months d4T/3TC/EFV</td>
<td>2 months ZDV 3 months ABC</td>
<td>51</td>
<td>M</td>
</tr>
<tr>
<td>2014</td>
<td>60 months ZDV/3TC</td>
<td>None</td>
<td>50</td>
<td>M</td>
</tr>
<tr>
<td>3015</td>
<td>9 months d4T/ddI/IDV</td>
<td>12 months ZDV/3TC 24 months ABC 9 months IDV/EFV</td>
<td>33</td>
<td>M</td>
</tr>
<tr>
<td>4016</td>
<td>60 months d4T/NFV 48 months ddI</td>
<td>12 months ZDV</td>
<td>42</td>
<td>M</td>
</tr>
<tr>
<td>5022</td>
<td>10 months d4T/ABC/EFV</td>
<td>108 months ZDV 42 months 3TC 9 months EFV</td>
<td>42</td>
<td>M</td>
</tr>
<tr>
<td>6027</td>
<td>1 month d4T/ZDV/NFV</td>
<td>24 months ZDV 12 months 3TC/NFV</td>
<td>41</td>
<td>F</td>
</tr>
<tr>
<td>7031</td>
<td>24 month ZDV/3TC/IDV</td>
<td>None</td>
<td>35</td>
<td>M</td>
</tr>
<tr>
<td><strong>Lipoatrophic (n=7)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1002</td>
<td>102 months ZDV</td>
<td>24 months d4T 18 months NFV</td>
<td>40</td>
<td>M</td>
</tr>
<tr>
<td>2003</td>
<td>144 months ZDV 60 months 3TC 48 months IDV</td>
<td>None</td>
<td>47</td>
<td>F</td>
</tr>
<tr>
<td>3004</td>
<td>96 months ZDV 18 months 3TC 1 month ABC</td>
<td>25 months NFV</td>
<td>45</td>
<td>M</td>
</tr>
<tr>
<td>4009</td>
<td>36 months ZDV/ddC/NFV</td>
<td>24 months d4T/3TC</td>
<td>36</td>
<td>M</td>
</tr>
<tr>
<td>5017</td>
<td>42 months d4T 48 months 3TC/iDVI</td>
<td>84 months ZDV 30 months ddI</td>
<td>42</td>
<td>M</td>
</tr>
<tr>
<td>6018</td>
<td>72 months ZDV/iDVI</td>
<td>None</td>
<td>55</td>
<td>M</td>
</tr>
<tr>
<td>7020</td>
<td>24 months d4T/ddI/NFV</td>
<td>36 months ZDV/3TC</td>
<td>59</td>
<td>F</td>
</tr>
</tbody>
</table>

ABC, abacavir; ART, antiretroviral therapy; ddI, didanosine; d4T, stavudine; EFV, efavirenz; IDV, indinavir; NFV, nelfinavir; RTV, ritonavir; 3TC, lamivudine; ZDV, zidovudine
and standard was run in triplicate (50 µl reaction volume) containing: 2X SYBR Green SuperMix, 0.5 µM of each primer, and 10 ng sample of DNA. PCR cycling conditions were: denaturation for 1 cycle 95˚C for 14:30 min, amplification for 40 cycles 95˚C for 30 sec, 58˚C for 1 min, and 72˚C for 30 sec, final extension for 1 cycle at 72˚C for 6:30 min, melt for 80 cycle at 55˚C for 30 sec, cool 1 cycle at 4˚C for infinity. The results were then analysed with version 3.0 iCycler software (Bio-Rad Laboratories).

Statistical analysis
Statistical analysis was done using the Mann–Whitney rank sum test for non-parametric data with the threshold for significance set at $P=0.05$. The assays were compared by linear regression analyses using Sigma Stat 3.0 (SPSS Inc, IL, Chicago, USA).

Results

Competitive PCR of abdominal subcutaneous fat was quantitated measuring mtDNA and gDNA titration curves of pMITO (Figure 1A). The mean mtDNA content and standard deviation were 1057 ±737 copies/cell in the HIV seronegative group ($n=7$), 280 ±110 copies/cell in the antiretroviral naive cohort ($n=5$), 219 ±355 copies/cell in the non-lipoatrophic cohort ($n=7$) and 59 ±64 copies/cell in the lipoatrophic cohort ($n=10$). Examples of low and high mtDNA copies/cell are demonstrated in Figure 1B and C, respectively. A statistically significant 94% decrease in mtDNA was found in the HIV-1 seropositive lipoatrophic cohort compared to the seronegative cohort ($P=0.001$).

Similarly, real-time PCR comparisons were also comparable (Table 2) in the abdomen (although, the two assays were not comparable by linear regression analyses). mtDNA copies/cell in abdomen, thigh, and neck fat were decreased in the HIV-1 seropositive lipoatrophic cohort compared to the HIV-1 seropositive and seronegative cohorts ($P=0.05$) by real-time PCR. Also, thigh and neck HIV-1 seropositive non-lipoatrophic mtDNA copies/cell were decreased compared to HIV-1 seropositive naive and the seronegative controls ($P=0.05$). These mtDNA decreases varied in the HIV-1 seropositive lipoatrophics from 40% in the thigh, 70% abdomen and 80% neck compared to the seronegative controls. There were no statistically significant differences in mtDNA copies/cell in the fat depots between the lipoatrophic and non-lipoatrophic seropositive patients or the seronegative and naive cohorts. NRTI exposure time between the lipoatrophic cohort ($90 ±39$) and non-lipoatrophic ($60 ±50$) was not statistically different [12]. Additive NRTI exposure time between the lipoatrophic cohort ($101 ±40$) and non-lipoatrophic ($51 ±50$) was not statistically different [12] (Table 1). Another interesting finding was variation in mtDNA copies/cell in the thigh, abdomen, and neck fat in the HIV-1 seronegative patients.
cohort. The thigh fat had 45–55% less mtDNA copies/cell than the abdomen and neck. Additionally, mtDNA copies/cell were evaluated in PBMCs from these cohorts and ranged from 105–263 copies/cell. No statistically significant differences in PBMC values were observed among the cohorts and there was no correlation with lipoatrophy state or HIV-1 serostatus (data not shown).

**Conclusion**

This study was designed to measure quantitatively mtDNA copies/cell from PBMCs and different subcutaneous fat depots in a previously described cohort [12]. Our previous study measured mtDNA copies/cell in the fat tissue using semi-quantitative PCR by amplifying three different mtDNA fragments and analyzing them by visual interpretation of ethidium-stained gels. The conclusion of that study showed a decrease in mtDNA content in HAART treated HIV-1 infected patients with peripheral fat wasting in comparison with subjects in the control cohorts. The findings in this current protocol confirm our previous study and show that significant mtDNA depletion is evident regardless of which PCR method was used. We observed significant differences by real-time PCR in mtDNA copies/cell in the subcutaneous adipose tissue from thigh, abdomen, and neck of seronegative subjects. Additionally, mtDNA depletion was observed in thigh and neck fat from both lipoatrophic and non-lipoatrophic cohorts on NRTIs compared with NRTI naive controls. In contrast, we found that Ficoll-isolated PBMC mtDNA copies/cell did not vary significantly among the cohorts. This lack of differences may be due to the small number of samples available particularly in the HIV (+) naive group, n=3. Thus in our study, PBMC mtDNA copies/cell did not correspond with HIV status, current NRTI exposure, or the presence of self-reported lipoatrophy.

Consistent with our findings, other investigators have found a substantial decrease in mtDNA content in fat tissue in association with HIV-1 lipoatrophy. This mtDNA depletion has been observed in vitro with different NRTIs affecting DNA polymerase γ [9] and is more profound in subjects on stavudine compared to zidovudine [15,19,20]. More recently, the partial reversal of lipoatrophy following 'switch' substitution of thymidine analogue NRTIs to a 'more mitochondrial friendly' non-thymidine analogue NRTI regimen or to a completely NRTI-sparing regimen also supports the hypothesis that NRTIs and thymidine analogues in particular have a role in the development of lipoatrophy [15,21]. In our study, both the non-lipoatrophic (five of the seven patients) and lipoatrophic (four of the seven patients) cohorts had been treated with stavudine and mtDNA depletion was

### Table 2. MtDNA copies/cell in subcutaneous fat and PBMCs

<table>
<thead>
<tr>
<th></th>
<th>HIV(-)</th>
<th>HIV (+) naive</th>
<th>HIV (+) no lipoatrophy</th>
<th>HIV (+) lipoatrophy</th>
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<tbody>
<tr>
<td><strong>Thigh</strong></td>
<td></td>
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<tr>
<td>435 ±63†</td>
<td>489 ±100</td>
<td>267 ±136*</td>
<td>255 ±124*</td>
<td></td>
</tr>
<tr>
<td>n=4†</td>
<td>n=5</td>
<td>n=6*</td>
<td>n=6*</td>
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<tr>
<td><strong>Abdomen</strong></td>
<td></td>
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<tr>
<td>790 ±292</td>
<td>545 ±190</td>
<td>335 ±158*</td>
<td>244 ±148*</td>
<td></td>
</tr>
<tr>
<td>n=5</td>
<td>n=5</td>
<td>n=6*</td>
<td>n=7*</td>
<td></td>
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<tr>
<td><strong>Neck</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>976 ±292</td>
<td>676 ±271</td>
<td>396 ±249*</td>
<td>205 ±78*</td>
<td></td>
</tr>
<tr>
<td>n=5</td>
<td>n=5</td>
<td>n=6*</td>
<td>n=7*</td>
<td></td>
</tr>
<tr>
<td><strong>PBMC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>201 ±62</td>
<td>105 ±48</td>
<td>157 ±49</td>
<td>148 ±53</td>
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</tbody>
</table>

All values are representative as mtDNA copies/cell (×±SD) and statistical significance are described. *Statistical significance between HIV (-) compared to either HIV(+) no lipoatrophy or lipoatrophy. Thigh HIV (+) no lipoatrophy and lipoatrophy were compared to HIV (-), P=0.05 and P=0.04 respectively. Thigh HIV (+) no lipoatrophy and lipoatrophy were compared to HIV (+) naive, P=0.05 and P=0.04 respectively. Thigh HIV (+) no lipoatrophy and lipoatrophy were compared to HIV (+) naive, P=0.05 and P=0.04 respectively. Thigh HIV (+) no lipoatrophy and lipoatrophy were compared to HIV (+) naive, P=0.05 and P=0.04 respectively. Thigh HIV (+) no lipoatrophy and lipoatrophy were compared to HIV (+) naive, P=0.05 and P=0.04 respectively. †Statistical significance between the HIV (-) thigh in comparison to abdomen (P=0.05) or neck (P=0.05).
observed in both cohorts, adipose tissues, but we did not observe mtDNA depletion in PBMCs. Other studies with HIV-1 lipoatrophic cohorts have confirmed these findings [22].

One of the most interesting findings from this study is the difference in mtDNA copies/cell in the HIV (-) thigh, neck, and abdomen, with mtDNA copies/cell being lowest in the thigh. These results suggest there may be mitochondrial metabolic differences in different fat depots. Studies comparing visceral and subcutaneous fat in humans have shown gene expression and biochemical differences. Gene expression profiles of subcutaneous and visceral adipose tissue from 10 non-diabetic, normolipidemic obese men showed increased expression in subcutaneous fat of genes in the Wnt signalling pathway, CEPBA, HOX, lipolytic stimuli, and cytokine secretion [23]. Furthermore, uncoupling protein 2 mRNA expression has been shown to be increased in the omentum vs subcutaneous adipocytes from patients undergoing elective intra-abdominal surgery [24]. Tumour necrosis alpha levels have been shown to be lower in visceral fat in women compared to subcutaneous adipose tissue in patients undergoing elective intra-abdominal surgery [25]. All these pathways, genes, and proteins affect mitochondrial function. For instance, uncoupling proteins are mitochondrial carrier proteins that catalyze a regulated proton leak across the inner mitochondrial membrane, diverting free energy from ATP synthesis by the mitochondrial F_{0}F_{1}-ATP synthase to the production of heat [26]. Therefore, further investigations are warranted in understanding the differences in subcutaneous fat depots and their roles in HIV lipodystrophy.

Acknowledgements

We would like to thank Duy Tran and Elizabeth Westgard for their technical assistance, Dr Christopher J O’Callaghan, Queen’s University, Kingston, Ontario, Canada for his statistical analyses and Drs Andrea Cossarizza and Marcella Pinti, University of Modena and Reggio Emilia School of Medicine, Modena, Italy for the plasmids and methodologies for quantitating mtDNA. Funding for this research was provided by the National Institutes of Health, USA, grant numbers: AI34853 and M D000173.

References


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Altered mitochondrial RNA production in adipocytes from HIV-infected individuals with lipodystrophy

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L Galluzzi and M Pinti contributed equally to this work.

Background: Damage to mitochondria (mt) is a major side effect of highly active antiretroviral therapy (HAART) that includes a nucleoside reverse transcriptase inhibitor (NRTI). Such damage is associated with the onset of lipodystrophy in HAART-treated HIV+ patients. To further investigate mt changes during this syndrome, we analysed the expression of mtRNA in adipocytes from lipodystrophic HIV+ patients taking NRTI-containing HAART and compared it with similar cells from healthy individuals.

Materials and methods: Total RNA was extracted from adipocytes collected from different anatomical locations of 11 HIV+ lipodystrophic patients and seven healthy control individuals. RNA was reverse transcribed and Taqman-based real-time PCR was used to quantify three different mt transcripts (ND1, CYTB and ND6 gene products). mtRNA content was normalized versus the housekeeping transcript L13.

Results: ND1, CYTB and ND6 expression was significantly reduced in HIV+ lipodystrophic patients. HIV+ men and women did not differ in a statistically significant way regarding the levels of ND1 and ND6, whereas the opposite occurred for CYTB.

Conclusions: Lipodystrophy following treatment with NRTI-containing HAART is associated with a decrease in adipose tissue mtRNAs.

Introduction

It has been proved that lipodystrophy (central obesity and peripheral lipoatrophy), together with hyperlipidaemia and insulin resistance, is associated with highly active antiretroviral therapy (HAART) [1,2]. Although the first studies focused on the role of protease inhibitors (Pis), subsequent analyses revealed that nucleoside analogue reverse transcriptase inhibitors (NRTIs) are equally, if not more, involved [3-5]. Indeed, it has been suggested that NRTI-induced mitochondrial (mt) toxicity may be one cause of HAART-associated lipodystrophy. In particular, as evidenced by several clinical studies, these drugs are the main cause of the onset of lipoatrophic forms: a strong association exists between NRTI-containing therapeutic regimens and either lipoatrophy or markers of mt toxicity such as lactic acidemia, hepatic impairment and changes in mtDNA content ([6-12]; reviewed in [13]). Mitochondrial activity could theoretically be impaired even in the absence of systemic abnormalities that give clinical signs, or when the mtDNA content of cells is still unaffected, at least in the case of cell lines in vitro exposed to NRTIs [14]. Thus, it could be of interest to look for other markers of mt damage, such as the expression of mtRNAs.

mtRNAs are synthesized, completely processed and translated within the organelle by a dedicated machinery made both of nuclear-encoded components (for example, mtRNA polymerase) and of mt-encoded ones (tRNAs) [15,16]. Human mtDNA is a double-stranded, circular molecule of 16 571 base pairs encoding 13 proteins, 22 tRNAs and two rRNAs. It is transcribed as two large polycistronic units from both template DNA strands (H or heavy and L or light); an endonuclease processes these large molecules and gives origin to all functional mtRNA species [17-19].

To conveniently analyse the transcription of the whole mt genome, we chose to quantify three different mt transcripts: ND1, encoding for subunit 1 of the NADH-dehydrogenase complex and lying at the 5′ end of the H strand; CYTB, encoding for cytochrome B and lying at 3′ end of the H strand; and ND6, encoding for subunit 6 of the NADH-dehydrogenase complex,
and being the only mRNA encoded by the L strand (5’ end). This study was designed to assess mtRNA content in the adipocytes from seropositive individuals who have developed lipodystrophy following NRTI-containing HAART and to compare the results with those from seronegative subjects.

Materials and methods

Studied subjects

The present study was performed on samples among HIV+ patients with lipodystrophy selected from the patients of the Metabolic Clinic of the ‘Azienda Policlinico di Modena’ Hospital and from healthy controls undergoing minor plastic surgery. The HIV+ subjects, who gave informed consent for the analysis reported in the present study, underwent surgical procedures for the amelioration of the physical changes caused by lipodystrophy. Fat samples were obtained from different subcutaneous body areas (buffalo hump, back, breast and abdomen) by mechanical liposuction, performed in some cases to obtain a fat graft for autologous fat transplant (in the case of HIV+ patients, to correct facial lipoatrophy) or for aesthetic purposes (healthy controls).

The lipodystrophic HIV+ group was formed by six men (mean age ± SD: 46.3 ± 8.9 years) and five women (mean age 41.4 ± 10.0). All these patients had a long history of exposure to NRTIs, including D-drugs (stavudine (d4T) and didanosine (ddI)), and were currently taking HAART including one or more NRTI. The control HIV− group was composed of one man (aged 63) and six women (mean age 52.3 ± 9.8).

RNA extraction and reverse transcription

Total RNA was extracted from adipocytes of study subjects by means of RNeasy Lipid Tissue Mini Kit from Qiagen (Hilden, Germany). Frozen biotptic samples of subcutaneous adipose tissue from different anatomical locations were the source of adipocytes from both HIV-infected patients and healthy control individuals. Represented locations included breast, abdomen, buffalo hump (from HIV-infected patients only) and lower limbs. Subsequently, 1 µg of the extracted RNA was subjected to a standard random-primed reverse transcription reaction [20,21].

Construction of the standard plasmid for mtRNA quantification

The amplicons of interest (L13, ND1, ND6 and CYTB, whose sequences are reported in Table 1) were cloned together in a plasmid in order to prepare a single, reference DNA molecule that could be used as the standard in real-time PCR quantification of all four transcripts (patent PCT/NL03/00545). The use of a single, cleaved plasmid was preferred to guarantee the ratio among amplicons to be fixed at the value of 1:1:1:1. As described below, the plasmid was constructed using classical molecular biology techniques [20], and its sequence checked by an automatic DNA sequencer (Abi Prism 310 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA). A human cDNA sample, obtained from the random-primed reverse transcription of total RNA from peripheral blood mononuclear cells, was used as a template to amplify the four amplicons of interest by PCR (primers are reported in Table 1). Each primer was designed to contain convenient linker regions useful for the subsequent cloning steps. Tails to be added to primers in order to produce linkers were selected from the genome of an evolutionarily distant organism (Cryptococcus neoformans) and blasted against the human genome to check for absence of homology. This was necessary to avoid unspecific annealing between the tailed primers and human cDNAs, which would have lead to unspecific PCR products.

All primers, as well as probes used in the quantification reactions, were purchased from Qiagen Operon (Hilden, Germany). Primers SP1/SP2 created L13 amplicon (72 bp) with a PsI restriction site immediately upstream and a linker (L1, 20 bp) immediately downstream; primers SP7/SP8 lead to ND6 amplicon (109 bp) with a different linker (L2, 21 bp) immediately upstream and a HindIII restriction site immediately downstream. Primers SP3/SP4 were used to amplify CYTB amplicon (107 bp) with L1 immediately upstream and a ‘false linker’ introducing a SacI restriction site immediately downstream. This ‘false linker’ contains the SacI restriction site and part of the sequence of ND1 amplicon (78 bp), which in turn was obtained with primers SP5/SP6, and serves only construction purposes. ND1 amplicon had L2 immediately downstream and a SacI restriction site immediately upstream, in the context of the false linker (in this case part of CYTB amplicon).

These four PCR products were used together as templates in a single PCR reaction to obtain the full insert of interest. A small amount of each amplicon was used in a multitemplate reaction (based upon an original strategy) as follows. The starting reaction mixture included <0.5 µl of the products of each described single reaction (templates); MgCl2-free buffer 1× (Promega, Madison, WI, USA); MgCl2 1.5 mM, Taq polymerase 2 U (Promega), dNTPs mix 0.2 mM (Fermentas, Vilnius, Lithuania) and water up to 49.4 µl (total starting volume). No primers were present at this stage. The mixture then underwent the following thermal protocol, to fill in the gaps between templates partially annealed because of the complementary tails: starting denaturation (95°C, 5 min), two main cycles

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### Table 1. Primers, probes and amplicons

<table>
<thead>
<tr>
<th>Name</th>
<th>5′-3′ Sequence/labels</th>
<th>Length, bp</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amplicons</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>A1–L13</td>
<td>5′-GCGGAAGTACGGACGAGGAGGCCACCCTTGAGGAGAAGGAAAGCAAGATCCACTACCCGF-3′</td>
<td>72</td>
<td>[21]</td>
</tr>
<tr>
<td>A2–ND1</td>
<td>5′-CTTTCGCTTTACACCACATAAACTCCTGCAACGGACCCCTTTAAAAACCCCCACATCCCATACCCTCCTACATACCCGG-3′</td>
<td>78</td>
<td>Present work</td>
</tr>
<tr>
<td>A3–CYTB</td>
<td>5′-AGTCCCCACCCCTCCTTTCATCTCAGTCTACCTTCCATTTATGAGCCAGACACTCCACCTTATCTTCCAGGAACAGGATCAAAGAA-3′</td>
<td>107</td>
<td>Present work</td>
</tr>
<tr>
<td>A4–ND6</td>
<td>5′-AACCCCACCCCTCCTTCTCTTCAATTTATGACCTTCCATTTATGAGCCAGACACTCCACCTTATCTTCCAGGAACAGGATCAAAGAA-3′</td>
<td>109</td>
<td>Present work</td>
</tr>
<tr>
<td><strong>Standard plasmid construction primers – tailed primers</strong> (Qiagen Operon)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP1–L13 dir/PstI</td>
<td>5′-AAACTGCAGCTGGAAGTACGGACGAGGAGGCCACCCTTGAGGAGAAGGAAAGCAAGATCCACTACCCGF-3′</td>
<td>30</td>
<td>Present work</td>
</tr>
<tr>
<td>SP2–L13 rev/L1</td>
<td>5′-TCCAGACACCCAACAGAGGCACGGTAGTGACGTCCTGTGCF-3′</td>
<td>41</td>
<td>Present work</td>
</tr>
<tr>
<td>SP3–CYTB dir/L1c</td>
<td>5′-GACCGTTGAGGCTGTCGGAGTACGGACGAGGAGGCCACCCTTGAGGAGAAGGAAAGCAAGATCCACTACCCGF-3′</td>
<td>40</td>
<td>Present work</td>
</tr>
<tr>
<td>SP4–CYTB rev/SacI</td>
<td>5′-GACCGTTGAGGCTGTCGGAGTACGGACGAGGAGGCCACCCTTGAGGAGAAGGAAAGCAAGATCCACTACCCGF-3′</td>
<td>40</td>
<td>Present work</td>
</tr>
<tr>
<td>SP5–ND1 dir/SacI</td>
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<td>39</td>
<td>Present work</td>
</tr>
<tr>
<td>SP6–ND1 rev/L2</td>
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<td>41</td>
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</tr>
<tr>
<td>SP7–ND6 dir/L2c</td>
<td>5′-AGGCGGGGAGGAGCTTGACAGAGCCACGGACCCTCTCCCTCCGTCTGAGGCCATATA-3′</td>
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<td>Present work</td>
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<tr>
<td>SP8–ND6 rev/HindIII</td>
<td>5′-CCAGATGTCAGTAGGATGGTCTCG-3′</td>
<td>28</td>
<td>Present work</td>
</tr>
<tr>
<td><strong>Real-Time PCR primers</strong> (Qiagen Operon)</td>
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<tr>
<td>P1–L13 dir</td>
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<td>[21]</td>
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<td>21</td>
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<td>P3–ND1 dir</td>
<td>5′-CTTTCGCTTTACACCACATAAACTCCTGCAACGGACCCCTTTAAAAACCCCCACATCCCATACCCTCCTACATACCCGG-3′</td>
<td>19</td>
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<td>P4–ND1 rev</td>
<td>5′-CTTTCGCTTTACACCACATAAACTCCTGCAACGGACCCCTTTAAAAACCCCCACATCCCATACCCTCCTACATACCCGG-3′</td>
<td>20</td>
<td>Present work</td>
</tr>
<tr>
<td>P5–CYTB dir</td>
<td>5′-AGTCCCCACCCCTCCTTCTCTTCAATTTATGACCTTCCATTTATGAGCCAGACACTCCACCTTATCTTCCAGGAACAGGATCAAAGAA-3′</td>
<td>20</td>
<td>Present work</td>
</tr>
<tr>
<td>P6–CYTB rev</td>
<td>5′-TGGAGGTAGGATGGTCTCG-3′</td>
<td>20</td>
<td>Present work</td>
</tr>
<tr>
<td>P7–ND6 dir</td>
<td>5′-AACCCCACCCCTCCTTCTCTTCAATTTATGACCTTCCATTTATGAGCCAGACACTCCACCTTATCTTCCAGGAACAGGATCAAAGAA-3′</td>
<td>20</td>
<td>Present work</td>
</tr>
<tr>
<td>P8–ND6 rev</td>
<td>5′-TGGAGGTAGGATGGTCTCG-3′</td>
<td>20</td>
<td>Present work</td>
</tr>
<tr>
<td><strong>Probes</strong> (Qiagen Operon)</td>
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<td></td>
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<tr>
<td>S1–L13</td>
<td>5′-TCTTTCGCTTCTCCTCCTCAGGGGTGCGF-3′ Texas Red – BHQ1</td>
<td>27</td>
<td>[21]</td>
</tr>
<tr>
<td>S2–ND1</td>
<td>5′-CGAAGGGGCGGGCTAAACCCCGGCT-3′ FAM490 – BHQ1</td>
<td>23</td>
<td>Present work</td>
</tr>
<tr>
<td>S3–CYTB</td>
<td>5′-CAAGCTTACCCACGGACACTCCACCTC-3′ Texas Red – BHQ1</td>
<td>27</td>
<td>Present work</td>
</tr>
<tr>
<td>S4–ND6</td>
<td>5′-CAAGCTTACCCACGGACACTCCACCTC-3′ FAM490 – BHQ1</td>
<td>23</td>
<td>Present work</td>
</tr>
</tbody>
</table>

*Tail nucleotides are reported in italics and inserted restriction sites are underlined. Gene bank accession numbers: NM_0012423 for L13, AAO88540 for ND1, AY339474 (region 14747–15887) for CYTB, AAO88837 for ND6. dir, direct; rev, reverse.
(94°C, 15 sec; 63°C, 30 sec; 72°C, 30 sec) and two additional main cycles (94°C, 15 sec; 72°C, 1 min). After this, one additional unit of Taq polymerase and primers SP1 and SP8 were added up to a final concentration of 0.4 µM (final volume 50 µl). The thermal protocol was then continued as follows: two main cycles (94°C, 15 sec; 64°C, 30 sec; 72°C, 30 sec), 35 additional main cycles (94°C, 15 sec; 70°C, 30 sec; 72°C, 30 sec) and final elongation (72°C, 7 min). Electrophoresis on 3% agarose gel of the products confirmed the amplification of a single fragment of 430 bp (see Figure 1, which also shows the localization of the genes we studied on the mtDNA molecule).

The 430 bp insert was then cloned as a PstI-HindIII fragment into the vector pQE-81L (Qiagen). The deriving plasmid (pQE-81L-mtRNA), extracted from recipient Escherichia coli JM109 cells with QIAprep Spin Miniprep Kit (Qiagen), was digested with SacI to obtain the final standard mixture, composed of two equimolar linear fragments of 4937 and 227 bp, respectively. The digestion pattern is due to the presence of two SaeI restriction sites: the first one within the insert and the second one as part of the polylinker of the parental vector pQE-81L. The products were then quantified by comparing their intensity after 1% agarose gel electrophoresis with a molecular weight marker of known concentration. The software Quantity One (Bio-Rad, Hercules, CA, USA) was used for this analysis. The digested plasmid was used in 10-fold dilutions for the construction of the standard curve for the real time PCR quantification.

Real-time PCR
Each different transcript was quantified in a separate reaction to avoid non-specific amplifications and to ensure the maximal reliability of the study. Each reaction entailed the following reagents: 1 µl of cDNA solution (sample); PCR Mg2+-free buffer 1× (Promega); MgCl2 1.5 mM; Taq polymerase 2 U (Promega); dNTPs mix 0.2 mM (Fermentas), direct and reverse primers 0.4 µM for mt transcripts, 0.2 µM for L13 (primers vary according to the measured RNA, see Table 1); Taqman probe 0.2 µM (probes vary according to the measured RNA, see Table 1); and water up to 50 µl.

Reactions were performed in PCR multiwell plates (Bioprintics, North Ridgeville, OH, USA). iCycler (Bio-Rad) was the real-time thermal cycler of choice and was set as follows: starting denaturation (95°C, 5 min) followed by 45 cycles (94°C, 15 sec; 60°C, 30 sec; fluorescence reading). The reading wavelength depended on the probe in use: FAM 490-labelled probes are detected at 520 nm, while those labelled with Texas Red are detected at 615 nm. Baseline cycles, threshold and threshold cycles (Ct) are calculated automatically by selecting the maximum correlation coefficient approach in the cycler software.

Data handling and statistical analysis
Each real-time PCR quantification reaction, including those run on the standard mixture at different dilutions, was performed in triplicate. The iCycler software, at the end of the run, automatically compares the mean Ct values with the created standard curve and gives results in terms of absolute copies and standard deviation for each group of triplicate samples. Nevertheless, absolute cDNA copies are not a suitable parameter for analysis. Indeed, to have interpretable data, it is recommended that the mtRNA content of each sample is normalized to its content of a housekeeping transcript. We chose L13, which encodes a protein belonging to the large ribosomal subunit and is widely considered as a housekeeping gene [22], whose

Figure 1. mtDNA map and insert for mtRNA standard plasmid

(A) Map of mtDNA that evidences the position of the genes chosen for the analysis of mtRNA production (in grey) and the sense of their transcription. Black lines indicate genes for tRNAs. Note that ND1 and CYTB genes are encoded by the H strand (clockwise arrows), while ND6 gene by the L strand (anticlockwise arrow). (B) Grey boxes represent the four amplicons involved in the quantification reactions. Arrows depict the annealing positions of the primers (indicated as SP) used for the construction of the molecule. Black lines show relevant restriction sites (enzymes used are in italics).
transcription is, in practice, constant in all functional moments of the cell. The normalization to a housekeeping RNA avoids further mathematical analyses that would otherwise be necessary to consider the different efficiency of RNA isolation and reverse transcription in different samples. Thus, in this study the results are shown as copies of each mt transcript per copy of L13.

Studied subjects were grouped into two main categories according to their HIV seropositivity. Infected individuals were subsequently divided according to sex. This was not done for healthy controls because of the large preponderance of women. Groups were compared for the expression of mtRNAs in the following ways: HIV+ patients versus HIV– controls, HIV+ men versus HIV+ women and HIV+ women versus HIV– women (for CYTB only). Statistical significance was determined by using two-tailed non-parametric test (Mann–Whitney test), with confidence intervals of 95%.

Results

Mitochondrial transcription in healthy adipocytes
Firstly we analysed the adipocyte mtRNAs from healthy subjects and found that the transcription levels of the three genes under investigation are different. Indeed, ND1 is expressed at much lower levels (130.5 copies/L13 copies, mean value) than the other two transcripts (573.1 and 1043.0 copies/L13 copies, mean values for ND6 and CYTB, respectively). No significant differences were present among fat samples collected in different sites.

mtRNA depletion in HIV+ patients
The quantification of mtRNAs in samples obtained from HIV+ individuals revealed a dramatic decrease of their expression. This decrease was similar in samples collected from different sites. In cells from HIV+ men, ND1 had a concentration of 65.6 copies/L13 copies, corresponding to a 50% reduction with respect to the controls. We also demonstrated a similar fall for HIV+ women (52.5 copies/L13 copies corresponding to a 60% reduction). The comparison between the control group and all HIV+ patients (P=0.0021), whereas HIV+ men and women did not differ (P=0.3290) (Figure 3).

The concentration of CYTB transcripts in adipocytes from HIV+ men was 359.0 copies/L13 copies (66% reduction vs controls). mtRNA levels in adipose tissue from HIV+ women were much lower (63.9 copies/L13 copies, which corresponds to a 94% reduction vs controls). As a consequence, statistical analysis revealed a significant difference not only between the control group and all HIV+ patients (P=0.0104, similar to ND1 and ND6), but also between HIV+ men and women (P=0.0303). Finally, we compared CYTB expression in HIV+ and HIV– women (who were represented by six individuals, aged 52.3 ±9.8), and found a statistically significant difference (P=0.0079) (Figure 4).

![Figure 2. ND1 expression in studied subjects](image)

**Figure 2. ND1 expression in studied subjects**

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>HIV+ Men</th>
<th>HIV+ Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND1 copies/L13 copies</td>
<td>130.5 ±20.2</td>
<td>65.6 ±13.2</td>
<td>52.5 ±12.3</td>
</tr>
</tbody>
</table>

Results are reported as ND1 copies/L13 copies in terms of mean value and standard deviation. Statistical significance is indicated by P value.

![Figure 3. ND6 expression in studied subjects](image)

**Figure 3. ND6 expression in studied subjects**

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>HIV+ Men</th>
<th>HIV+ Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND6 copies/L13 copies</td>
<td>573.1 ±93.7</td>
<td>183.5 ±31.4</td>
<td>107.8 ±19.0</td>
</tr>
</tbody>
</table>

Results are reported as ND6 copies/L13 copies in terms of mean value and standard deviation. Statistical significance is indicated by P value.
Discussion

Considerable attention has been paid to the role of mitochondria in the pathogenesis of lipodystrophy, and several authors have demonstrated a significant, dramatic depletion of mtDNA content in adipose tissue of lipodystrophic HIV+ patients compared with controls [23–26]. The determinant role of D-drugs, especially d4T, in causing lipoatrophy has been well defined by several sets of clinical data. The functional consequences of mtDNA depletion are complex. It is known from classic studies on genetic disorders affecting the mt genome that an mtDNA decrease of the order of 80% is necessary to evidence functional alterations. However, in treated HIV+ patients, even smaller decreases in mtDNA content are significantly associated with relevant alterations in the distribution of fat tissue [3,11,27,28]. Depletion of adipocyte mtDNA positively correlates with evidence of tissue, cell and mt toxicity, including decreased expression of mt proteins, increased adipocyte pleiomorphism, infiltration of macrophages and non-adipocyte inflammatory cells, and increased apoptosis [29]. Mitochondrial damages are not irreversible, since improvements in lipoatrophy, mtDNA content and fat tissue apoptosis can be obtained after replacing a constituent drug with a less toxic one [30–32]; damage can also be reduced by regimen alternations [33].

At the functional level, studies on frozen adipocyte biopsies from HIV+ individuals demonstrated that NRTI-induced mtDNA depletion can result in a decreased enzymatic activity of the respiratory chain complex IV [34]. It is not known, at least in fat cells, what relationship exists between changes in mtDNA content and changes in the number of mitochondria. Nevertheless, it has been shown that mtDNA depletion is associated with an increased organelle mass, suggesting that this may represent a compensatory response to decreased mtDNA [25].

The mechanisms that regulate the replication of mtDNA and the relationship among mtDNA levels, mtRNA levels and the levels of the encoded proteins are not completely clear. On one hand, it could be hypothesized that the reduction in mtDNA leads to a parallel decrease in its transcription and translation; on the other hand, in cell cultures, mitochondria could compensate mtDNA loss with a concomitant increase in its transcriptional rate [35]. Clinical data showed that the treatment with d4T plus ddI was associated with decreased mt mass and mtDNA content, but that the expression and activity of mtDNA-codified enzymes such as COXII remained unaltered [36]. This suggests that up-regulatory transcriptional or post-transcriptional mechanisms could compensate for mtDNA depletion before profound mtDNA depletion occurs.

In cultured mouse muscle cells treated with zidovudine (AZT), the disruption of mt cristae occurred after 4 weeks of treatment and was not accompanied by changes in mtDNA content [37]. Cytochrome b and cytochrome c oxidase I mRNA significantly increased (64% and 31%, respectively), while no changes were present as far as mtDNA was concerned. This suggests that AZT can provoke complex alterations in the production of nucleic acids, that can affect also mtRNA transcription. In patients, it was reported that dual NRTI therapy decreases mtRNA production after 2 weeks of therapy, before changes in fat mass or changes in metabolic parameters [38]. There are very few data, in addition to ours, about the quantification of mtRNAs in human cells [35,38–40]. The analysis of mtRNA levels, besides those on mtDNA, probably provides additional information on the consequences of mtDNA depletion. This approach could also help to clarify if and how this depletion effectively leads to a decline in terms of functionality of the mt machinery.

The real-time PCR technique that we describe in this paper measures the concentration of three mt transcripts encoded at different positions in the mt genome. We selected these mtRNAs in order to conveniently represent the whole transcriptional process from the mtDNA molecule. It is indeed known that the mt genome is transcribed from both mtDNA strands into two large polycistronic units, which are cleaved by specific endonucleases to produce the mature mtRNAs [18,19]. According to this model, an equal amount of ND1 and CYTB mtRNAs should be found, since they are encoded on the same template strand. This is not
the case, probably because the amount of each mtRNA is determined not only by its transcription (presumably occurring at the same rate for ND1 and CYTB genes) but also by its degradation, which probably occurs at different rates, according to the intrinsic stability of each transcript and the cellular functional microenvironment. Our analysis allows the quantification of mtRNAs using nuclear RNA as a reference. Even if quite unlikely, because the nuclear gene we chose as reporter is quite stable [22], it cannot be excluded that modifications in nuclear transcription could influence the data we obtained.

We have found different expression of CYTB in HIV+ male and females. Since CYTB is the last gene transcribed in the heavy chain and since no main differences were observed among fat samples collected in different sites, one could speculate that the efficiency of the transcription is lower in women, or that they are more prone to mt toxicity. Since the number of patients we have studied is relatively low, further data are required to investigate this aspect.

We have previously shown that the relative ratios among these three mtRNAs is different in various experimental models [14]. This suggests that it cannot be exclusively due to different transcription rates, but is probably the result of a dynamic process involving rate of mtRNA synthesis, editing, stability and degradation, which all vary between different cells [14]. What we observed in ex vivo lymphocytes and in vitro models (U937, CEM and HUT-78 cell lines) clearly indicates that the relative ratio among ND1, ND6 and CYTB is highly variable, but is usually kept constant in a particular type of cell.

The data we report in this paper reveal the presence of an association between NRTI-induced lipodystrophy in HIV+ patients and significantly reduced levels of ND1, ND6 and CYTB mtRNAs. However, we are well aware that the analysis of HIV+ patients without lipodystrophy is urgently needed to ascertain which is the role of the viral infection per se in provoking mtRNA alterations, as observed for mtDNA [41]. For this reason, we are in the process of selecting naive HIV+ patients whose adipocytes will be studied.

When chronically exposed to NRTI drugs, adipose tissue from HIV+ patients with lipodystrophy shows a general, dramatic decrease in the mtRNA levels for all of the three messengers analysed. However, even in these cells, the ratio between the mtRNAs was roughly maintained, suggesting that NRTIs act by provoking a general drop in the efficiency of the transcriptional apparatus of mitochondria. Since the decrease in mtRNA levels does not affect one single transcript, we are tempted to assume that NRTIs interfere with the overall transcriptional process rather than affecting the stability of mature molecules. It will be interesting to know whether this decrease is an indirect consequence of mtDNA depletion in these cells (a well-known phenomenon described by several authors in adipose tissue), or if it is due to a direct action of NRTIs on the transcriptional machinery.

In a previous work, we demonstrated that mtRNA levels can decrease in cell lines treated with NRTIs even when mtDNA content is still preserved [14]. This additionally stresses the importance of mtRNA quantification, which is able to reveal mt impairment before a considerable decrease in mtDNA content can be measured. Direct studies on mtDNA transcription in the presence or absence of different drugs/drug combinations, as well as sequential studies in patients who start NRTI-based therapy, could clarify the causes of this phenomenon and help in a better identification of the molecular target(s) of their toxic action.

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**References**


Methodological considerations in human studies of gene expression in HIV-associated lipodystrophy

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HIV-associated lipodystrophy (HIVLD) is a complex syndrome of multifactorial aetiology and, although prevention strategies have yielded some encouraging results [1], no effective treatment exists. Lipodystrophy takes months to develop [2,3] and, even when the causal agents are substituted, undergoes only a slow and incomplete reversal [4]. In vitro, protease inhibitors (PIs) disrupt the action of sterol regulatory element binding protein 1 (SREBP1), a transcriptional activator that mediates expression of peroxisome proliferator-activated receptor gamma (PPARγ) [5,6]. The thymidine analogue nucleoside reverse transcriptase inhibitors (NRTIs) zidovudine and stavudine decrease expression of mitochondrial genes and PPARγ in human adipose tissue [7] and have been linked with mitochondrial dysfunction [8,9] and depletion [10,11]. As there is no effective way to predict or monitor the onset of HIVLD before it becomes clinically apparent, a fuller understanding of drug-induced molecular changes in vivo is important if the joint goals of effective prevention and treatment of HIVLD are to be realized.

In contrast to laboratory experiments using cell lines, where conditions and interventions can be tightly regulated, studies of gene expression in vivo are complicated by numerous potential confounding factors that can complicate the detection or interpretation of changes in gene expression. In the case of lipodystrophy, the incidence and severity of the syndrome can be significantly influenced by factors such as age, gender, presence of coinfection, and pre-treatment weight and immune status [12]. Many of these factors may influence the molecular effects of antiretrovirals and complicate gene expression studies in this clinical setting.

With any target tissue under investigation, such as subcutaneous adipose tissue, appropriate sample collection and processing is of the utmost importance. This is because the amount of tissue required is dictated by the number of planned investigations and the RNA yield from the target tissue, which varies considerably between tissues and, in the case of human subcutaneous adipose tissue, is relatively low [7,13,14]. In addition, certain genes of interest, such as transcription factors, are expressed at very low levels within tissues [7] making it more difficult to detect subtle but potentially clinically important changes in their expression. Biological and pharmacological confounders, such as ongoing opportunistic infections or use of polypharmacy, which can affect overall cellular gene expression in a target tissue, may make...
identifying an appropriate housekeeping gene difficult. Despite these obstacles, with careful attention to study design and sample processing, it is possible to limit the impact of these factors.

**Sample collection and processing**

RNA yields from adipose tissue are lower per weight of tissue than other tissues such as liver and muscle. In contrast to muscle and liver, which are expected to yield 100–600 µg of RNA per 100 mg tissue [15], adipose tissue yields are only in the region of 2–3 µg per 100 mg tissue [7,11,13,14]. Given the rapid degradation of RNA, in particular messenger RNA (mRNA), careful sample collection and rapid processing, such as immediate fixing and snap-freezing of biopsied samples in liquid nitrogen, can limit degradation and maintain optimal RNA yields (Figure 1).

Careful consideration also needs to be applied to the quality of tissue biopsied. Collection techniques such as needle biopsies [11,13] or liposuction [14], are often well tolerated and easy to perform, but the overall yields of tissue can be low. In addition, the blinded nature of the sampling increases the risk of contamination of biopsied fat with unwanted tissues such as connective tissue and muscle, especially in subjects with low subcutaneous fat volumes, such as those seen in patients with lipoatrophy. Open biopsy techniques [7,10] (Figure 2) have the potential advantage of yielding larger amounts of tissue, with operators being able to carefully biopsy adipose tissue, thus limiting contamination. However, these procedures are arguably more invasive and complicated than needle-based procedures.

**RNA extraction**

Although isolation reagents based on phenol and guanidine isothiocyanate, such as TRIZol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), are commonly used to fix tissues, avoiding RNA degradation and maintaining RNA expression as close as possible is crucial. A flowchart illustrates the process of adipose tissue preparation for use in gene expression experiments.

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**Figure 1. Processing of adipose tissue for use in gene expression experiments**

1. **Adipose tissue**
2. Fix in TRIZol, snap freeze and store at −70°C
3. Homogenize (100 mg tissue per ml TRIZol)
4. Extract RNA, DNase treatment, purify and store in 200 ng aliquots
5. **4 X RNA (200 ng)**
6. **4 X RT (oligo dT)**
7. **4 X cDNA (20 µl)**
8. Check PCR (β-actin)
9. **Low expression = fail (discard aliquots)**
10. **β-actin**
11. **Gene of interest**
12. **2 µl**
13. **PCR (duplicates)**
14. **cDNA pool (80 µl)**
15. **Results = gene of interest**
16. **β-actin**

RT, reverse transcriptase.
possible to that seen in vivo at the time of sample collection, high lipid levels within the target tissue (such as seen in adipose tissue) can inhibit the chloroform/ethanol extraction process and reduce the yield of RNA obtained using this method. In the case of human adipose tissue using the TRIzol extraction protocol, RNA yields tend not to increase if more than 400 mg adipose tissue is fixed per ml of TRIzol (Figure 3), presumably because of the inhibitory effects of higher lipid concentrations on the reagent and subsequent precipitation process.

Problems with RNA yield are not limited to adipose tissue. We have evaluated monocytes as a potential peripheral blood surrogate for mitochondrial toxicity [7]. As monocytes make up only a fraction of the total cell population in whole blood, we found the RNA yield from extracted monocytes also to be relatively low – in the region of 2 µg per monocyte sample. In monocytes, the RNA yield from extracted samples significantly correlated with the number of monocytes isolated from the original whole blood sample (Figure 4). It is therefore important to ensure that the volume of blood collected is enough to ensure an adequate number of monocytes to give an appropriate RNA yield. In the majority of cases, more than 3 million monocytes [extracted from 18 ml blood in acid citrate dextrose (ACD) buffer] provide in excess of 2 µg total RNA, enough to examine the expression of more than 10 genes of interest.

RNA and cDNA preparation

For real-time quantitative PCR, it is important that the starting RNA is of the highest purity. As a minimum, RNA samples should undergo DNase treatment and column purification. Contamination with genomic DNA can give false positive results, especially for genes that are expressed at very low levels, whilst contamination with ethanol-based buffers used in RNA extraction can inhibit subsequent PCR reactions [16]. After RNA extraction, it is usual to store RNA at −70°C for use in future PCR reactions or gene arrays. As RNA is

**Figure 2. Open subcutaneous adipose tissue biopsy**

The biopsy is taken from the lateral aspect of the buttock. The procedure is performed in an ambulatory setting under local anesthetic.

**Figure 3. RNA yields from adipose tissue using TRIzol extraction protocol**

RNA yields begin to plateau if RNA is extracted from more than 400 mg adipose tissue per 1 ml TRIzol. This is probably due to inhibition of the reagent due to high lipid levels within the sample. RNA was extracted from both visceral and subcutaneous adipose tissue samples.

**Figure 4. Relationship between RNA yield and monocyte cell count**

RNA yields rise proportionally to the number of monocytes in the starting sample. Starting with at least 3 million cells (extracted from 18 ml ACD blood) will give a yield >2 µg RNA in more than 90% cases. ACD, acid citrate dextrose (buffer).
unstable and tends to degrade easily with every freeze-thaw episode, adequate storage of RNA is important. Storing RNA at pre-determined concentrations (either ng or µg aliquots, depending on whether the planned investigations include PCR or gene array) avoids multiple freeze-thaws. Ideally, laboratory protocols should be developed to limit the number of freeze-thaw events between RNA extraction and cDNA preparation to only one.

Various methods are used to quantify extracted RNA, the commonest of which are absorbance assays [17] and fluorescent nucleic acid stains [7,13]. Spectrophotometers measure the ability of RNA to absorb ultraviolet light. Maximum absorption occurs at 260 nm and the absorption can be used to calculate the concentration of RNA within a sample. Due to the potential for contaminants such as protein and DNA to affect absorbance, this method is relatively insensitive, especially when measuring low concentrations of RNA, such as those extracted from adipose tissue (for example, an A260 of only 0.1 represents 4 µg/ml RNA). Fluorescent nucleic acid stains, such as SYBR® Green (Applied Biosystems, Foster City, CA, USA) [7] and RiboGreen® (Molecular Probes, Eugene, OR, USA) [13], rely on the use of standard curves constructed from serial dilutions of RNA of a known quantity. This method is more specific for RNA and is accurate at measuring even small quantities.

First strand complementary DNA (cDNA), used in real-time PCR experiments, can be prepared from RNA using oligo dT [7,13] or random hexamer primers [17] and the reverse transcriptase (RT) enzyme. The reaction involves the RT enzyme elongating single strands of DNA from a site where the primer anneals to the RNA, using the RNA as a template. As their name suggests, random hexamer primers comprise short lengths of random nucleic acids capable of annealing to all types of RNA, while oligo dT primers are designed to specifically anneal to the poly-A tails of mRNA. For this reason, there are differences in the cDNA resulting from the use of these two primers. RT using oligo dT should result in transcription to cDNA of only transcribed genes (mRNA) and some ribosomal RNA (rRNA), whilst use of random hexamers results in the additional transcription to cDNA of non-coding RNA, transfer RNA (tRNA) and most rRNA. The presence of cDNA transcribed from non-messenger RNA introduces the risk of interference by these molecules in any subsequent PCR reaction, increasing non-specific annealing of PCR primers and the potential for false positive results.

Regardless of the primers used, the efficiency of the RT reaction can vary considerably, both between samples and between reactions. If equal amounts of the same RNA sample undergo RT, the yield of cDNA, as measured by β-actin expression, can vary by more than 60% (Figure 5A). The variability introduced by the RT step can significantly affect results when RT is combined with PCR in a single-step RT-PCR reaction [16]. If efficiency of a particular RT reaction is low, it may be difficult to measure the expression of a gene of interest, such as a transcription factor, that is normally expressed at much lower levels than β-actin; sometimes greater than 100-fold lower for genes such as those encoding PPARγ co-activator 1 or the uncoupling proteins UCP1 and 2 [7,13]. Separating the RT step from the PCR reaction, performing multiple RT reactions on an individual sample, testing the efficiency of the RT by examining the expression of a gene known to be ubiquitously expressed in the target tissue (such as β-actin) and pooling the multiple cDNAs to establish a ’pool’ of adequately transcribed cDNA can help overcome much of this type of variability (Figure 5B). In this way, a large amount of cDNA of adequate quality can be made from which expression of multiple genes of interest can be determined (Figure 1).

Analysis

Given the variability introduced by, among other things, RNA extraction and RT techniques, careful interpretation of results is required. Although various corrections can be made along the way to attempt to adjust for within-sample variation, such as quantification of and correction for starting RNA or cDNA concentrations, these analyses are relatively insensitive when using nanogram quantities for real-time PCR assays. As such, it is important to perform a final correction prior to presentation of data – the correction for the housekeeping gene.

The principal is simple. Find a gene that is ubiquitously expressed in the tissue of interest and which isn’t affected by the intervention under investigation, and present any changes in a gene of interest relative to changes in the housekeeping gene. By doing so, an investigator can say with some confidence that the intervention under investigation is the cause of the change in expression of the gene of interest, rather than other potential confounders such as insensitive quantification of RNA, failed reverse transcription or interference in cellular gene expression introduced by environmental or laboratory factors.

This argument for use of a housekeeping gene carries special significance in cross-sectional human studies that measure changes in gene expression in disease states. For example, in studies in HIV-positive populations, confounders such as age, weight, cortisol and insulin responsiveness and the effects of HIV, antiretroviral therapy and changes in body composition all have the potential to individually alter cellular gene expression.
Figure 5. (A) Intra-sample variation in reverse transcriptase and (B) post-pooling gene expression

(A) shows the considerable inter-RT variability (measured by expression of β-actin in the cDNA) when equal amounts of starting RNA from the same sample undergo RT. The lower limit of this assay is $1 \times 10^8$ ng/µl. Therefore, if the ‘check RT’ result falls below $1 \times 10^6$ ng/µl (dotted line) then the sample would be unsuitable for measuring expression of genes such as transcription factors, that are expressed at much lower quantities than β-actin and should be considered to have ‘failed’ the RT. (B) shows duplicate results for β-actin expression after discarding the failed samples and pooling the remaining cDNA. Much of the intra-sample variability is removed and a large amount of good quality cDNA remains from which expression of multiple genes of interest can be measured. cv, coefficient of variation; ID, identification; RT, reverse transcriptase.
Even exercise can significantly alter the expression of some commonly used housekeeping genes [22]. As a result, some authors recommend that, due to inherent changes in gene expression between systems, use of two [23] or even multiple [24] housekeeping genes should be employed in any analysis of such studies. In such diverse populations as those seen in studies of lipodystrophy, identification of a true housekeeping gene can be difficult and any results presented without correcting for expression of a housekeeping gene need to be interpreted with caution.

Various housekeeping genes have been used in human studies of gene expression. These include genes encoding β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β2-microglobulin and 18S ribosomal RNA [7,13,14,17]. An in-depth knowledge of the molecular characteristics of the tissue under investigation, including the likely effect of any intervention on the expression of a housekeeping gene and the laboratory processes used, helps in the choice of an appropriate housekeeping gene. For example, RT using oligo dT primers, although more specific in transcribing mRNA, may not transcribe rRNA as efficiently. As a result, most studies using 18S rRNA as a control use random hexamers to prepare cDNA. In addition, as HIVLD arises as a result of disturbances in adipose tissue lipid metabolism, use of genes encoding components of the glycolytic pathway, such as GAPDH, may be inappropriate as it is likely that their expression will be affected by disturbances in lipid metabolism.

To illustrate these difficulties, we performed a cross-sectional study examining the expression of the transcription factor SREBP1 in monocytes extracted from whole blood, as previously described [7]. We examined monocyte gene expression using real-time PCR (Lightcycler, Roche Applied Science, Mannheim, Germany) from samples of pooled cDNA synthesized from individual starting quantities of 20 ng RNA using oligo dT primers. Samples from a total of 53 subjects were analysed: 39 HIV-infected subjects with lipodystrophy and 14 HIV-negative healthy controls. Initial, uncontrolled analysis showed a significant decrease in SREBP1 expression in those subjects with HIVLD (Figure 6A), which would support a drug-induced down-regulation of SREBP1 in monocytes similar to that observed in fat. However, β-actin expression was also significantly down-regulated in those with HIVLD compared with healthy controls (Figure 6B). When the SREBP1 results were reanalysed correcting for β-actin expression, no significant difference in SREBP1 expression was detected between the two groups (Figure 6C). Similar results were found when GAPDH was used as an alternative housekeeping gene (data not shown). As such, these results tell us very little about the relationship between HIVLD and gene expression in monocytes.

HIVLD, HIV-associated lipodystrophy; SREBP1, sterol-regulatory element binding protein 1.
as it is not possible to control for the inherent differences in gene expression between the two diverse populations studied using these housekeeping genes. This is a limitation seen in many cross-sectional studies and underlies the need for appropriate study design when examining tissue gene expression in human disease.

**Population selection and study design**

Cross-sectional human studies of gene expression in lipodystrophy, especially those involving small numbers of subjects, are prone to significant bias introduced by the many potential confounding factors described above. To overcome these difficulties in a cross-sectional setting, studies need to be carefully and adequately powered for expression of each gene of interest before comments relating to the clinical or biological relevance of negative results can be made.

The target tissue under examination can also differ significantly in structure and cellular content between cross-sectional groups. In the case of subcutaneous adipose tissue, tissue biopsies from patients affected by HIVLD have been shown to differ at a microscopic level from HIV-negative control subjects, with smaller adipocytes and infiltrates of inflammatory cells observed in some diseased adipose tissue [10,14]. Although, as previously described, adequate sampling can help reduce contamination by unwanted tissue, the potential influence of tissue infiltration by cells other than adipocytes on gene expression within adipose tissue needs to be carefully considered when interpreting the results of cross-sectional studies.

Prospective studies using well-defined populations can overcome many of these difficulties. As many antiretroviral drug (ARV) toxicities originate at the molecular level, changes in expression of biologically relevant genes would be expected to occur early after exposure to ARVs. By examining changes in gene expression induced by ARVs in a prospective randomized setting, environmental and disease-related factors may have less of an impact on the overall results, as any potential error introduced by these confounders would be present in both baseline and post-intervention samples, provided that the interval between sampling is relatively close. At present, the number of such studies is relatively small, but is increasing.

**Limitations**

Molecular studies on human tissue offer the ability to demonstrate clinically relevant changes in gene expression due to ARVs. However, this approach has several important limitations. As previously mentioned, many molecular pathways involved in HIVLD are under complex regulatory control and, given the many variables HIV introduces, it can be difficult to determine definite cause and effect relationships between exposure to ARVs and subsequent effects on gene expression in vivo. In addition, when expression of multiple genes changes in response to ARV exposure [7,13,14,25], it is almost impossible to elicit an exact sequence of events, that is, which changes arise directly from antiretroviral drug effects and which arise as a result of dysregulation induced by changes in other genes, without very detailed and sample-intensive time-course studies. Human studies involving multiple biopsies, although ideal in principle, would be both difficult to recruit to and ethically challenging. For these reasons, in vitro experiments remain a vital resource for robustly testing the many hypotheses that the limited in vivo gene expression studies generate.

**Summary**

Molecular studies of lipodystrophy in populations of HIV-infected subjects have the potential to reveal aspects of the syndrome that may help prevent the syndrome occurring and aid in the development of new, safer therapies. Effective research in this field requires an effective communication between clinical and laboratory researchers to enable them to design and implement clinical trials and laboratory assays capable of overcoming the many pitfalls inherent in this type of research. Further research is vital, particularly using newer technologies such as microarray that are able to characterize genomewide responses to exposure to ARVs, if the goal of safe, lifelong ARV therapy for HIV is to be realized.

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The role of hepatitis C virus (HCV) in mitochondrial DNA damage in HIV/HCV-coinfected individuals

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Oxidative stress accompanying hepatitis C virus (HCV) infection seems to result in mitochondrial (mt) dysfunction. In HIV/HCV-coinfected individuals, HCV-related mt damage could be further enhanced and clinical manifestations of mt damage may appear, particularly following exposure to some antiretroviral drugs. Furthermore, when HCV medications are used together with certain antiretrovirals, the risk of developing mt adverse events may be particularly frequent, such as development of pancreatitis when ribavirin and didanosine are coadministered. The management of HIV/HCV-coinfected individuals needs to consider the high risk of mitochondria-associated toxicities in this population, which may significantly influence treatment decisions and therapeutic modalities.

Introduction

Around 200 million people are infected with hepatitis C virus (HCV) worldwide. The prevalence of HCV infection among HIV-positive individuals varies widely and mainly depends on the risk category. In regions such as southern Europe, the east coast of North America and South East Asia, where intravenous drug users represent a large proportion of HIV-infected individuals, the rate of HIV/HCV co-infection is extremely high [1–3].

The involvement of antiretroviral therapy (ART) drugs in mitochondrial (mt) damage, especially nucleoside analogues, has been highlighted in recent years [4–6]. This is clear even when taking into consideration that HIV infection itself may cause mtDNA depletion in at least some cell compartments such as peripheral blood mononuclear cells (PBMCs) [7–9]. On the other hand, some co-morbidities may be particularly prone to mt damage in this population. There is no doubt that ART-related complications such as lipoatrophy and hyperlactataemia are more frequent among individuals co-infected with HCV [10,11].

In this review, we have tried to summarize the information available regarding the role of HCV in mt damage, especially in the setting of HIV infection. The involvement of HCV drugs and their interactions with antiretrovirals leading to further mt dysfunction in HIV/HCV-coinfected patients will be discussed further.

HCV and mt dysfunction — aetiological and pathophysiological aspects

Despite recent advances in the pathogenesis of HCV-related liver disease, many questions remain unanswered. There are many challenges to reproducing in vitro HCV results due to difficulties in producing HCV in viral cultures. For this reason, it is still unclear how HCV persists and replicates in hepatocytes or how it produces cellular injury. Ultrastructural studies have shown that mt abnormalities are common in liver biopsies from HCV-infected patients [12]. Mitochondrial alterations due to enhanced oxidative stress caused by HCV infection are associated with a depletion of the tissue glutathione store, which is a key cellular antioxidant [13], and with a reduction in the mtDNA/nuclear DNA ratio.

In the light of various findings, Rust & Gores [14] have suggested that HCV could induce mt dysfunction by the following mechanisms: 1) direct effect of viral proteins on mitochondria; 2) inducing a persistent intracellular oxidative stress, which may lead to secondary mt dysfunction; 3) immune-mediated activation of the cell death pathways, such as the Fas system; and 4) stimulation of inflammatory cells that could target mitochondria through further oxidative stress (see Figure 1).

Oxidative injury may occur as a direct result of the HCV core protein expression both in vitro and in vivo [15] and may involve a direct effect of the HCV core protein on mitochondria. HCV replication might also lead to an increase in intracellular oxidant production, damaging the mitochondria of infected cells. Accordingly, reactive oxygen species have been associated with disease activity in chronic hepatitis C [16].

In relation to the apoptotic pathways, similar assertions have been made to explain why mtDNA depletion
is seen in drug-naive, HIV-infected individuals and subjects with chronic HCV infection alone [7–9]. Briefly, mtDNA depletion in drug-naive, HIV-infected individuals seems to be related to cell apoptosis [17,18]. HIV-1 infection may cause apoptosis through different mechanisms, some of which rely on the intricate virus-host cell interaction while others involve activation of the host's inflammatory and immune responses. Soluble HIV-1 products, such as the accessory proteins Tat, Vpr and gp120, directly promote cell death through interactions with CD4, CXCR4 and other uncharacterized receptors [19] or by induction of the caspase cascade [20]. By similar mechanisms, immune-mediated apoptotic pathways are activated in chronic HCV infection. Expression of the death receptor Fas is significantly higher in HCV core antigen-positive hepatocytes compared with uninfected cells [21]. Fas expression promotes the formation of a death complex, which activates the caspase-8 cascade, which eventually leads to apoptotic cell death [22]. Likewise, a recent study has shown that expression of the HCV NS3 protein, or the NS2/NS3 precursor protein, results in induction of apoptosis and activation of the caspase-8 cascade [23].

Chronic HCV infection is characterized by inflammatory liver damage and is associated with a significant risk of liver cirrhosis and hepatocellular carcinoma in the long term. There is increasing evidence suggesting that liver cell damage in chronic hepatitis C is mediated by the induction of apoptosis [24]. Indeed, a marked increase in caspase activity is noticed in the sera of HCV-infected patients [25].
Although one quarter of individuals with chronic HCV infection may show persistently normal serum alanine aminotransferase (ALT) levels, inflammatory and fibrotic lesions in the liver may appear in more than half of these patients, which in turn exhibit elevated serum caspase activity. Moreover, 30% of patients with normal ALT levels but elevated caspase activity show a significant fibrosis (F2–F4) in the liver biopsy. This is why some authors have claimed that measurement of caspase activity in serum might be a useful indirect tool for assessing liver damage in chronic hepatitis C, particularly in patients with normal ALT levels [25,26].

Although the association between HCV and mt damage seems to be clear, it remains unclear how HCV could cause mt dysfunction. An increased production of free radicals by virus-related inflammation is most probably the main cause of HCV-associated mt damage [27].

Clinical manifestations of mt damage in HIV/HCV coinfection

The first mention of mt dysfunction in HIV infection appeared in the early 1990s, when several reports linked zidovudine (AZT)-associated myopathy with mt damage [28–30]. Since Brinkman proposed his theory in 1998 [31], mt damage in HIV infection has been directly related to nucleoside reverse transcriptase inhibitor (NRTI) use. However, there is evidence suggesting that HIV infection itself may deplete mtDNA in some cell compartments [7,8], causing clinical symptoms in the absence of any ART [32].

Mitochondrial dysfunction in HIV-infected individuals may be enhanced by the presence of HCV coinfection. Lipodystrophy, pancreatitis, peripheral neuropathy, myopathy, hyperlactataemia and lactic acidosis are the most important adverse events of ART due to mt damage [33]. HCV infection seems to increase the incidence of some of these events. In a transverse study conducted at our institution, coinfection with HCV and/or hepatitis B virus (HBV) was associated with a higher decrease in the mtDNA content of PBMCs than in patients infected with HIV alone [8]. A more detailed investigation showed that HCV infection itself led to a significant depletion of mtDNA in PBMCs, which was more pronounced in patients who underwent treatment with pegylated interferon plus ribavirin (RBV) for chronic hepatitis C [34] (Figure 2).

Lipoatrophy

In a cohort of 226 HIV-infected patients, HCV infection was significantly more frequent in lipoatrophic patients (46%) than in subjects with adiposity or the mixed syndrome (15%). It was also more prevalent than in patients without lipodystrophy (20%) [11]. Thus, HCV infection may be associated with the atrophic form of lipodystrophy in HIV-infected patients, especially after extended periods of exposure to nucleoside analogues.

Hyperlactataemia and lactic acidosis

Mitochondrial toxicity causing lactic acidosis is a rare but fatal complication, which has been described in some HIV-infected patients exposed to nucleoside analogues. It has been reported in individuals receiving both single and dual nucleoside combinations, including AZT or stavudine (d4T) with didanosine (ddI), zalcitabine (ddC) or lamivudine. The overall prevalence is in the range of five per 1000 patients on therapy per year [35]. The risk of lactic acidosis is somewhat more common in women, the obese and those with viral hepatitis coinfections [30]. Analysing the drugs in use, the combination of d4T plus ddI is associated with the highest risk [8,35–37]. In a retrospective study performed in France, nine cases of severe lactic acidosis were identified [38]. The incidence was 0.9/1000 patient-years of exposure to nucleoside analogues. Six patients were coinfected by HCV and/or HBV. More recently, a case of lactic acidosis in an HIV-infected patient receiving ART after liver transplantation for HCV-induced liver disease has been reported [39]. All these observations support HCV increasing the incidence of mitochondria-related complications in the setting of HIV infection.
Liver-related mt damage

Grade 3 or 4 liver enzyme elevations occur on average in 5–10% of patients who initiate triple ART [40]. The rate is significantly higher in HIV-infected individuals with underlying chronic hepatitis C [41–45].

Four main mechanisms of drug-related liver toxicity have been recognized in HIV-infected individuals exposed to ART: i) direct drug toxicity, ii) immune reconstitution following initiation of highly active antiretroviral therapy (HAART) in the presence of HCV and/or HBV coinfections, iii) mt toxicity and iv) hypersensitivity reactions with liver involvement. Chronic hepatitis C is known to increase the toxic effects of drugs in the liver, most probably by impairing the mechanisms of cytoprotection in liver cells [46]. This is why exposure to drugs like ritonavir at full doses (600 mg twice daily) is associated with a high risk of hepatotoxicity [43].

Immune reconstitution may explain some episodes of liver enzyme elevations following the initiation of HAART, particularly in severely immunosuppressed patients with chronic hepatitis B or C [47–49]. The rationale behind this phenomenon is that the inhibition of HIV replication with HAART leads to rapid immune recovery and, consequently, to an immune response against HBV and/or HCV antigens exposed in the surface of hepatocytes, which are destroyed [50]. Mitochondrial toxicity of some nucleoside analogues is a well-known cause of liver damage [51]. As we have discussed previously, exposure to HIV nucleoside inhibitors (particularly ddI and/or d4T), female sex and obesity are some of the predisposing factors, and underlying chronic HCV infection may increase the chance of this complication [46,52–55]. Finally, hypersensitivity reactions to drugs such as nevirapine, abacavir or amprenavir may involve multiple organs including the liver. In this situation, liver enzyme elevations occur within the first days or weeks following the initiation of HAART and tend to resolve upon drug discontinuation. As expected, allergic reactions may be more frequent in patients with high CD4 counts and do not seem to be favoured by the presence of HCV coinfection [56].

HCV may induce mt alterations in the liver of patients with hepatocellular carcinoma [57,58]. Similar ultrastructural liver mt abnormalities were observed in a group of 30 HIV/HCV-coinfected patients under HAART [59]. The main ultrastructural abnormalities of mitochondria were reduction or loss of cristae, decrease in matrix density and glycogen accumulation.

There is evidence of mtDNA depletion in the liver of coinfected individuals in association with the use of nucleoside analogues. For instance, the liver mtDNA content was decreased by 47% in HIV/HCV-coinfected individuals taking d4T, ddL or ddC compared with subjects never exposed to these NRTIs [60]. However, the authors of this study did not observe a clear association between increases in serum lactate levels and the extent of liver mtDNA depletion. Lactate elevations may occur only in the presence of significant reductions in the hepatic mtDNA content.

Finally, a higher risk of metabolic abnormalities, including insulin resistance and changes in body composition, have been reported in HIV/HCV-coinfected individuals receiving ART, compared with HIV-monoinfected individuals exposed to the same medications [10,61–64].

Interactions between HCV medications and antiretroviral drugs

The combination of pegylated interferon plus RBV, a guanosine nucleoside analogue, is the current recommended therapy for chronic hepatitis C in HIV/HCV-coinfected individuals [65]. Sustained virological response to this combination is around 50% in HCV-monoinfected individuals [60] but much lower in HIV/HCV-coinfected subjects [67,68]. Besides an intrinsically lower efficacy of HCV medication in the HIV setting, the co-administration of HCV medication and antiretroviral drugs has resulted in a substantial number of side effects that often lead to premature drug discontinuation, precluding the achievement of response to HCV therapy. Among the most important drug interactions are those of RBV with ddI and d4T, whose pathogenic mechanism relies on an enhancement of mt toxicity (Figure 3).

HIV nucleoside analogues require intracellular phosphorylation to become active. The deoxyribonucleoside kinases are located in the cell cytosol as well as within the mitochondrion. The mechanism of inhibition of phosphorylated nucleoside analogues is by inhibitory competition with the natural nucleoside for

![Figure 3. Interactions between RBV and nucleoside analogues](https://example.com/figure3.png)

AZT, zidovudine; ddL, didanosine; d4T, stavudine; RBV, ribavirin.
binding to the HIV reverse transcriptase, ultimately leading to premature DNA chain termination. Nucleoside analogues are also substrates of DNA polymerase γ, the enzyme responsible for mtDNA replication. This affinity explains the depletion and/or mutation of genes encoded by mtDNA in subjects exposed to these drugs [31,69]. The final consequence of mt dysfunction is a reduction in ATP production and the release of reactive oxygen radicals, which ultimately affect the mitochondrial structure. Of note, RBV is also a nucleoside analogue and might cause mt damage by the same mechanism as the HIV inhibitors, although their affinity for polymerase γ has not yet been determined.

The concomitant use of ddl and RBV is particularly risky as it favours the development of pancreatitis [61,62,70]. RBV inhibits inosine monophosphate (IMP) dehydrogenase, promoting the phosphorylation of ddl [71]. This effect leads to an increase in intracellular and also mt concentrations of ddATP, which inhibits DNA polymerase γ (Figure 4) [72]. Regarding a different mechanism, a recent report has highlighted a potential synergistic interaction between d4T and RBV, leading to a rapid and severe weight loss in HIV-infected patients receiving both d4T and/or ddl along with HCV medications [10]. Interestingly, more pronounced weight loss, and lactate and amylase elevations were found in patients taking d4T or ddl compared with those taking other nucleoside analogues along with RBV.

**Figure 4.** Metabolic pathways involved in the potentiation of ddl by RBV. The increased concentrations of ddATP due to the inhibition of the IMP dehydrogenase by RBV inhibits the DNA polymerase γ.

<table>
<thead>
<tr>
<th>RBV → IMP</th>
<th>Physiological nucleosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMP → ddl</td>
<td>ddIM + ddAMP + ddADP</td>
</tr>
<tr>
<td>HIV-RT</td>
<td>DNA synthesis</td>
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**Summary**

HCV infection seems to be associated with mt alterations either causing it directly or in the context of indirect induction of cell death mediated by oxidative stress. In HIV/HCV coinfection, mt damage could be further enhanced and clinical manifestations may become more apparent when using certain antiretroviral drugs and when HCV medication is used with these antiretrovirals. The management of HIV/HCV-coinfected individuals needs to consider the high risk of mitochondria-related toxicities in this population, which may influence treatment decisions and therapeutic modalities significantly. Given the deleterious influence of HCV on HIV, efforts to eradicate hepatitis C with specific therapy will be of great value in the coinfected population.

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1st Meeting on Mitochondrial Toxicity & HIV Infection: Understanding the Pathogenesis for a Therapeutic Approach
Uridine in the prevention and treatment of NRTI-related mitochondrial toxicity

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Long-term side effects of antiretroviral therapy are attributed to the mitochondrial (mt) toxicity of nucleoside analogue reverse transcriptase inhibitors (NRTIs) and their ability to deplete mtDNA. Studies in hepatocytes suggest that uridine is able to prevent and treat mtDNA depletion by pyrimidine NRTIs (zalcitabine (ddC) and stavudine (d4T)) and to fully abrogate hepatocyte death, elevated lactate production and intracellular steatosis. Uridine was also found to improve the liver and haematopoietic toxicities of zidovudine (AZT), which are unrelated to mtDNA depletion, and to prevent neuronal cell death induced by ddC. Most recently, uridine was found to prevent the onset of a lipoatrophic phenotype (reduced intracellular lipids, increased apoptosis, mtDNA depletion and mt depolarization) in adipocytes incubated long-term with d4T and AZT. Various steps of mt nucleoside utilization may be involved in the protective effect, but competition of uridine metabolites with NRTIs at polymerase γ or other enzymes is a plausible explanation. Pharmacokinetic studies suggest that uridine serum levels can be safely increased in vitro (50–200 µM). Uridine was not found to interfere with the antiretroviral activity of NRTIs. Mitocnol, a sugar cane extract which effectively increases uridine in human serum, was beneficial in individual HIV patients with mt toxicity and is now being tested in placebo-controlled randomized trials. Until these data become available, the risk–benefit calculation of using uridine should be individualized. The current safety data justify the closely monitored use of uridine in individuals who suffer from mt toxicity but who cannot be switched to less toxic NRTIs.

Introduction

More than 8 years after the widespread introduction of highly active antiretroviral therapy (HAART), it has become clear that antiretroviral drugs have long-term effects on organs and body metabolism. Nucleoside reverse transcriptase inhibitors (NRTIs) within the antiretroviral cocktail are associated with hyperlactataemia and organ toxicities such as damage to the liver, peripheral nerves and skeletal muscle. The choice of NRTI also determines an individual’s risk of developing lipoatrophy, a clinically irreversible loss of subcutaneous tissue. The main mechanism of these NRTI-related side effects has been identified as mitochondrial (mt) toxicity [1–7].

Pathogenesis of NRTI-related mt toxicity

NRTIs are activated by phosphorylation and then they inhibit polymerase γ, the enzyme which replicates mtDNA [3,8]. Polymerase γ inhibition is a result of several distinct steps [3]. The first step involves competition of NRTI triphosphates with the natural nucleoside triphosphates. If this competition is successful, the NRTIs are incorporated into the nascent mtDNA strand. This second step causes chain termination. As a result of polymerase γ impairment, mtDNA depletion (a quantitative reduction of the mtDNA copy number) ensues. The relative potency of activated nucleoside triphosphates to inhibit polymerase γ is not the same among all NRTIs. In vitro data indicate a relatively strong inhibitory effect of the ‘d-drugs’, that is, zalcitabine (ddC), didanosine (ddI) and stavudine (d4T), whereas abacavir, emtricitabine, lamivudine and tenofovir do not impair mtDNA replication in clinically relevant concentrations [3,8,9].

Zidovudine (AZT) is a special case because this NRTI is a mt toxin despite the fact that AZT triphosphate only has a low potency to affect polymerase γ and mtDNA content in clinically relevant and cytotoxic concentrations, at least in proliferating cells [8–11]. On one hand, the mt toxicity of AZT may, in part, involve binding to adenylate kinase (an enzyme involved in ATP formation) and inhibition of the mt ADP/ATP translocator [12–14]. These mechanisms may explain why some toxicities have been observed relatively early after AZT exposure [9,13]. On the other hand, mtDNA depletion has indeed been
observed with AZT in vivo [6,15–17]. Two observations may explain why mtDNA depletion may also occur in the absence of direct polymerase γ inhibition. Firstly, it has been shown in vivo that some of the administered AZT can be non-enzymatically converted into d4T, and thus a stronger polymerase γ inhibitor [18]. Secondly, mtDNA depletion may result from a another mechanism, namely from AZT-mediated inhibition of thymidine kinase (TK) type 2 [19]. This TK is expressed in mitochondria and responsible for the intramitochondrial phosphorylation of pyrimidine nucleosides (deoxythymidine, deoxyctydine and deoxyuridine). In non-replicating cells, the cytosolic TK type 1 (TK1) is down-regulated, making the pyrimidine supply for mtDNA synthesis dependent on the activity of TK2. Such reduced supply of the normal deoxypyrimidine phosphates limits mtDNA replication, especially in skeletal muscle, as evidenced by a mt myopathy in subjects carrying TK2 mutations [20].

As mtDNA encodes for subunits of the mt respiratory chain, mtDNA depletion therefore results in respiratory chain dysfunction. Any respiratory chain dysfunction may promote electron leakage in the mt matrix and thus the generation of reactive oxygen species (ROS). Such increased ROS formation may then in turn damage the lipid architecture of the mt membrane, attack respiratory chain proteins or damage polymerase γ and mtDNA itself, thereby closing several vicious circles that promote even more ROS formation [21,22]. There is also evidence for additional mechanisms of ROS formation [1]. Markers of oxidative damage and heteroplasmic mtDNA point mutations have indeed been shown to increase in patients treated with NRTIs [23,24].

Respiratory chain dysfunction also leads to the secondary impairment of several metabolic pathways. Firstly, ATP can no longer be synthesized efficiently through oxidative phosphorylation and glycolysis has to be relied upon. Secondly, the block of NADH utilization in the respiratory chain increases the intracellular NADH/NAD + ratio. This alteration of the redox status promotes the conversion of pyruvate to lactate and inhibits key enzymes of beta oxidation, resulting in the intracellular accumulation of triglycerides [25].

The mt respiration also has a third important task: an efficient electron-flux through the respiratory chain is essential for the activity of dihydroorotate-dehydrogenase (DHODH; E.C. 1.3.99.11), an enzyme located in the inner mt membrane and necessary for the de novo synthesis of all (intramitochondrial and intracytoplasmic) pyrimidines [26]. This is because DHODH catalyses the oxidation of dihydroorotate to orotate from which uridine monophosphate (UMP) and intracellular pyrimidines are synthesized (Figure 1).

A defect in the respiratory chain therefore results in pyrimidine depletion. The indirect inhibition of DHODH by NRTI-related mt toxicity is likely to be similar to those caused by direct DHODH inhibitors [27]. Research into leflunomide [27,28], a direct DHODH inhibitor and a licensed immunosuppressive drug has taught us about the in vitro and in vivo consequences of DHODH inhibition (Figure 2). The depletion of UMP and derived intracellular pyrimidines activates p53 and its immediate transcriptional target p21 [27,29]. p53 also regulates the activation of Rb protein and thus of cyclins via phosphorylation [30]. Through this mechanism, the pyrimidine depletion inhibits the transition to the S-phase of the cell cycle and leads to a mitotic arrest in the G1 phase. p53 can also activate the transcription of Bax [31] and promote apoptosis. These molecular mechanisms may explain why cells with mtDNA depletion stop dividing and then die.

The importance of the intracellular pyrimidine pools for the survival of cells without a functional respiratory chain is supported by the fact that cells without a single molecule of mtDNA (rho0-cells) are rescued from cell death. The mt DNA depletion may result from a secondary impairment of several metabolic pathways. Firstly, ATP can no longer be synthesized efficiently through oxidative phosphorylation and glycolysis has to be relied upon. Secondly, the block of NADH utilization in the respiratory chain increases the intracellular NADH/NAD + ratio. This alteration of the redox status promotes the conversion of pyruvate to lactate and inhibits key enzymes of beta oxidation, resulting in the intracellular accumulation of triglycerides [25].

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The biosynthetic pathway starts with the formation of carbamoyl phosphate. DHODH (an enzyme which is inhibited by respiratory chain dysfunction in mt toxicity of NRTIs and by leflunomide) then catalyses the synthesis of orotate. Orotate is then anabolized to UMP, which can be used to produce RNA, DNA, glycosylation products or membrane constituents. Uridine can be salvaged into UMP by uridine kinase or degraded into beta-alanine, which enters the tricarboxylic acid cycle. Dashed arrows signify pathways involving intermediate metabolites. mt, mitochondrial; CDP, cytidine diphosphate; DHODH, dihydroorotate dehydrogenase; dCTP, deoxycytidine triphosphate; dTTP, deoxythymidine triphosphate; UDP, uridine diphosphate; UMP, uridine monophosphate; UTP, uridine triphosphate.

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**Figure 1. Simplified scheme of pyrimidine metabolism**
death and grow virtually normally if the intracellular pyrimidine pools are replenished by substances that can be salvaged into pyrimidines by being converted into UMP distal to DHODH. One such substance that can bypass the block in the de novo synthesis of pyrimidines is uridine [32].

Uridine abrogates mt toxicity in vitro

The relationship between respiratory chain dysfunction and pyrimidine metabolism makes uridine an attractive candidate to alleviate symptoms of NRTI-related mt toxicity. Early work has demonstrated that neuronal cells exposed to ddC are rescued from death and improve in proliferation and neurite outgrowth if the medium was supplemented with uridine (50 µM) [33]. Uridine in concentrations of 50 µM also completely reversed the haematopoietic toxicity of AZT (5 µM) on normal human granulocyte-macrophage progenitor cells [34]. Similar strategies in mouse models of AZT-induced bone marrow suppression reversed anaemia, leucopenia, increased peripheral reticulocytes and increased bone marrow cellularity [35]. The mechanism for the beneficial action of uridine on AZT is still unclear. As discussed above, AZT has many effects on cell metabolism [8,19]. It is conceivable that uridine or its derived pyrimidines may compete with AZT for one or several of these metabolic steps or, alternatively, for kinases or transporters responsible for the intramitochondrial presence of triphosphorylated AZT [8,34].

Investigations into a model of d-drug-related hepatotoxicity made the surprising discovery that uridine was not only able to prevent cell death (an expected finding), but also to prevent the onset of a severe mtDNA depletion and thereby normalize the synthesis of mtDNA-encoded respiratory chain subunits. This also normalized the rate of lactate production and the intracellular triglyceride content [36]. Importantly, uridine was only able to improve the mtDNA depletion caused by pyrimidine NRTIs, not that caused by purine analogues such as ddI.

The ability of uridine to antagonize the polymerase γ inhibition by pyrimidine d-drugs may be explained by its ability to disrupt the following vicious circle (Figure 3): as discussed above, polymerase γ inhibition involves competition of NRTIs with the natural nucleotides as a first step. mtDNA depletion, respiratory dysfunction, DHODH inhibition and pyrimidine depletion ensue. The decrease in intracellular pyrimidines most probably allows for a more efficient competition of the exogenous nucleoside analogue at polymerase γ. Thus, a vicious circle is closed and drives the cell into further mtDNA depletion. We hypothesize that this circle is disrupted by supplying uridine as an exogenous source of intracellular pyrimidines. The data also suggest that the ability of uridine to abrogate mt toxicities was proportional to the concentration of uridine [33,34,36], underlining the hypothesis of a competitive process. Alternatively, uridine may compete with antiretrovirals at steps of intracellular NRTI transport and phosphorylation.

Most recently, long-term exposure of adipocytes to d4T (10 µM), ddC (0.2 µM) or AZT (1 µM) was shown to induce a lipoatrophic phenotype consisting of apoptosis, loss of lipids, mtDNA depletion, loss of mtDNA-encoded respiratory chain subunits and disruption of the mt membrane potential [37]. The addition of uridine (200 µM) completely abrogated all these effects on adipocytes.
Notably, uridine was not only able to prevent the onset of mt toxicity but also to treat toxicities that were already established [35,36]. Interestingly, in the absence of uridine it took considerably longer for mtDNA depletion to develop (weeks), than it took for uridine to revert such mt toxicity (days) [36]. This relatively quick therapeutic effect of uridine relative to the more prolonged development of mt toxicities may allow for intermittent uridine dosing in order to ‘reset the mitochondrial clock’.

Metabolism, pharmacokinetics and safety of uridine in humans

Normal uridine concentrations range from 3–8 µM in human blood plasma, bone marrow and cerebrospinal fluid [38]. Although uridine is part of our everyday food, diet is not an important source of uridine [39,40]. Clinical studies and animal models suggest that uridine is mostly produced in the liver and that erythrocytes serve as carriers for distributing the uridine throughout the body [38]. Exogenous uridine rapidly disappears from plasma (t1/2=2 min), reflecting a concentrative and, under physiological conditions, unsaturated entry into tissue cells, as well as catabolism by the liver [41]. Subsequently, the tissue uridine pools turn over with half-lives of 13 to 18 h [41]. The physiological range of uridine in the human plasma was shown not to completely satisfy the pyrimidine requirements of dividing cells, making some de novo synthesis necessary for optimal proliferation [42]. Circulating uridine may nevertheless be of physiological importance by allowing dividing cells to utilize their salvage pathway [42].

Uridine has several metabolic fates in the cell (Figure 1). Exogenous uridine is rapidly incorporated into nucleotides in nucleated cells [43]. Uridine can be converted to dTTP and dCTP, which are used to produce DNA. UTP is used for the synthesis of RNA. UTP can also be converted into CTP, which upon conjugation of lipids forms cellular membrane constituents such as CDP ethanolamine. In the form of UDP sugars, uridine may help in the production of glycogen and in protein glycosylation. Uridine is degraded into beta-alanine, which can enter the tricarboxylic acid cycle (See Figure 1 for abbreviations) [38].

Pharmacokinetic and safety data for uridine were collected in several human Phase 1 and 2 trials. Parenteral administration of uridine as a 1 h infusion (8 g/m²) resulted in plasma levels in the millimolar range, far above those required to abrogate NRTI-related mt toxicity [44]. Half-life, volume of distribution (634 ml/kg) and total clearance (4.98 ml/kg/min) of uridine appear to be independent of dose, whereas Cmax and AUC increase with dose in a linear fashion [44]. In subjects given uridine at doses of 2–12 g/m² as a single 1 h infusion, about a fourth of the administered dose was excreted in the urine [44]. Uracil, a uridine catabolite, accounted for 3% of the uridine dose [44]. These intravenous doses were tolerated without side effects. However, transient fever, lasting for 15 min, occurred with higher doses [44,45]. Intermittent infusion schedules with 3 g/m²/h for 3 h, alternating with a 3-h treatment-free interval, resulted in plasma levels of 138–335 µM during the treatment-free period and were tolerable over the 72-h study period [45]. Parenteral uridine necessitates central venous administration due to the onset of phlebitis if given through a peripheral vein [45]. Uridine can also be administered orally and is generally tolerated without any side effects. However, excessive oral dosing (12 g/m²) is limited by mild and reversible osmotic diarrhoea due to the relatively poor bioavailability of uridine (7%) [46]. Using other pyrimidine precursors, for example, triacetyluridine [34,47,48] or inhibitors of uridine catabolism or excretion may also be envisaged [35,49,50].

Human uridine serum levels can now be effectively increased with mitocnol, a sugar cane extract with a high content (17%) of nucleosides [51]. 24-hour pharmacokinetic data indicate that consuming 36 g of a powder that contains mitocnol increases human uridine serum levels from baseline values (5.6 µM) to mean uridine serum concentrations (Cmax) of 152.0 µM [51]. Adverse events were not observed. It is recommended that three sachets of mitocnol are taken on three consecutive days per month, taking into account the relatively quick improvement of mt toxicity from in vitro studies.

In summary, the current data indicate that uridine concentrations that are protective in vitro can be safely achieved with oral and parenteral dosing. Oral uridine supplementation (150 mg/kg/d) is also recommended and has been safely used long-term in patients with hereditary orotic aciduria, an inborn error of pyrimidine de novo synthesis, in which uridine reverses mega-loblastic anaemia and other symptoms [52].

Interaction of uridine with antiretroviral nucleotides

If uridine or its metabolites are able to compete with NRTIs at the level of polymerase γ, they may also do so at the level of HIV reverse transcriptase (RT). This poses a theoretical risk for the antiretroviral efficacy of nucleoside analogues. The efficiency of RT inhibition is dependent on the ratio between the normal deoxynucleoside triphosphates and the NRTI triphosphates at the enzyme. For example, mycophenolate mofetil, an inhibitor of purine synthesis, depletes intracellular deoxyguanosine triphosphate and decreases plasma HIV-1 RNA in patients treated with the guanosine...
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analogue abacavir [53,54]. Uridine may thus theoretically have an opposite effect on RT by increasing the normal deoxypyrrimidine triphosphates.

Such an effect of uridine on the antiretroviral activity of pyrimidine analogues was first analysed with regard to AZT [34]. Phenotypic HIV resistance assays demonstrated that uridine did not interfere with viral suppression [34]. Importantly, uridine did not impair the antiretroviral activity even in a 10 000-fold molar excess, whereas the maximal therapeutic effects of uridine were already achieved with a 10-fold molar surplus. Investigations in mice also came to the same conclusion [35].

The potential interference of uridine with the antiretroviral activity of NRTIs was also extensively examined in phenotypic HIV resistance assays using nucleoside analogues alone and in combinations [55]. Both X-4 tropic and R-5 tropic HIV isolates were tested and three different detection systems including primary human peripheral blood mononuclear cells were used. Uridine was added in concentrations up to 615 µM. Additionally, these investigations no effect of uridine on NRTI-mediated viral suppression was detected. Enhancement of the normal intracellular pyrimidine stores therefore does not seem to have a crucial effect on HIV replication.

Taken together, the data suggest that the interaction between uridine and NRTIs in the prevention of mt damage does not necessarily imply a reduced antiretroviral efficacy. Explanations for this selectivity include a separate regulation of mt and cytoplasmic dNTP pools, either at the level of mt transport [56] or by the presence of disparate kinases in both compartments [57]. The differential action of uridine on the mt and antiretroviral replication enzymes may also be caused by differences between the polymerases in selecting the natural nucleotide over the activated NRTI.

Uridine in HIV-infected patients

The selective effect of exogenous uridine on NRTI-inhibited mtDNA replication, but not on NRTI antiretroviral action, implies that HIV-infected patients under treatment with pyrimidine NRTIs and suffering from mt toxicity may benefit from strategies aimed at increasing uridine. Mitocnol was used in an HIV patient with progressive hyperlactataemia, mt steatohepatitis and symptomatic elevation of creatine kinase (CK) under long-term antiretroviral treatment with d4T [58]. The patient was started on mitocnol (three sachets/day for four consecutive days). After 2 weeks, at his next visit, liver and muscle enzymes, as well as the myalgias had improved rapidly, despite unchanged medication. Lactate had normalized after 7 weeks and HIV replication remained below the limit of detection. d4T was then switched to tenofovir with no subsequent clinical or laboratory abnormalities.

Mitocnol is now widely used in Germany. Several and in-part randomized and placebo-controlled clinical trials are currently being conducted to formally analyse whether mitocnol is able to prevent and treat mt toxicities such as lipoatrophy, polyneuropathy, hepatic steatosis and myopathy. Virological failure has not been reported (UA Walker, personal communication).

Perspective

The issues discussed above have several further implications. Uridine supplementation may be used to enhance the therapeutic index of pyrimidine NRTIs and thus allow higher dosing to overcome multidrug resistance in salvage therapy. The available data also suggest the possibility of mtDNA depletion in blood [17,59,60]. If the detected mtDNA depletion in blood secondary to pyrimidine NRTIs indeed also reflects reduced mtDNA copy numbers in lymphocytes and if it exceeded a certain threshold, it would have effects similar to those of the direct DHODH inhibitor leflunomide and of inherited defects in pyrimidine synthesis. From the clinical experience with leflunomide as a licensed immunosuppressive antirheumatic drug, it could then be predicted that the mt toxicity in lymphocytes impairs the proliferation of lymphocytes in response to mitotic stimuli, interferes with CD4 recovery and thus is immunosuppressive. Impaired cell-mediated immune responses and reduced CD4 and CD8 lymphocyte subsets were also observed in several patients with an inherited defect in the de novo synthesis of pyrimidines; their immunodeficiency improved upon uridine therapy [52,61]. It was also shown that uridine antagonized the inhibition of lymphocytes by leflunomide [62,63]. Most recent in vitro and in vivo observations in HIV patients also support the view of mt toxicity as being immunosuppressive [11,64,65]. This also offers the potential for uridine to enhance the CD4 cell recovery of patients under antiretroviral treatment.

Strategies aimed at increasing uridine also improved symptoms in patients harbouring a qualitative defect in mtDNA by carrying inherited mtDNA mutations [66]. In patients with such mtDNA mutations, however, uridine would be predicted to ameliorate only one aspect of respiratory chain dysfunction, namely the consequences of DHODH inhibition and, in contrast with antiretroviral-treated HIV patients, not to improve the underlying mtDNA pathology. Therefore, patients with mtDNA mutations are likely to have a continued defect in ATP synthesis and hyperlactataemia under uridine. Further clinical data on this group of patients are eagerly awaited.
M any aspects of uridine are still poorly understood. For example, an oral dose of 300 mg three times daily for 6 months also improved diabetic neuropathy in a well-conducted trial [67]. Therapeutic uses of uridine were also proposed in cardiovascular disease, hypertension, liver disease and infertility, among others [38].

Until the data from formal clinical studies are available, the risk–benefit calculation of using uridine in HIV-infected patients should be individualized. The current safety data justify the current use of uridine in individuals suffering from mt toxicity who are closely monitored and who cannot be switched to an anti-retroviral regimen with a lower potential of mt toxicity.

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Uridine in mitochondrial toxicity


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HIV-associated antiretroviral toxic neuropathy (ATN): a review of recent advances in pathophysiology and treatment

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Introduction

Nucleoside analogue reverse transcriptase inhibitors (NRTIs) disrupt neuronal mitochondrial DNA synthesis, impairing energy metabolism and resulting in a distal symmetrical polyneuropathy (DSP), categorised as an antiretroviral toxic neuropathy (ATN) that causes significant morbidity in HIV disease [1].

Drugs used to treat many HIV-related conditions, such as isoniazid, vincristine, lithium carbonate, dapsone, pyridoxine (vitamin B6) and thalidomide, and opportunistic illnesses, for example, cytomegalovirus infection and syphilis, may also cause neuropathy. Other possible causes of these symptoms include alcohol and some recreational drugs such as heroin, cocaine, or amphetamines.

It seems that these different causes of neuropathy can interact. People with mild neuropathy symptoms often find that they intensify after they start taking treatments such as didanosine (ddI) or stavudine (d4T). Neuropathy has been associated with a deficiency of the B vitamins, especially vitamin B12. Factors associated with the development of peripheral neuropathy in a large cohort of HIV-infected patients include older age, diabetes and white race.

Pathophysiology

Antiretroviral toxic neuropathy (ATN) is the commonest HIV-associated DSP [1] causing significant morbidity in 10–35% of HIV positive patients [2–4], and occurring in 11–66% of patients [1,5] on NRTI drug therapy. Dideoxynucleotide analogue agents such as zalcitabine (ddC), d4T and ddI are implicated in its pathogenesis [1,4,6] and changes in the prescribing pattern of these drugs, including dose reduction, have led to incidence reduction in antiretroviral toxic neuropathy (ATN) [4]. A feature of HIV-associated neuropathy is development of dyseaesthetic pain [4] whose severity is associated with elevated plasma HIV-1 RNA [7] levels, and which may be unresponsive to analgesia, even in combination with anticonvulsants [1], tricyclic antidepressants [3], mexiletine [3], or gabapentin [8]. Withdrawal of certain NRTIs often becomes necessary [1,4] since there are no licensed effective therapies, although lamotrigine [9] and recombinant nerve growth factor (rhNGF) [10] have shown some benefit. Ideally a pathogenesis-based treatment for ATN would allow patients to continue NRTI therapy, still the keystone of current highly active antiretroviral therapy regimes [11].

ATN is thought to result from disrupted mitochondrial oxidative metabolism [6,12] secondary to reduction in neuronal mitochondrial DNA content [6,13,14]. Consequently, neurons are unable to meet the metabolic requirements of their long peripheral axons which undergo die-back, resulting in the glove and stocking distribution of ATN [1]. In keeping with this die-back hypothesis, epidermal innervation is reduced in ATN [15,16].

Symptomatic treatment

If the neuropathy is caused by a drug, symptoms usually occur after a few weeks. Depending on severity of the neuropathy and other treatments available, the patient may be advised to stop or to reduce the dosage of the offending drug. The main purpose of treating neuropathy is to relieve symptoms. In mild cases, where the symptoms are not affecting daily life, standard painkillers such as ibuprofen may be all that is necessary.

If the symptoms start to be disruptive, tricyclic antidepressants such as amitriptyline may help. These drugs can take a couple of weeks to show any effects, and may cause side-effects of a dry mouth, difficulty...
urinating, high blood pressure and drowsiness. People with severe symptoms may require stronger pain killers such as fentanyl or other opiates. The anti-convulsants, phenytoin and carbamazepine, can be useful.

La Spina and co-workers assessed the efficacy and safety of gabapentin as a sole analgesic in patients with HIV-related painful neuropathy [8]. Nineteen patients with HIV-related painful neuropathy were administered gabapentin. Efficacy was evaluated with two 100 mm visual analogue scales (VAS) (0: no symptom; 100: worst symptom), rating pain and interference of pain with sleep, performed at baseline and monthly intervals. Mean pain VAS score decreased from a baseline of 55.7 ±19.1 mm to a final 14.7 ±18.6 mm (P<0.0001) and mean sleep interference VAS score decreased from a baseline of 60.4 ±31.9 mm to a final 15.5 ±27.7 mm (P<0.0001).

Hahn [17] carried out a multicentre, prospective, randomised, double-blind placebo-controlled study of gabapentin in 26 patients with HIV sensory neuropathy. Fifteen patients received gabapentin at 400 mg per day before being increased to 1200 mg per day over 2 weeks. This dose was maintained or increased to 2400 mg per day if not beneficial. There was a significant decrease in pain score in the gabapentin group (-44%) but not the placebo group (-12%). Somnolence was reported in 80% of the gabapentin group.

Capsaicin has been found to be effective in relieving pain associated with other neuropathic pain syndromes, and is mentioned as a possible topical adjuvant analgesic for the relief of DSP [18]. This multicentre, controlled, randomized, double-masked clinical trial studied patients with HIV-associated DSP and compared measures of pain intensity, pain relief, sensory perception, quality of life, mood and function for patients who received topical capsaicin to the corresponding measures for patients who received the vehicle only. Twenty-six subjects were enrolled in the study. At the end of 1 week, subjects receiving capsaicin tended to report higher current pain scores than did subjects receiving the vehicle (P=0.042). The dropout rate was higher for the capsaicin group (67%) than for the vehicle group (18%) (P=0.014). A further study showed a 40% reduction in pain in capsaicin treated individuals [19].

**Pathophysiologic treatments**

Vitamin B₁₂ deficiency is a cause of neuropathy and some individuals with HIV have such a deficiency, which may cause symptoms such as fatigue, poor memory and low levels of red blood cells. A vitamin B complex supplement may be taken to relieve vitamin B₁₂ deficiency although overdosing of vitamin B₆ may exacerbate nerve damage. Recombinant human (rh) nerve growth factor (NGF) has been used to treat HIV-related and diabetic peripheral neuropathy.

One study in HIV-associated neuropathy found that NGF reduced pain and improved sensitivity [20]. A total of 270 patients with HIV-associated sensory neuropathy (SN) were randomized to receive placebo, 0.1 µg/kg rhNGF, or 0.3 µg/kg rhNGF by double-blinded subcutaneous injection twice weekly for 18 weeks. The primary outcome was a change in self-reported neuropathic pain intensity (Gracely pain scale). In a subset, epidermal nerve fibre densities were determined in punch skin biopsies. Both doses of NGF produced significant improvements in average and maximum daily pain compared with placebo. Positive treatment effects were also observed for global pain assessments (P=0.001) and for pin sensitivity (P=0.019). No treatment differences were found with respect to mood, analgesic use or epidermal nerve fiber densities. Injection site pain was the most frequent adverse event, and resulted in un-blinding in 39% of subjects. Schifitto [21] reported that symptoms of pain improved in 200 people with HIV-associated distal symmetrical polyneuropathy (DSP) who were treated with neurotrophin (NGF) for 48 weeks in an open-label study. However, there was no improvement as measured by neurologic examination, quantitative sensory testing, and epidermal nerve fibre density. Following disappointing results in treating diabetic neuropathy, Genentech have ceased development of NGF [22].

Research into diabetic neuropathy has suggested that supplements of γ-linolenic acid (GLA), α-lipoic acid, magnesium and chromium may help relieve pain due to neuropathy, although none of these supplements have been tested among people with HIV-related neuropathy.

A proposed therapeutic agent for DSP is acetyl-L-carnitine (ALCAR), the acetyl ester of L-carnitine. ALCAR is vital for normal mitochondrial function, being a transport molecule for free fatty acids, and an important acetyl-group donor in high energy metabolism and free fatty acid β-oxidation [23]. Also, ALCAR potentiates NGF actions [24], promotes peripheral nerve regeneration [25], is neuroprotective in vitro [26], in vivo [27] and in animal models of diabetic neuropathy [28]. ALCAR has analgesic properties, possibly mediated by increasing ACTH and β-endorphin levels [29], whilst its non-acetylated form, L-carnitine, also has favourable immunological benefits [30] in HIV infection. ALCAR has shown improvement in pain scores and electrophysiologic parameters in a placebo controlled study in diabetics with DSP [31]. Short term ALCAR treatment [32] has shown symptomatic benefits in ATN, and further studies are needed in people with HIV-related neuropathy.
underway to define whether this effect is long-lasting, due to neuronal regeneration, or merely to a result of analgesia.

Hart and co-workers reported on 21 patients with NRTI-associated DSP treated with 1500 mg ALCAR twice daily for up to 18 months [33]. Median baseline CD4 cell count was 286 cells/mm³ and 40% had HIV RNA <400 copies/ml. Eight started the study with grade 1 neuropathy, 10 with grade 2 and three with grade 3. Skin biopsies were taken from the leg at baseline and at months 6 and 12 and stained with antibodies for nerve fibres. The innervation of the epidermis increased by 34% and by 101% after 6 and 12 months. In the dermis, it increased by 65% and around the sweat glands by 75% after 6 months (Figure 1). Nerve fibre density approached normal levels after 6 months. The greatest increase was in small sensory nerve fibres, with 100% increase in the epidermis \( (P=0.006) \) and 133% in the dermis \( (P<0.001) \). Neuropathic pain improved in 76% of patients. No side-effects of ALCAR were experienced.

Discussion

Quantification of cutaneous innervation has previously been found to correlate with clinical tests of sensory function in leprosy [34] and diabetic neuropathy [32], where during disease progression, immunohistochemical changes precede those of sensory testing [31]. The morphology of cutaneous innervation in DSP has previously been described qualitatively, and the density of epidermal fibres shown to be reduced along a proximal-distal gradient in the limb in DSP [14] and ATN [15]. Epidermal nerve fibre density has recently been described as a therapeutic outcome measure in HIV [16], however fibre counts do not assess the health of surviving fibres, whose atrophy or regeneration can be determined by immunostaining area quantification, as used in this study.

In established ATN, the symptoms reflect a cutaneous innervation reduction of the lower leg, with epidermal innervation being most affected, consistent with the dieback hypothesis of sensory neuropathy [4]. There is also marked atrophy of dermal and sweat gland plexi. This cutaneous denervation may explain why neuropathic symptoms frequently do not begin to resolve for some weeks after starting ALCAR treatment, and may then continue to improve for many months, since this timeframe matches the slow rate at which peripheral nerves regenerate.

As expected from the ATN dysaesthetic algesic symptomatology, small sensory (C, Aδ) fibres are most affected, and it has been demonstrated that these fibres show the greatest reduction in immunostaining area when compared to controls [33]. Furthermore, the proportion of small sensory and structural fibres was markedly reduced in neuropathic patients' epidermis (ATN 4%; control 36%), implying loss of epidermal fibres and reduced function of the surviving sensory fibres. Six months of oral ALCAR treatment resulted in significant increases in the innervation in epidermis,

Figure 1. Plots of the mean of all treated individuals mean fractional immunostaining for protein gene product 9.5 in each area of cutaneous innervation at each time point up to 18 months
dernis and sweat glands, an improvement maintained throughout the treatment for all patients. The results demonstrate that dermal PGP-immunostaining nerve fibres regenerated sufficiently to reach the range found in normal skin. Intra-epidermal fibres also regenerated, although more gradually, suggesting a temporal relationship with improvement.

ALCAR treatment may counteract deoxyribonucleotide NRTI toxicity (ATN) by several mechanisms. Firstly, it may reduce mitochondrial DNA damage by direct antioxidant effect [34,35]. Secondly, by promoting glucose utilization and high-energy substrate oxidative metabolism, ALCAR improves neuronal metabolic capacity [36]. In addition ALCAR may facilitate distal neurotrophic support, particularly of myelinated (Aδ) and unmyelinated (C) fibres. Furthermore, ALCAR promotes peripheral nerve regeneration [31,37] independently of NRTI toxicity and function [38–41]. Patients with NRTI-associated peripheral neuropathy also have reduced serum ALCAR levels, contrary to asymptomatic HIV positive controls [42]. Although nucleoside analogue antiretroviral agents have been partly superceded by other agents such as protease inhibitors, they remain fundamental for combination therapy [4,11] and are likely to remain important components in HIV management for the foreseeable future [4]. Peripheral neuropathy has been the principal complication limiting the use of these agents [1,4] and pathogenesis-based therapies such as ALCAR may be promising effective management approaches, allowing patients to remain on NRTI therapy.

References


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