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Could Mitochondrial DNA Quantitation Be a Surrogate Marker for Drug Mitochondrial Toxicity?

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Abstract

Nucleoside reverse transcriptase inhibitors have been proven to inhibit mitochondrial DNA (mtDNA) polymerase gamma, resulting in decreased mtDNA synthesis and consequential risk for the development of mitochondrial dysfunction in HIV-infected individuals. The depletion of mtDNA seems to correlate with the development of symptomatic hyperlactatemia and lipoatrophy. A validated quantitative mitochondrial DNA assay could be useful to monitor and prevent mitochondrial damage in HIV-infected patients, especially in those under antiretroviral therapy with nucleoside analogues. This review analyzes the current methods to determine mitochondrial damage and the available data to support their utility in clinical practice. **(AIDS Reviews 2004;6:169-80)**

Key words

Mitochondrial DNA. HIV. Nucleoside reverse transcriptase inhibitors. Hyperlactatemia. Lipoatrophy.

Background

Damage to cellular mitochondria is one of the most important long-term toxicities of antiretroviral therapy and has only been fully accepted in recent years[1-3]. Its incidence and prevalence is variable, and several factors have been identified to affect mitochondria in the context of HIV infection[4].

In this review we will try to summarize the etiology of mitochondrial damage and the possible role of monitoring mitochondrial DNA (mtDNA) levels in HIV-infected individuals, particularly in those under antiretroviral therapy, as a marker of drug toxicity.

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Mitochondrial genetics and disease

The mitochondrion is the organelle responsible for the majority of cellular energy production through the generation of adenosine triphosphate (ATP), although is also implicated in pyruvate oxidation, Krebs cycle, and metabolism of amino acids, fatty acids and steroids[5]. Each cell contains hundreds to thousands of mitochondria, with each mitochondrion containing 2-10 mtDNA molecules. Because mtDNA has only 37 genes, most of the gene products in the organelle (approximately 900) are encoded by nuclear DNA (nDNA) and are imported from the cytoplasm. Defects in any of the numerous mitochondrial pathways can result in mitochondrial disease[6].

The DNA polymerase gamma is the enzyme responsible for the replication of mtDNA. It has a mutation rate 17 times higher than for nuclear polymerases[7]. However, pathogenic mutations are seen only in a few mitochondrial DNA molecules. For this reason, cells and tissues contain mutated and wild-type mtDNA genomes in a situation known

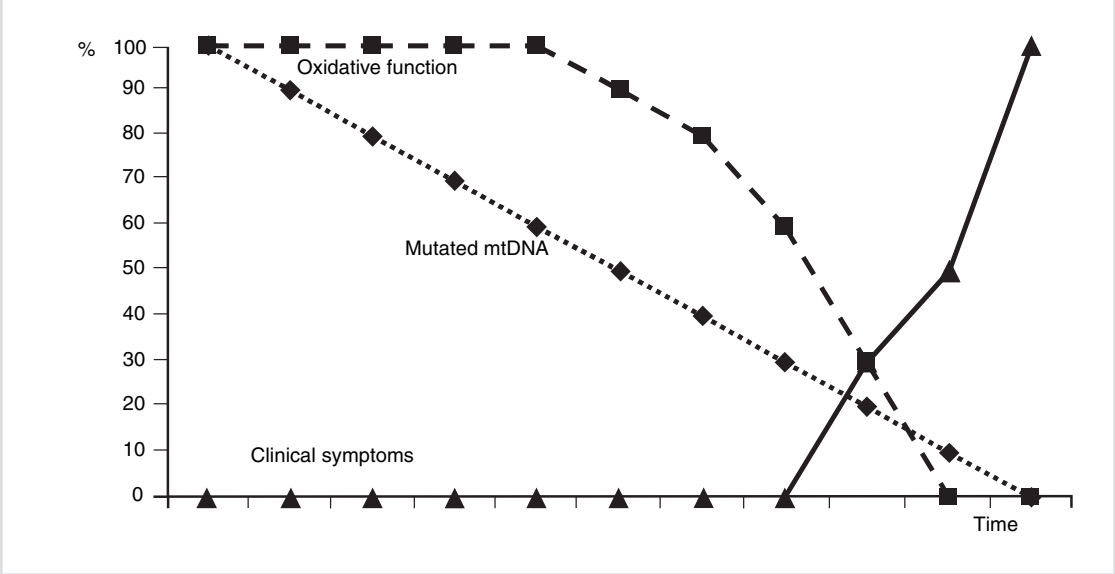


Figure 1. *Threshold effect. Appearance of clinical symptoms only in the presence of a high level of mutated mtDNA.*

as heteroplasmy. To observe a mitochondrial dysfunction, a minimal number of mutated mtDNA molecules should be present at cellular level as well as at tissue level. This phenomenon is known as a "threshold effect", above which clinical events may appear (Fig. 1)[8].

Mitochondrial diseases appear due to the presence of mutations or deletions in the mtDNA, affecting specific proteins, protein-coding genes or particular genes[8]. Other factors which have been associated with impairing of mitochondrial DNA synthesis include alcohol, drug abuse, obesity, and aging[9,10]. Since the introduction of antiretroviral therapy, many complications associated with mitochondrial dysfunction have also been recognized in HIV-infected individuals, but these dysfunctions are based mainly on depletion of mtDNA instead of mutations in mitochondrial genome.

Mechanisms of antiretroviral mitochondrial toxicity

A wide range of adverse events occurring in persons with HIV infection receiving nucleos(t)ide reverse transcriptase inhibitors (NRTIs) have been linked to mitochondrial dysfunction[3,4,11-14]. These drugs require intracellular phosphorylation to be active. Phosphorylation is catalyzed by deoxyribonucleoside kinases, which are located in the cytosol or mitochondria, depending on the cell type. The mechanism of reverse transcriptase (RT) inhibition after NRTI phosphorylation is by competition with the natural nucleoside for incorporation into the nascent DNA chain, leading to premature DNA chain termination. The NRTIs also are substrates for the DNA polymerase gamma, the enzyme responsible for mtDNA replication. This affinity results in a depletion or mutation in genes encoded by the mtDNA (Fig. 2)[11,15]. The consequence of these actions is a deficiency in ATP production, but also the emergence of reactive oxygen radicals, which ultimately may affect mitochondrion structure.

There are eight NRTIs so far approved for the treatment of HIV infection (zidovudine [AZT], zalcitabine [ddC], didanosine [ddI], stavudine [d4T], lamivudine [3TC], abacavir [ABC], tenofovir [TDF] and emtricitabine [FTC])[16]. *In vitro* inhibitory constants for DNA polymerase gamma have been determined for each of these drugs[17-19]. The results obtained show that the nucleoside with the highest affinity for this enzyme is ddC, being followed in order by ddI > d4T > 3TC > AZT > ABC. Results from the two last nucleos(t)ide analogues approved, TDF and FTC, indicate that their potential to interfere with mitochondrial function is relatively low[20,21]. The difficulty in extrapolating these data to clinical toxicity is the fact that the efficiency in phosphorylation also differs among NRTIs, which determines its affinity for the DNA polymerase γ.

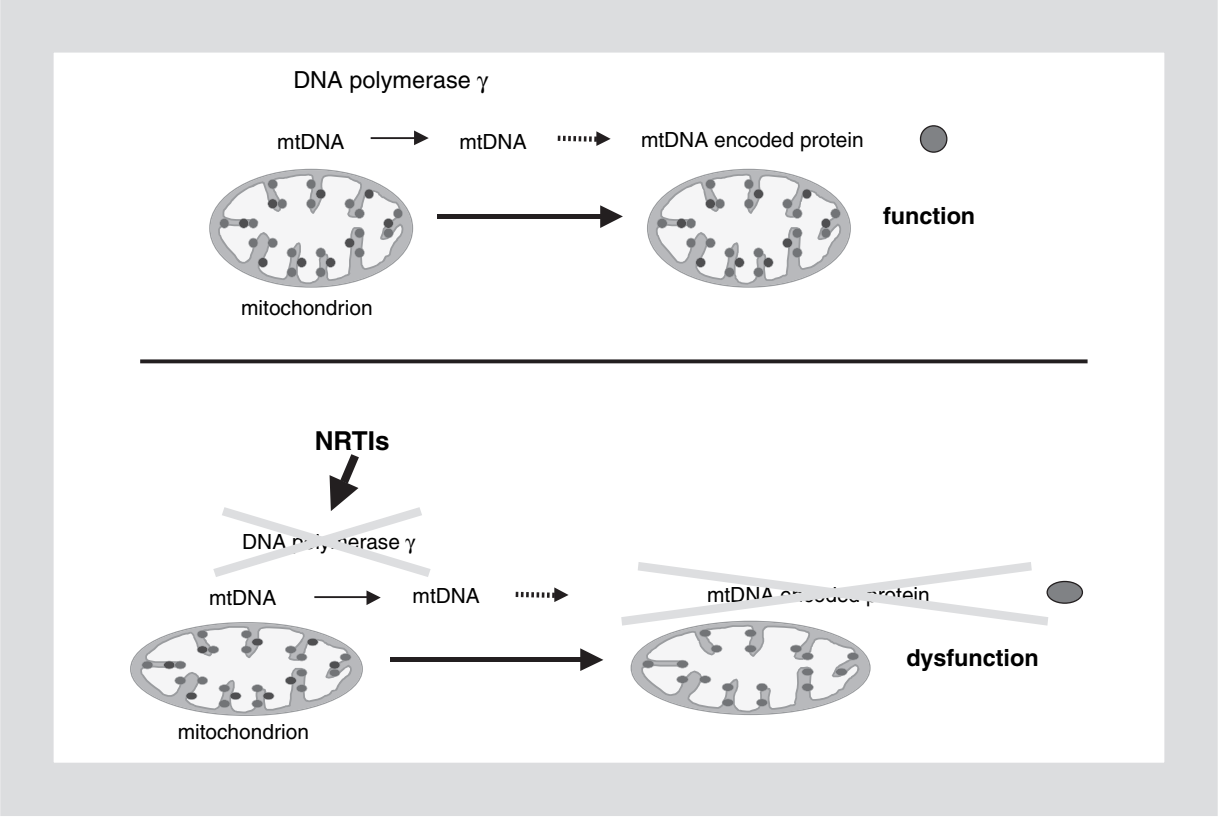


Figure 2. *Mechanism of mitochondrial toxicity by inhibition of DNA polymerase γ. Influence of NRTIs in the replication of mtDNA. Adapted from Brinkman, et al[13].*

Clinical evidence

The first mention of mitochondrial dysfunction appeared in the early 1990s, when several reports linked AZT-associated myopathy with mitochondrial damage[22-24]. Table 1 summarizes the clinical symptoms associated with mitochondrial dysfunction and adverse events of NRTIs ascribed to them. In 1998, Brinkman, et al.[11] proposed the theory of mitochondrial toxicity of antiretroviral drugs. When NRTIs inhibit DNA polymerase gamma, the production of mtDNA and mtDNA-encoded proteins is interrupted, finally leading to a dysfunctional mitochondria. A wide range of adverse events associated with NRTI therapy have similar etiology, due to impaired mitochondrial function in different organs and tissues.

The most serious presentation of NRTI-induced mitochondrial dysfunction is lactic acidosis, although it is relatively uncommon[25,26]. By contrast, elevations of plasma lactate levels are common, particularly since the wide use of triple combination therapy, ranging from 5 to 35% of patients on highly active antiretroviral therapy (HAART)[27]. Risk factors for the development of symptomatic hyperlactatemia are female sex, obesity, hepatitis C virus (HCV) infection, pregnancy, low CD4 counts, renal insufficiency, and any intercurrent illness[28,29]. However, in a recent study the only predictors for elevated lactate levels in 299 patients followed during 24 weeks were baseline cholesterol and the current use of d4T[30]. Only 16 patients maintained elevated lactate levels during the study follow-up and four of them developed lactic acidosis. One of these four subjects had normal lactate levels two months before symptoms appeared. The measurement of plasma lactate levels does not seem to be useful as a predictor of symptomatic hyperlactatemia[31], and so far there is not enough data to support that mild hyperlactatemia is a risk factor for developing lactic acidosis or liver disease. However, monitoring of serum lactate levels could alert clinicians to mitochondrial toxicity, and help them to take appropriate precautions.

Lipodystrophy is the other “big syndrome” linked to mitochondrial dysfunction in HIV-infected patients. The overall prevalence of at least one physical abnormality associated with this syndrome is around 50% in patients on stable HAART[32-35]. The main metabolic abnormalities observed in HIV-infected individuals with lipodystrophy include hypertriglyceridemia, hypercholesterolemia, low

Table 1. Clinical manifestations due to mitochondrial dysfunction and adverse events related to nucleoside analogue treatment in HIV-infected individuals

Organ and tissue	Symptoms and signs	NRTI
Central nervous system	Seizures	
	Ataxia	
	Myopathy	AZT
	Myoclonus	
	Psychomotor retardation	
	Psychomotor regression	
	Hemiparesis and hemianopia	
	Cortical blidness	
	Encephalomyopathy	AZT, 3TC
	Migraine-like headaches	
	Dystonia	
Peripheral nervous system	Peripheral neuropathy	d4T, ddI, ddC
Muscle	Weakness and exercise intolerance	
	Ophtalmoplegia	
	Ptosis	
Eye	Pigmentary retinopathy	
	Optic atrophy	
Blood	Sideroblastic anemia	AZT
	Pancytopenia	AZT
Endocrine system	Diabetes Mellitus	DdI
	Short stature	
	Hypoparathyroidism	
Heart	Conduction disorder	
	Cardiomyopathy	AZT, ddC, ddI
Gastrointestinal system	Intestinal pseudo-obstruction	
Kidney	Aminoaciduria	Adefovir, TDF?
	Renal tubular dysfunction	
	Fanconi's syndrome	
	Barter syndrome	
Pancreas and liver	Exocrine pancreatic failure	
	Pancreatitis	d4T,ddI
	Hepatocellular failure	AZT, ddI, d4T
	Lactic acidosis	AZT, ddI, d4T
Skin	Lipomatosis	d4T, all?

levels of HDL-cholesterol, insulin resistance, type 2 diabetes mellitus, lactic acidemia, and elevated hepatic transaminase[32,36-40]. Lipodystrophy was firstly associated with the use of protease inhibitors (PIs)[35,41], although patients never exposed to PIs and taking NRTIs during long periods soon were reported to develop lipoatrophy and buffalo hump[42-44].

The pathogenesis of HIV lipoatrophy remains unclear. Several evidences suggest that lipoatrophy may result from mitochondrial toxicity caused by NRTIs, especially when d4T is included in the combination[45-48]. The incidence of this syndrome differs for different NRTI combinations. In a cohort of 76 HIV+ drug-naive individuals who started HAART, the combination of d4T + ddI produced greater increases in serum lactate and lipoatrophy than therapies based on AZT and 3TC within the first year of therapy[49]. Moreover, the selective replacement of d4T with ABC reduces lactate levels

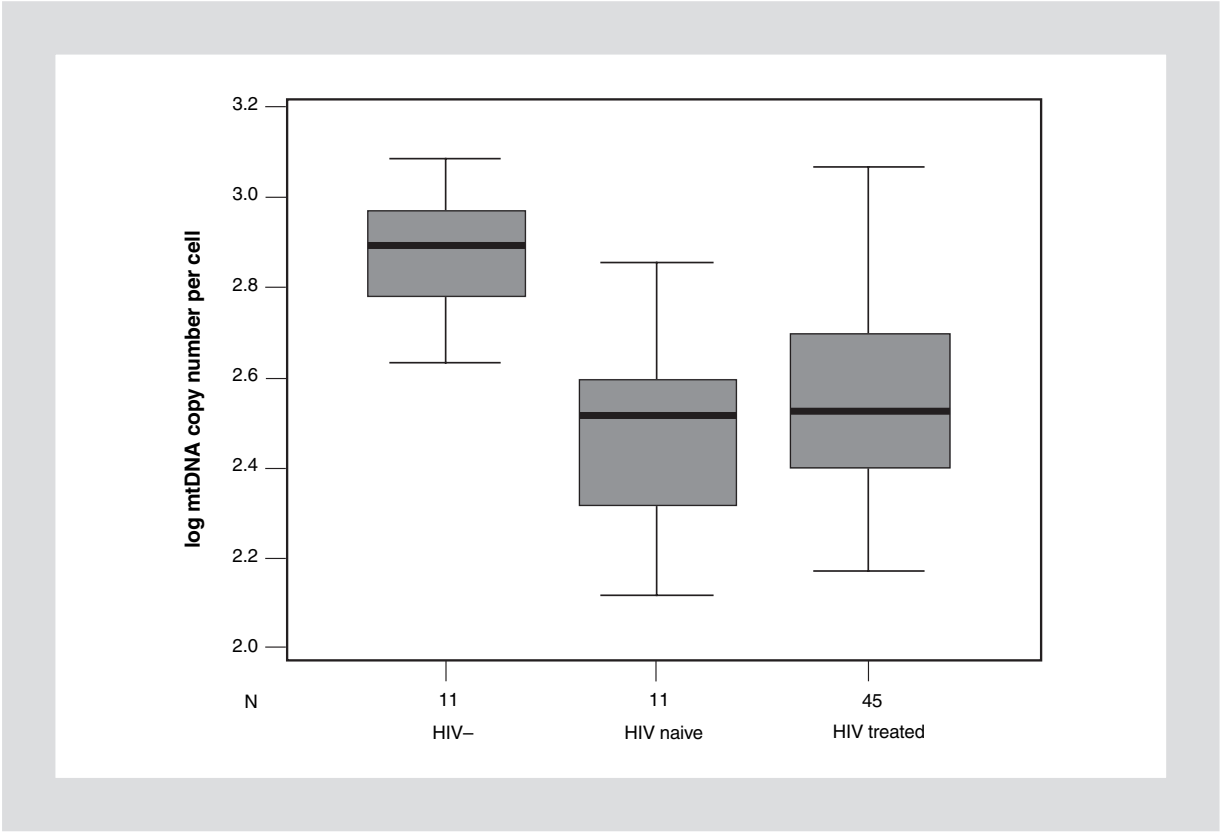


Figure 3. *Influence of HIV infection itself and antiretroviral therapy on the mtDNA copy number in peripheral blood mononuclear cells[54].*

and improves lipoatrophy[50]. In the Gilead 903 trial, in which d4T + 3TC + efavirenz (EFV) was compared with TDF + 3TC + EFV in drug-naive patients, the main difference after 144 weeks of treatment was a better lipid profile and less lipodystrophy in the TDF-containing arm[51] One hypothesis published last year is stating that the central nerve system is playing a major role in the body-fat distribution, wherein the sympatic and parasympatic nerve systems play a key role. The effects of the NRTIs, suggestively on the mitochondria of the nerve cells, would thereby indirectly influence the redistribution of adipose tissue. Clearly, although all NRTIs may affect mitochondria, not all do it to the same extent.

Depletion of mtDNA in HIV infection

The role of nucleoside analogues

Since the release of Brinkman's hypothesis, several studies have pointed out the association between the use of NRTIs and depletion of mtDNA. In 2002, Cote, et al. published for the first time that patients with symptomatic hyperlactatemia had significantly lower mtDNA levels in their peripheral blood mononuclear cells (PBMCs), taking as comparison HIV– and HIV+ individuals without symptoms[52]. Moreover, clinical manifestations resolved after discontinuation of antiretroviral of therapy. Surprisingly, HIV-infected subjects never exposed to antiretroviral drugs also showed significantly lower mtDNA levels than HIV– controls. More recent studies have confirmed this observation (Fig. 3), suggesting that HIV infection itself may produce depletion of mtDNA to some extent[53,54].

At least two possible mechanisms have been proposed to explain mtDNA depletion in HIV infection. First, HIV itself by shortening the half-life of lymphocytes might compromise mitochondrial replication, which is independent of cell division and occurs during the G0/G1 cellular phase (Fig. 4)[54]. The second mechanism might be related with cell apoptosis[55,56]. HIV infection may cause apoptosis through multiple mechanisms, some of which rely on the intricate virus host cell interaction, and some of which involve activation of the host's inflammatory and immune systems. Soluble HIV-1 products, such as the accessory proteins Tat, Vpr and gp120, have been shown to promote

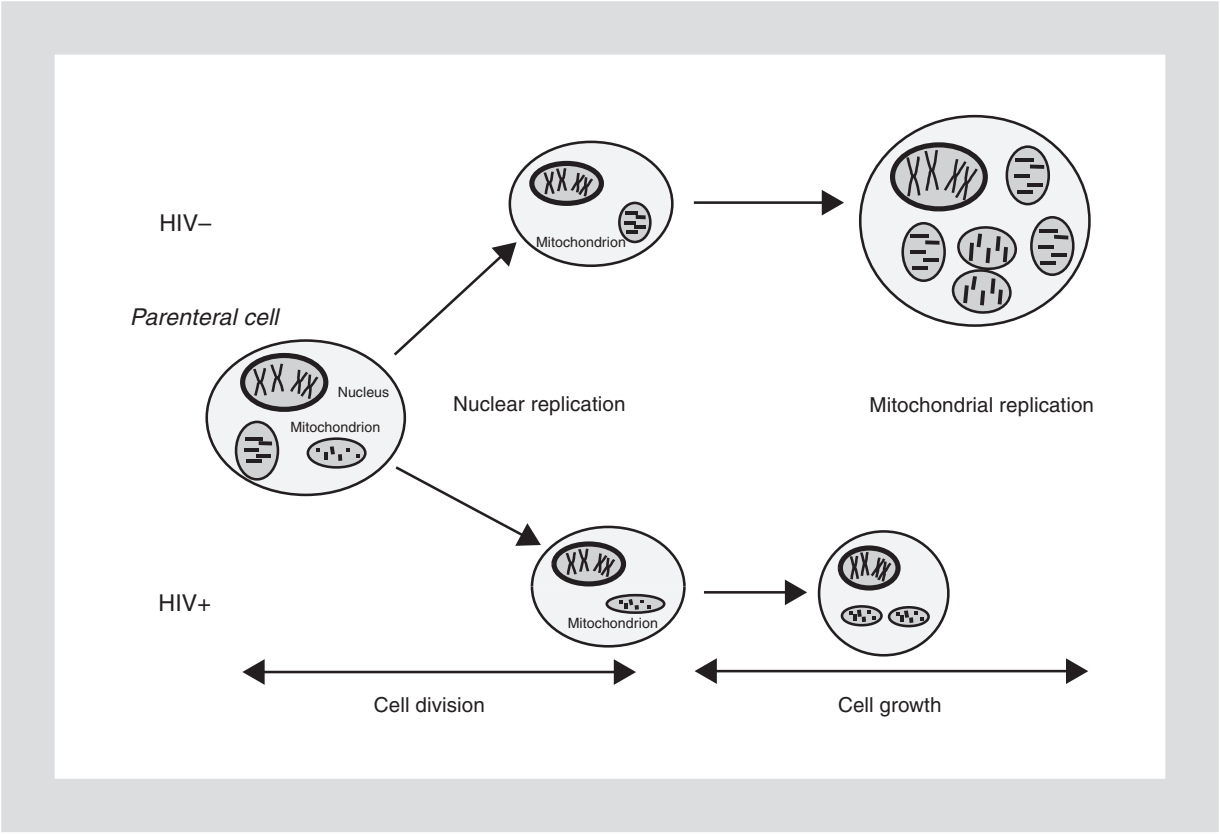


Figure 4. *Cell division and mitochondrial segregation. Influence of HIV infection an cell growth and total number of mitochondria per cell.*

cell death directly through interactions of gp120 with CD4, CXCR4 and other uncharacterized receptors[57], or by induction of the caspase via[58]. Another mechanism has been proposed in which the HIV infection results in changes in cytokine and interleukine profiles that play a role in the regulation of mtDNA transcription, eg. TNFα.

Recent reports have revealed that NRTI therapy is associated with mtDNA depletion in PBMCs[52-54,59-61], adipocytes[59,62] and the liver[63]. Depletion of mtDNA in PBMCs is much higher when d4T or the combination of d4T + ddI is used[52,54]. *In vitro*, the triphosphate form of d4T is incorporated into DNA more readily than other NRTIs, and exerts the greatest inhibition over the DNA polymerase gamma[15,64]. This fact may explain the strong association noticed between d4T and mitochondrial toxicity.

Although lactate levels have not been considered as an independent marker for mitochondrial toxicity, when a significant mtDNA depletion occurs, lactate levels tend to increase, and this is particularly true for patients with symptomatic hyperlactatemia[52].

Reports that have highlighted mtDNA depletion in adipocytes and adipose tissue[62,65] have confirmed that prolonged NRTI treatment is associated with reduced adipocyte mtDNA copies/cell. On average, mtDNA depletion in the treated group was 78% with respect to HIV+, untreated individuals. Significant differences were found between subjects treated with d4T (mean mtDNA depletion of 87%) vs. AZT (mean mtDNA depletion of 52%)[62]. These data support that mtDNA depletion contributes to the pathogenesis of subcutaneous fat loss associated with NRTI therapy, and that selected drugs within this family are associated with a higher relative risk of inducing adipocyte mtDNA depletion. In the study conducted by Walker[65], ultrastructural abnormalities of adipocytes were found, suggesting a link between mitochondrial damage, the use of NRTIs, and lipoatrophy in HIV-infected patients. Of note, these authors emphasized that their results were mainly associated with a depletion of mtDNA instead of a high rate of mutations and deletions, as previously reported by others[66].

Hepatitis C and mtDNA depletion

An association between hepatitis C and lipoatrophy has been suggested for a long time. In a cohort of

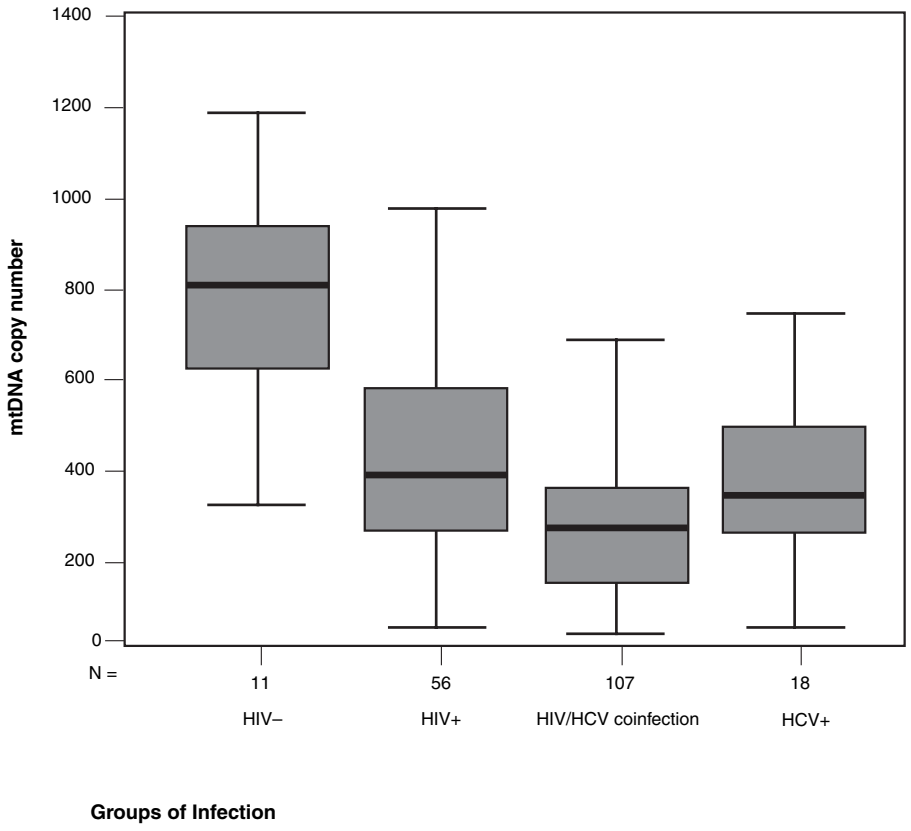


Figure 5. *Comparison of mtDNA copy number in PBMCs from different groups (HIV– individuals, HIV alone, HIV-HCV co-infected, and HCV alone)*[71].

226 HIV-infected patients, HCV infection was significantly more frequent among lipoatrophic patients than in subjects with adiposity, or the mixed lipodystrophy syndrome, or those without lipodystrophy (46.2 vs. 14.7% vs. 19.6%, respectively; $p < 0.03$)[67]. The authors concluded that HCV infection might be associated with the atrophic form of lipodystrophy in conjunction with a prolonged NRTI exposure. In favor of a role of HCV as a cause of mitochondrial damage in the liver are recent demonstrations of mitochondrial abnormalities in patients with hepatocellular carcinoma[68,69]. Additionally, a recent study has pointed out that liver mtDNA content may be decreased by 47% in HIV-HCV coinfected individuals on d4T, ddI or ddC compared to subjects not treated with these drugs[70].

In a cross-sectional study, coinfection with HCV and/or HBV was associated with a higher decrease of the mtDNA content in PBMCs than in HIV-monoinfected patients[54]. A more detailed investigation showed that HCV infection itself leads to a depletion of mtDNA in PBMCs, which could be further enhanced if treatment of chronic hepatitis C with pegylated interferon and ribavirin was used (Fig. 5)[71].

Ribavirin is a guanosine analogue that could also inhibit the DNA polymerase gamma. In HIV-HCV coinfected patients, the co-administration of anti-HCV therapy and antiretrovirals may synergistically enhance the toxic effects on mitochondria. Several reports have demonstrated that the concomitant use of ddI and ribavirin may result in a higher risk of pancreatitis[72-74]. Ribavirin inhibits the inosine monophosphate dehydrogenase (IMP), promoting the phosphorylation of ddI[75]. This results in an increase in the intracellular levels of ddATP, the active form of ddI, leading to further inhibition of the DNA polymerase gamma.

Table 2. Tests available to measure mitochondrial function and mitochondrial DNA

Information	Method	Measure
Measure of mtDNA depletion	Southern blot In-house real time PCR Retina Mitox	mtDNA/nDNA ratio mtDNA/nDNA rario
Deletions and mutations	Southern blot Sequencing	Molecular weight Sequence changes
Genomic expression of mRNA	Northern blot	mRNA density
Protein expression	Western blot	Protein density
Function of mitochondrial respiratory chain	Fluorimetric assays Polarographic assays Spectrophotometric	Oxidative damage Oxygen consumption Enzymatic activity

Another report has highlighted a potential synergistic interaction between d4T and ribavirin, leading to a rapid and severe weight loss in HIV-infected patients receiving both antiretrovirals and anti-HCV therapy[76]. A pronounced weight loss, and high lactate and amylase levels were found among patients taking d4T or ddI compared with those taking other NRTIs along with ribavirin.

Finally, a higher risk of metabolic abnormalities, including insulin resistance and modifications in body compositions, have been reported among HIV-HCV coinfected individuals receiving antiretroviral therapy compared to HIV-monoinfected subjects[72,73,76-78].

mtDNA depletion in children born from HIV-infected mothers

A lower mtDNA copy number has been observed in placenta and cord blood of pregnant women exposed to HAART compared to HIV– pregnant women[79]. Results from a cohort of 18 HIV– children whose mothers took Combivir during pregnancy revealed morphological and molecular evidence of mitochondrial damage, in spite of an absence of clinical symptoms[80]. However, persistent mitochondrial dysfunction has been reported more recently in a group of HIV-uninfected children born to HIV-infected mothers who had received either AZT or AZT + 3TC during pregnancy, and two infants died with severe persistent mitochondrial toxicity[81,82].

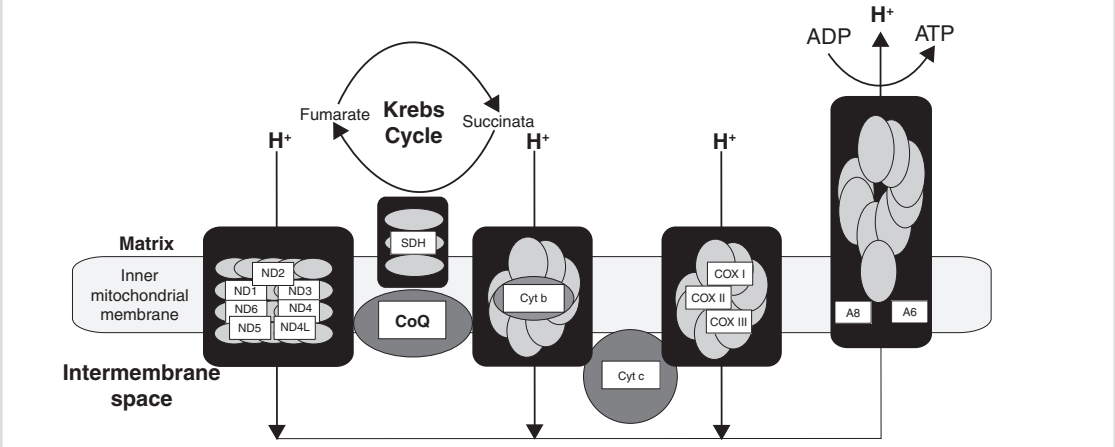
Since all mitochondria in the zygote derive from the ovum, mtDNA depletion or mutations present in the mother due to prior antiretroviral therapy may pass to the children, ultimately leading to mitochondrial dysfunction in the infant, even in those children without acquired HIV infection.

Methods for measuring mtDNA

If mitochondrial toxicity is the main pathogenic mechanism underlying the side effects of NRTIs, especially lipoatrophy and hyperlactatemia, a method to quantify mitochondrial damage would be helpful to monitor HIV-infected individuals on antiretrovirals. Different tools have been proposed in recent years to follow mitochondrial toxicity, from simple quantitative methods to more complicated ones, including those based on the analysis of phenotypes for each of the functions of mitochondria. In this section we will summarize all these methods (Table 2) in an attempt to identify which could be the most reliable for monitoring mitochondrial toxicity in different settings.

Biochemical methods: enzymatic function and oxygen consumption

The enzymatic analysis of mitochondrial respiratory chain (MRC) complexes is an important step in the diagnosis of mitochondrial disorders. The mtDNA contains 37 genes, and 13 of them encode subunits of the respiratory chain: seven subunits of complex I, one subunit of complex III (cytochrome b), three subunits of complex IV (cytochrome c oxidase), and two subunits of complex V (ATP synthase) (Fig. 6). The absolute enzyme activity of the complexes of the MRC as well as of the single enzymes that form each complex can be determined either by spectrophotometric or



Subunits	*Complex I*	*Complex II*	*Complex III*	*Complex IV*	*Complex V*
No. of nDNA encoded subunits	35	4	10	10	12
No. of mtDNA encoded subunits	7	0	1	3	2

Figure 6. *Mitochondrial respiratory chain (MRC) and subunits encoded by nuclear DNA (nDNA) and mitochondrial DNA (mtDNA).*

polarographic techniques[83-85]. These assays require the use of simple instruments such as spectrophotometers, spectrofluorimeters or plate readers, and provide quantitative measurements of the activity of a given enzyme.

The analysis of MRC oxidative phosphorylation (OXPHOS) has the advantage of providing a functional view of the respiratory chain. OXPHOS biochemical studies can be performed in tissue homogenates, in permeable whole cells, or in mitochondria isolated from tissue or cultured cells[85-87]. Adipose tissue, skeletal muscle and liver tissue as well as PBMCs would be the ideal tissues for the diagnosis of mitochondrial dysfunction due to antiretroviral therapy. Several reports have demonstrated alterations in the enzymatic activity of MRC and oxygen consumption in each of these tissues[88-90]. However, the accessibility for tissues like the liver, adipose tissue or muscle requires a biopsy, a bloody method, that makes its use difficult for treatment monitoring. Additionally, biochemical assays are neither standardized nor robust and therefore the interpretation of the data is complicated.

Genetic methods

Two different aproaches, qualitative and quantitative, are useful for the measurement of disorders related to mtDNA. The qualitative assays are based on sequence analysis and represent the best approach to identify specific mutations, causing illnesses such as mitochondrial encephalomyopathies, stroke-like episodes, retinitis pigmentosa, etc. or long deletions, such as in Kerns-Sayre syndrome, progressive external ophthalmoplegia, Pearson's syndrome, etc[8]. Mechanisms behind these disorders will not be further discussed, since this review focuses on acquired mitochondrial disorders.

For mtDNA secondary dysfunction due to the use of NRTIs in HIV-infected individuals, there is no evidence of one specific mutation that affects mitochondrial function. Martin, et al. reported the presence of multiple mutations after treatment with NRTIs, and this was especially significant in patients with lipoatrophy[91]. However, Walker, et al. failed to detect mutations or deletions in the adipose tissue of patients with lipoatrophy[65]. Since the recent introduction of quantitative methods using real-time PCR detection, the availability of accurately measured mtDNA has greatly improved. Several reports have described in-house, real-time PCR methods based on the detection of both nuclear and mtDNA genes[52,60,62]. The ratio established between nuclear DNA (nDNA) and mtDNA may allow to estimate the total copy number of mtDNA per cell. Based on this

principle, a commercial method for the quantitation of mtDNA has recently become available. The Retina DNA Mitox™ assay is based on the NASBA amplification principle[92], followed by molecular beacon, real-time detection. Both mtDNA and nDNA are amplified and detected in one tube. The one-tube format allows direct measurement of the ratio under the same amplification conditions, avoiding any external influence which might interfere when amplifications are done separately. This assay has been proven to be reproducible, with a low variability and high robustness[93,94].

The first step of the blood sample collection/preparation is critical for valid mtDNA quantitation in PBMCs, especially when depletion of mtDNA is expected to be found due to NRTI therapy. In PBMC specimens, platelets that contain mtDNA, but not nDNA, can influence the mtDNA/nDNA ratio, yielding higher results due to mtDNA contamination[93-96]. Platelet sorting using magnetic beads[96], or the use of special cell preparation tubes for blood collection that have cheaper than magnetic beads, may be useful to eliminate platelets from PBMCs. However, when the platelet content is lower than five times the PBMC content, the quantitation of mtDNA is only minimally affected and the results are reliable[93].

Current limitations and future prospects

Treatment with NRTIs has been associated with mitochondrial toxicity. Depletion of mtDNA can be found in PBMCs collected from HIV-infected patients on antiretroviral therapy with hyperlactatemia, and in adipose tissue of subjects with severe lipoatrophy. Therefore, quantitative mtDNA assays could be useful to monitor and evaluate mitochondrial toxicity in HIV+ patients on antiretroviral therapy. However, several aspects need to be resolved before the implementation of these tests. Firstly, changes in mtDNA in one tissue or cell line (PBMCs) may not accurately reflect what is happening in other tissues (adipocytes, muscle, liver,...). This observation makes it difficult to extrapolate changes in mtDNA, content in PBMCs for each of the pathologies associated with mitochondrial dysfunction. Secondly, for other inherited mitochondrial diseases, severe symptoms tend to occur when mtDNA levels go down 20% or less than normal. However, no threshold has been defined yet in HIV infection. Thirdly, the inter-individual variability for mtDNA content seems to be high, which may make it difficult to establish clinically significant thresholds, and monitoring including baseline measures may be necessary to assess changes over time. Fourthly, the various methods that have been developed to measure mtDNA in cells should be further harmonized and validated to enable a proper comparison between the various methods and studies. The recent introduction of a commercially available assay could be the first step to solve this problem.

An important question that clinicians have to answer regards whether we can prevent mitochondrial toxicity associated with NRTIs based on the results of mtDNA measurements. All the limitations discussed above should be solved before attempting to answer this question, in particular the clinical significance of mtDNA depletion, the relationship between mtDNA depletion in PBMCs and other tissues, and the prognostic value of low mtDNA contents in asymptomatic patients. Fortunately, many investigations are in progress in an attempt to answer these questions.

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