

DIAGNOSIS AND MONITORING OF MITOCHONDRIAL TOXICITY INDUCED BY NUCLEOSIDE-ANALOGUE REVERSE- TRANSCRIPTASE INHIBITORS IN HIV- INFECTED PATIENTS UNDERGOING ANTIRETROVIRAL THERAPY

CĂTĂLIN TILIȘCAN¹, VICTORIA ARAMĂ^{1,2}, RALUCA
MIHĂILESCU², ADRIAN STREINU-CERCEL^{1,2}, OANA
ANDREIA COMAN¹, DANIELA ION¹, ISABEL GHIȚĂ¹, ȘTEFAN
SORIN ARAMĂ^{1*}

¹University of Medicine and Pharmacy "Carol Davila", no. 37,
Dionisie Lupu str., 020021 Bucharest, Romania

²„Prof. Dr. Matei Balș” National Institute of Infectious Diseases, no.
1, Dr. Calistrat Grozovici str., 21106 Bucharest, Romania

*corresponding author: sorinarama@gmail.com

Abstract

Highly Active Antiretroviral Therapy (HAART) containing nucleoside reverse transcriptase inhibitors (NRTIs) has been associated with various side effects following mitochondrial toxicity. The most important clinical consequences of mitochondrial DNA (mtDNA) depletion are: lipodystrophy, hepatic toxicity, lactic acidosis, myopathy and polyneuropathy.

In order to prevent the onset of clinical and biological side effects during NRTIs treatment, different methods have been proposed to assess metabolic toxicity non-invasively and to adequately predict the metabolic risk. These methods have to be harmonized and validated, and there is a need for available commercial tests. Further studies are needed, in order to clarify whether clinicians can prevent mitochondrial toxicity based on the results of mtDNA measurements.

Rezumat

Terapia combinată antiretrovirală înalt activă cu inhibitori nucleozidici de revers-transcriptază (INRT) are numeroase efecte secundare, care sunt consecința depleției de ADN mitocondrial din diverse țesuturi. Cele mai importante consecințe clinice sunt: sindromul lipodistrofic, toxicitatea hepatică, acidoza lactică, miopatia și polineuropatia.

Au fost propuse diferite metode non-invazive pentru a diagnostica precoce riscul de apariție a acestor efecte secundare. Aceste metode se bazează pe evaluarea cantității de ADN mitocondrial, ca marker de toxicitate mitocondrială. Aceste metode nu sunt încă standardizate și nu există încă suficiente studii clinice care să confirme relația dintre scăderea cantității de ADN mitocondrial și apariția modificărilor clinice și metabolice, la pacienții cu infecție HIV.

Keywords: Mitochondrial DNA, HIV, Nucleoside reverse transcriptase inhibitors, Nucleoside reverse transcriptase inhibitors (NRTIs), Highly Active Antiretroviral Therapy (HAART)

Introduction

Over 75 diseases involve mitochondrial dysfunction. Many are characterized by multiorgan involvement, frequently targeting central nervous system, peripheral sensory and motor neurons and cardiovascular system [13].

Each cell contains hundreds to thousands of mitochondria and each mitochondrion contains about 2-10 mitochondrial DNA (mtDNA) molecules. Mitochondrial DNA is circular and has only 37 genes [10].

There are approximately 900 mitochondrial products. Most of them are coded by nuclear DNA (nDNA), which is imported from the cytoplasm [13].

Oxidative phosphorylation is responsible for approximately 95% of adenosine triphosphate (ATP) formation in animal cells under aerobic metabolism. Mitochondria convert energy released during substrate oxidation into forms available for cellular processes. Mitochondria is also implicated in calcium homeostasis, aminoacids, fatty acids and steroids metabolism [10].

The introduction of Highly Active Antiretroviral Therapy (HAART) in 1996 has fundamentally changed the evolution of HIV infection and led to a decrease in opportunistic infections and HIV-related cancer mortality. Unfortunately, most patients experience side effects during long-term antiretroviral treatment. Clinical and metabolic changes are known to result from overlapping but distinct effects of the various drugs within the HAART protocol. The primary pathogenetic mechanism through which HAART precipitate metabolic changes and organ toxicities is *via* mtDNA depletion [18].

Highly Active Antiretroviral Therapy (HAART) toxicity pathogenesis

Mitochondrial toxicity is the main pathogenic mechanism of side effects during HAART.

Nucleoside reverse transcriptase inhibitors (NRTIs) have been proven to decrease mtDNA synthesis resulting in consequential risk for the development of mitochondrial dysfunction [18]. The degree of mtDNA depletion seems to correlate with the development of symptomatic hyperlactatemia and lipoatrophy. NRTIs mitochondrial toxicity is the result of mtDNA depletion, not of specific mutations [6].

In order to become active, NRTIs (Table I) require phosphorylation in the cell, before they are able to inhibit HIV reverse transcriptase. As a side effect, the phosphorylated NRTIs also inhibit polymerase γ , which is responsible for the replication of mtDNA. The result of polymerase γ impairment is a quantitative reduction of the mtDNA copy number, which causes the impairment of the cellular respiratory chain [6].

Table I
Nucleoside Analog Reverse Transcriptase Inhibitors

NRTI	Abbreviation	Analogue of
Zidovudine	AZT	Thymidine (Pyrimidines)
Stavudine	d4T	Thymidine (Pyrimidines)
Zalcitabine	ddC	Cytidine (Pyrimidines)
Emtricitabine	FTC	Cytidine (Pyrimidines)
Lamivudine	3TC	Cytidine (Pyrimidines)
Abacavir	ABC	Guanosine (Purines)
Didanosine	ddI	Inosine (Purines)
Tenofovir	TDF	Adenosine (Purines)

The respiratory chain is responsible for ATP synthesis. It is also involved in the fatty acid oxidation. The impairment of fatty acid beta-oxidation is responsible for the accumulation of intracellular triglycerides [12].

Normal respiratory chain function is essential for the *de novo* synthesis of all intracellular pyrimidine nucleosides. Pyrimidine nucleosides depletion leads to further impairment of mtDNA synthesis, establishing a vicious circle [7].

The onset of mitochondrial toxicity requires prolonged exposure. Clinical changes are observed at least a few months after HAART initiation [9].

Some NRTIs induce more profound mtDNA depletion than others. The degree of mitochondrial toxicity was determined *in vitro* as follows:

$$\text{zalcitabine} > \text{didanosine} > \text{stavudine} > \text{lamivudine} \geq \text{abacavir} \geq \text{tenofovir} \geq \text{emtricitabine} \quad [18].$$

These data do not correlate well with the clinical toxicity as the efficiency in phosphorylation differs among NRTIs, which determines its affinity for the DNA polymerase γ [10].

Clinical manifestations of mitochondrial toxicity

Mitochondrial toxicity may have a wide range of clinical consequences.

Lipodystrophy

Lipodystrophy is the most important syndrome linked to mitochondrial dysfunction in HIV-infected patients. Some subjects may develop subcutaneous fat wasting at the Bichat's fat pad, of temporal fat, or at the buttocks and extremities [18]. Other patients may experience abnormal fat accumulation intraabdominally and in the dorsocervical region. The overall prevalence of at least one physical abnormality associated with this syndrome is around 50% in patients on stable HAART.

The main metabolic abnormalities observed in lipodystrophy include: hypertriglyceridemia, hypercholesterolemia, low levels of HDL-cholesterol, insulin resistance, type 2 diabetes mellitus, lactic acidemia, elevated hepatic transaminases [10].

Hepatic toxicity

Mitochondrial liver toxicity is associated with hepatomegaly, steatosis and elevated serum liver transaminases. Steatohepatitis may rarely progress to liver failure. Liver complication have been associated mainly with didanosine, stavudine, and zalcitabine [13].

Elevations of plasma lactate levels are common (5-35%), but lactic acidosis is rare. Symptoms associated with hyperlactatemia are nonspecific: nausea, abdominal tenderness, myalgias [2].

Mitochondrial myopathy

Myopathy is mainly associated with zidovudine therapy and may occur as weakness, ophtalmoplegia and ptosis [14]. Serum creatine kinase (CK) is often normal. Mitochondrial myopathy must be distinguished from HIV myopathy. The histochemical examination shows ultrastructurally abnormal mitochondria (NRTI myopathy) or CD8-positive T lymphocytes infiltrate (HIV myopathy) [1].

Mitochondrial neuropathy

NRTI neuropathy occurs weeks or months after the initiation of HAART (mainly linked to zidovudine and lamivudine). The mitochondrial axonal toxicity may manifest predominantly as a sensitive, distal, simmetrical polyneuropathy of the extremities [18].

Hematologic toxicity

The most important mtDNA depletion in peripheral blood mononuclear cells was observed in patients exposed to zidovudine, didanosine, stavudine and zalcitabine. By reduction of cellular mtDNA

content, NRTIs increase apoptosis and inhibit lymphocyte proliferation, acting as immunosuppressive drugs [11]. Zidovudine can also inhibit hematopoietic progenitor cells, causing anemia, neutropenia and thrombocytopenia [15].

Kidney toxicity

Mitochondrial depletion in kidney is still controversial. There have been reported isolated cases of aminoaciduria, renal tubular dysfunction, Fanconi's syndrome, Barter syndrome during treatment with HAART regimens containing adefovir and tenofovir [17].

Diagnosis and monitoring of mitochondrial toxicity

In symptomatic patients, histological examination is the most accurate test for the diagnosis of mitochondrial toxicity. The following findings indicate a mitochondrial etiology: ultrastructural abnormalities of mitochondria, intracellular microvesicular steatosis, diminished histochemical activities of cytochrome c oxidase, ragged-red fibers in skeletal muscle [18].

Different methods have been proposed to assess metabolic toxicity noninvasively and to predict the metabolic risk adequately prior to its onset.

These biochemical and clinical instruments vary from quantitative methods to those based on the analysis of phenotypes for the functions of mitochondria (table II) [10]. Still, there is currently no reliable method to predict the risk of mitochondrial toxicity.

Table II

Methods available to measure mitochondrial function and mitochondrial DNA [10]

Information	Method	Measure
Measure of mtDNA depletion	In-house real time PCR Southern blot Retina Mitox	mtDNA/nDNA ratio mtDNA/nDNA ratio mtDNA/nDNA ratio
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 Spectrophotometric	Oxydative damage Oxygen consumption Enzymatic activity

Lactate levels in asymptomatic patients cannot predict clinical changes, so routine lactate screening is not recommended for these patients. In contrast, most symptomatic patients have elevated serum lactate [9].

Biochemical methods

The mtDNA contains 37 genes, and 13 of them encode enzymes of the respiratory chain. The biochemical methods can determine the overall enzymatic activity of the mitochondrial respiratory chain as well as the activity of every single subunit. There have been developed spectrophotometric and polarographic techniques [3], which can provide quantitative measurements, using simple instruments. However, there are two main disadvantages: the necessity of a biopsy and the lack of standardization of the biochemical assays [18].

Genetic methods

Both qualitative and quantitative standardized tools have been developed.

The sequence analysis is useful in diagnosing specific mutations, that are characteristic to mtDNA genetic disorders (such as retinitis pigmentosa, mitochondrial encephalomyopathies, etc.) [4].

NRTIs mitochondrial toxicity is the result of mtDNA depletion, not of specific mutations. Several available methods are based on the quantification of both nuclear and mtDNA genes, establishing a ratio between nuclear DNA and mtDNA [10]. This ratio may allow the estimation of the total copy number of mtDNA per cell.

Peripheral blood mononuclear cells (PBMCs) and subcutaneous fat may be accessible sources of mtDNA for examining toxicity.

A number of in-house assays have been developed to quantify mitochondrial DNA. These methods are based on real-time polymerase chain reaction (PCR) and nucleic acid sequence base amplification (NASBA).

The blood sample preparation is very important for valid mtDNA quantitation in PBMCs, as platelets contain mtDNA, but not nDNA. Platelet mtDNA contamination may determine higher results, especially when depletion of mtDNA is expected to be found due to NRTI therapy.

The quantitation of mtDNA is only minimally affected when the PBMC content is five times higher than the platelet content.

The platelets may be sorted using magnetic beads or special cell preparation tubes for blood collection. [10] The samples can also be washed with a medium of phosphate-buffered saline containing 20 mL/L fetal calf serum and subsequently centrifuged for 15 min at 100g. [16]

Real-time polymerase chain reaction is currently a standard technology for effective quantification of RNA and DNA in human cells.

Real-time PCR assays use primers and polymerase in thermocycling reactions to generate an exponential accumulation of the

target DNA. The PCR amplified nucleic acid is detected and quantified in real time by monitoring the increase in the fluorescence signal.[5] Several reports have described in-house, real-time PCR methods based on the detection of both nuclear and mtDNA genes.

Nucleic acid sequence based amplification (NASBA) technology is an alternative method to standard amplification procedures.

In combination with fluorescent probes, NASBA also allows real-time quantification of mitochondrial nucleic acids in relation to nuclear RNA or DNA. The product of NASBA is single-stranded RNA of opposite sense to the original target. Advantages of NASBA over reverse transcriptase PCR include selective amplification of RNA in a background of DNA and fast amplification kinetics. NASBA assay is isothermal and thus there is no requirement for thermocycling during the procedure.[5]

Primagen's Retina™ Mitox™ is the first available commercial assay to quantify mitochondrial DNA. The Retina™ Mitox™ test measures the mitochondrial DNA content per cell in a real-time duplex NASBA (Retina) assay. Real-time duplex NASBA is a modification of the standard NASBA, in which both mitochondrial and nuclear DNA targets are simultaneously amplified in a single-tube format. The simultaneous amplification allows direct measurement of the mtDNA to nDNA ratio under the same conditions, thus avoiding any external factors that might occur when amplifications are performed separately. [16]

The monitoring of mtDNA in PBMCs could be an important non-invasive early detection tool of NRTIs toxicities, before the onset of clinical manifestations.

Before quantitative mtDNA assays could be implemented in clinical practice there are important aspects that need to be clarified:

- The various methods developed for mtDNA measurement have to be standardized and harmonized and there is a need for available commercial tests.
- There is yet to establish a threshold for mtDNA reduction, in order to quantify the risk of mitochondrial toxicity.
- It has not yet been established a definite association between the level of mtDNA and the risk of clinical and metabolic manifestations.
- The PBMCs mtDNA modifications may not properly reflect the changes of mtDNA in other tissues.

Conclusions

Highly active antiretroviral therapy (HAART) containing nucleoside reverse transcriptase inhibitors (NRTIs) has been associated with various side effects following mitochondrial toxicity. The most important clinical consequences of mtDNA depletion are: lipodystrophy, hepatic toxicity, lactic acidosis, myopathy and polyneuropathy.

In order to prevent the onset of clinical and biological side effects during NRTIs treatment, different methods have been proposed to assess metabolic toxicity non-invasively and to predict the metabolic risk adequately. These methods have to be harmonized and validated, and there is a need for available commercial tests. Further studies are needed, in order to clarify whether physicians can prevent mitochondrial toxicity relying on the results of mtDNA measurements. There is yet to establish a threshold for mtDNA reduction, in order to quantify the risk of mitochondrial toxicity.

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